Optimization of process parameters for enzymatic hydrolysis of tuna red meat protein with emphasis on specific applications

Thesis submitted to

Cochin University of Science and Technology

In partial fulfillment for the degree of

DOCTOR OF PHILOSOPHY

in

MARINE SCIENCES

Faculty of Marine Sciences

Cochin University of Science and Technology

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Certificate

This is to certify that the research work presented in the PhD thesis entitled **"Optimization of process parameters for enzymatic hydrolysis of tuna red meat protein with emphasis on specific applications"** is a bona fide record of research carried out by Mrs. Parvathy U. (Reg. No. 5023), under my guidance and supervision and that no part therefore has been formed the basis of award of any degree, diploma, associateship, fellowship or other similar titles or recognitions of this or any other Universities. It is also certified that all the relevant corrections and modifications suggested by the audience during the pre-synopsis seminar and recommended by the Doctoral committee of the candidate has been incorporated in the thesis.

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Kochi
May, 2019

Dr. A. A. Zynudheen
Co-guide

DECLARATION

I, Parvathy U. (Reg. No. 5023), Ph.D candidate registered under the Faculty of Marine Sciences, CUSAT hereby declare that, my PhD thesis entitled **"Optimization of process parameters for enzymatic hydrolysis of tuna red meat protein with emphasis on specific applications"** is a genuine record of research carried out by me under the guidance of Dr. George Ninan, Principal Scientist, Fish Processing Division, ICAR-CIFT, Kochi and the co-guidance of Dr. A.A. Zynudheen, Head of Division (i/c) and Principal Scientist, Quality Assurance and Management Division, ICAR-CIFT, Kochi. No part of this work has previously formed the award of any degree, associateship, fellowship or any other title or recognition of any other University or Society.

Kochi
May, 2019

Parvathy U.

Dedicated

to

My Family and Friends

With

Love and Gratitude

Acknowledgement

I wish to express my deepest regards and profound sense of gratitude to my major advisor Dr. George Ninan, Principal Scientist, Fish Processing Division, ICAR-CIFT for his inspiring guidance and constant encouragement which helped in the timely completion of my research work. His keen attention and advice helped me a lot in preparation of the thesis and submitting it in the present form.

I am grateful and indebted to Dr. A. A. Zynudheen, Co-guide and HOD (i/c), Quality Assurance and Management Division, ICAR-CIFT, for his valuable help and support during the course of my study and the constructive suggestions given for the successful completion of thesis.

I place on record my sincere gratitude to Dr. C. N. Ravishankar, Director, ICAR-CIFT for all his encouragement and incredible support given and providing necessary facilities for the successful conduct of the research work.

I express my gratitude to Dr. K. Ashok Kumar, HOD, Fish Processing Division for his support and encouragement for the successful completion of my research work.

I owe a great deal to Dr. Binsi P. K, in being a great support during the course of my research work. Her helpful advice and constructive suggestions given during the course of the experiment and preparation of the thesis is commendable and I sincerely thank her for all the ideas and contributions made for preparing the thesis in the current structure.

My sincere gratitude to Dr. T.K. Srinivasa Gopal, Former Director, ICAR-CIFT for his valuable and constructive suggestions given during the study. I also place on record my deepest regards to Dr. Sajan George, my MFSc research guide, whose aptitude and dedication towards work has always inspired me to work hard.

I wish to place on record my sincere thanks to all staffs of Mumbai Research Centre, where major part of my research work was carried out. Thanks to Dr. L. N. Murthy, for his support and providing the facilities to carry out my work. I deeply acknowledge Dr. A. Jeyakumari for her constant encouragement, incredible support and contributions. I also sincerely thank Dr. S. Visnuvinayagam for his support and contributions. All technical, supporting and

administrative staffs are also greatly acknowledged: Smt. Sangeeta, Smt. Priyanka, Smt. Triveni, Smt. Girija, Smt. Hema, Shri Waghmare, Shri Vinod, Shri Avinash and all other staffs.

My sincere thanks are also due to Dr. Joshy C. G. for the help in statistical analysis of the experiment, results and also for his cordial and timely help.

I place on record my sincere gratitude to my senior colleagues of Fish Processing Division, Dr. J. Bindu, Dr. C. O. Mohan and all my colleagues, Sreelakshmi, Sarika, Sreejith, Mandakini, Elavarasan and Satheesh for the help extended by them during course of my work. All the staffs of fish processing division are also greatly acknowledged for their direct and indirect support and assistance. Special thanks to Shri. Noby, Smt. Susmitha and Smt. Priyanka for the timely and cordial help in case of immediate assistance. I place on record my gratitude to all Heads of the Division, ICAR-CIFT and all staffs of ICAR-CIFT who have directly or indirectly helped and supported me in completion of this study.

I would like to wholeheartedly thank my CIFT colleagues Anupama, Laly, Renuka, Jesmi, Rehana, Priya and all other colleagues who have directly or indirectly helped me during my research work. Special thanks to my colleague, Manju for her moral support which has helped me a great in the successful completion of my research work.

Cooperation from Smt. Silaja and all the library staff of ICAR-CIFT, Kochi has helped me to a great extent in successful compilation of my PhD thesis. I express my heartfelt thanks to them. I also place on record my gratitude to ICAR and CUSAT for the permission and facilities given in carrying out this research work successfully.

Most often words fail to express the feelings. The support and care given by my family is incredible and can never be acknowledged through words. They have been a great deal of support to me throughout my study. I express my sincere gratitude to my family for all the encouragement and continuous support for the successful completion of my work.

My homage to the Almighty for what I am today and for all the blessings in my life.

Parvathy U.

Abstract

Globally, tuna resources have high commercial value on account of its demand for thermally processed delicacies. Reports reveal that tuna canning industry generate an estimate of 4,50,000 tons of processing discards globally per year. Dark muscle from tuna is rich in proteins and is an important edible fish by-product from tuna cannery. However, on account of low market recognition, it is currently being utilized for preparation of fertilizer, animal feed etc. Recovery and utilization of this biomass to bioactive protein hydrolysate is a promising alternative as it facilitates food and pharmaceutical applications.

Numerous studies have been reported on the characterization of fish protein hydrolysates derived from various sources under different hydrolytic conditions and have suggested the possible areas of application. However no comprehensive studies have been reported on the optimization of protein hydrolysate properties viz., functional and antioxidative activity, separately from same source, with emphasis to protein recovery for its end application and further commercialization potential. Hence a study was proposed with the aim of standardization of enzymatic hydrolytic conditions to obtain protein hydrolysate from yellowfin tuna red meat with specific properties for their potential applications, characterization and storage stability of the derived tuna protein hydrolysate and their performance evaluation in the incorporated food formulations.

Initially, a comparative evaluation of the peptides from white and red meat of yellowfin tuna (*Thunnus albacares*) was carried out to explore the extent to which the properties vary in red meat derived hydrolysate in comparison to its white meat. The findings from the study indicated the nutritional composition of tuna red meat comparable to that of white meat with abundance in recoverable proteins. Assessment of the peptide properties indicated better antioxidative activity for tuna

white meat protein hydrolysate. However, except oil absorption capacity, other functional properties were higher for tuna red meat protein hydrolysate. Further detailed studies are required to reveal the extent of variations that the properties may exhibit with respect to white and red meat of tuna, as it is influenced by intrinsic as well as extrinsic factors.

Process optimisation studies for the selective extraction of functional and antioxidant hydrolysates from cooked tuna red meat (*Thunnus albacares*) using RSM with a central composite design, with emphasis on protein recovery was carried out. The optimum hydrolytic conditions for superior functional properties were achieved at an E/S ratio of 0.34 % for hydrolysis duration of 30 minutes, referred to as functional tuna protein hydrolysate. Similarly, the optimum conditions to exhibit the maximum antioxidative properties were: 0.98 % E/S and 240 minutes of hydrolysis time, referred to as antioxidant tuna protein hydrolysate. Further the optimized spray dried hydrolysates were comprehensively characterized and their storage stability studies were carried out at ambient (28^{o}C) and chill storage conditions (4^{o}C) for up to six months. Storage studies indicated an uptake of moisture, increase in oxidative indices as well as changes in functionality which was more prominent under ambient conditions. Efforts were also made in the investigation to develop and upscale the laboratory outcomes to facilitate industrial production of fish protein hydrolysate.

Similar to hydrolysis optimization carried out for cooked tuna red meat protein, studies were conducted for separate extraction of functional and antioxidant hydrolysates from raw yellowfin tuna red meat for a comparative evaluation. Under this study, the optimum hydrolytic conditions to get hydrolysates having superior functional properties were E/S ratio of 0.41 % and 30 minutes hydrolysis time whereas hydrolysates derived under conditions: 1.28 % E/S and 240 minutes hydrolysis time exhibited the highest antioxidative properties. Studies indicated protein recovery during hydrolysis to be higher from raw tuna red meat than from cooked meat. Hydrolysate from cooked tuna red meat exhibited superior functional properties except OAC, whereas except ABTS radical scavenging activity, hydrolysates from raw tuna red meat exhibited dominance with regard to antioxidative activities.

Application potentials of derived hydrolysates were explored by microencapsulation of fish oil. Studies were carried out to compare the efficacy of yellowfin tuna red meat hydrolysate (optimized for antioxidative properties) in protecting the core sardine oil, when used as wall and core polymer during encapsulation. Their storage stability was also compared under accelerated (60^{o}C), chilled (4^{o}C) and ambient conditions (28^{o}C). Current observations suggest the advocation of protein hydrolysate as core material along with sardine oil for obtaining shelf stable spray dried oil encapsulates.

Fortification and stabilization of mayonnaise by incorporating functionally optimized tuna protein hydrolysate as a partial replacer of egg yolk in the product was done. Results indicated a replacement ratio of 1:2::TPH:egg yolk, as desirable and hence opted for further stability studies. The storage stability parameters of the samples under chilled conditions (4^{o}C) indicated better oxidative and physicochemical stability for fortified samples compared to control.

Utilization of protein hydrolysate from yellowfin tuna red meat for formulation of a health beverage mix was carried out. Tuna protein hydrolysate (TPH), optimized for functional and antioxidative properties using papain under a hydrolytic condition viz., E/S of 1.08 %, 30 minutes hydrolysis time, temperature and pH of 60^{o}C and 6.5, respectively was used. Health beverage base mix was formulated based on RSM and the sensorily selected base mix formulation was further incorporated with different levels of TPH. Sensory studies indicated highest acceptability for $HM_{2.5}$ (base mix added with 2.5 % TPH) and further storage studies of $HM_{2.5}$ samples under ambient conditions (28^{o}C) indicated good stability throughout the study period of six months.

Present work addresses process modifications to reduce production cost of protein hydrolysate by optimization of enzymatic processing conditions based on protein recovery, functionalities and sensory attributes. Further the study paves scope for the development of innovative fish protein hydrolysate fortified food products.

ABBREVIATIONS

AAN	:	Alpha amino nitrogen
ABTS	:	2,20 -azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
ANOVA	:	Analysis of variance
AOAC	:	Association of official analytical chemists
AR	:	Analytical grade reagent
AU	:	Activity unit
BHA	:	Butylated hydroxy anisole
BHT	:	Butylated hydroxy toluene
CCD		Central composite design
Da	:	Dalton
DH	:	Degree of hydrolysis
DPPH	:	2,2-diphenyl-1-picryl hydrazyl
DSC	:	Differential scanning calorimetry
EAA	:	Essential amino acid
EAI	:	Emulsifying activity index
EE	:	Encapsulation efficiency
ESI	:	Emulsion stability index
E / S	:	Enzyme to substrate ratio
FAO	:	Food and agriculture organization
FC	:	Foaming capacity
FFA	:	Free fatty acid
FMOC	:	9-fluorenylmethyl-chloroformate
FPH	:	Fish protein hydrolysate
FRAP	:	Ferric reducing antioxidant power
FS	:	Foam stability
FT-IR	:	Fourier transform infra red analysis
g	:	Gram
h	:	Hour
HPLC	:	High performance liquid chromatography
ICP-OES	:	Inductivity coupled plasma-optical emission spectrometer
IC_{50}	:	Half maximal (50 %) inhibitory concentration
kCal	:	Kilocalorie
kDa	:	Kilodalton
kg	:	Kilogram
kV	:	Kilovolt
mEq	:	Milliequivalents

mg	:	Milligram
min	:	Minutes
ml	:	Millilitre
mmol	:	Millimole
MSE	:	Mean square error
MW	:	Molecular weight
NEAA	:	Non essential amino acid
nm	:	Nanometer
NPN	:	Non-protein nitrogen
OAC	:	Oil absorption capacity
OPA	:	O-phthalaldehyde
PA	:	Proteolytic activity
PG	:	Propyl gallate
PR	:	Protein recovery
PV	:	Peroxide value
RDA	:	Recommended daily intake
RP	:	Reducing power
rpm	:	Rotations per minute
RSM	:	Response surface methodology
SDS	:	Sodium dodecyl sulphate
sec	:	Seconds
SEM	:	Scanning electron microscope
SGF	:	Simulated gastric fluid
SIF	:	Simulated intestinal fluid
TCA	:	Trichloroacetic acid
TMA	:	Tri-methyl amine
TPH	:	Tuna protein hydrolysate
TRPH	:	Tuna red meat protein hydrolysate
TVBN	:	Total volatile base nitrogen
TWPH	:	Tuna white meat protein hydrolysate
UV	:	Ultraviolet
v/v	:	Volume by volume
w/v	:	Weight by volume
α	:	Alpha
µl	:	Microlitre
µm	:	Micrometer
µM	:	Micromole

Contents

List of Tables

List of Figures

Chapter 1
Introduction

Nutritional insecurity is a major problem faced by the modern society and in this context marine resource is considered as a safe source of nutrition that provides rich amounts of protein having good pattern of essential amino acids. A major share of the marine biomass is being discarded as byproduct with low market realization. Awareness about the potential recovery of nutrients from fish waste has created increased interest in exploiting these resources. Tuna resources, which includes tuna (*Thunnus* spp.) as well as tuna-like species are significant sources of food and hence play a vital role in the economy of many countries. More than about 48 species of tuna are widely distributed in the Atlantic, Indian, Pacific oceans and the Mediterranean sea. The two major products that drive tuna production are the traditional thermally processed delicacies and sashimi/sushi. These commodities exhibit relevant differences with regard to the species utilized, quality requirements as well as production systems. Canning industry preferably demands light meat species like skipjack and yellowfin, while in the sushi and sashimi market, the fatty ones like bluefin and other red meat species like bigeye are preferred.This widespread economic significance of tuna and their contribution to international trade has made tuna waste of particular interest to upgrade. Tuna market mainly utilizes the white meat during canning operations thus resulting in the under utilization of protein rich by-products viz., red meat, head, skin, trimmings, viscera etc. that are discarded without recovery attempts and accounts for about 50–70 % of biomass (Guerard et

al., 2002; Chalamaiah et al., 2012; Saidi et al., 2014). Reports by Sutanbawa and Aknes (2006), revealed an estimate of 4,50,000 tons per year of processing discards globally from the tuna canning industry. Of these, 10-12 % is the dark or red meat portion which has nutrients especially proteins, of high quality comparable to that of the white meat (Nishioka et al., 2007). Currently, the red meat generated during tuna canning operations is usually discarded as waste or is converted to low value by-products like animal feed, fertilizers etc with negligible market value (Herpandi et al., 2011). Hence utilization of these dark meat proteins is a serious matter to be addressed on account of the limited food resources, for meeting the nutritional security and increasing environmental pollution issues.

Seafood proteins, on account of its structural diversification as well as nutritional, functional, and biological properties, can be effectively exploited for their recovery to different forms viz., concentrates, isolates, hydrolysates, protein fractions like collagen, gelatin etc. In this regard, these protein rich fish processing discards could be enzymatically converted into its hydrolysates, facilitating its effective utilization. Protein hydrolysates are the breakdown products of proteins viz., smaller peptide chains with 2-20 amino acids obtained by hydrolysis either chemically or enzymatically. This process facilitates recovery of essential nutrients viz.,amino acids as well as has immense scope in food, nutraceutical and pharmaceutical industry on account of the excellent physicochemical, functional as well as bioactive properties they possess (He et al., 2013; Halim et al., 2016). Based on the extent of hydrolysis that the parent protein undergoes, the properties exhibited by the hydrolysates vary considerably.

One of the major characteristics of protein hydrolysates is the functional properties, which are those physicochemical properties that affect the behaviour of proteins in food systems during storage, processing, preparation and consumption

(Kinsella, 1982; Hall and Ahmad, 1992; Phillips et al., 1994). These characteristics influence the quality and organoleptic attributes in food and hence are important particularly if they are used as ingredients in food products. Functional properties are related to protein structure viz., the sequence and composition of amino acids, molecular weights, conformation and the net charge distributed on the molecule (Damodaran, 1996; Casarin et al., 2008). Hydrolysis of proteins generates a mixture of free amino acids, di-, tri- and oligopeptides, increasing the number of polar groups and hydrolysate solubilities thereby modifying the functionalities and bioavailability (Adler-Nissen, 1986; Kristinsson and Rasco, 2000). Functional properties are important when the fish protein hydrolysates interact with other components of food such as oil and water. Reports suggest that fish protein hydrolysates (FPH) showed enhanced functional properties, in comparison with the parent protein, or other commercial food-grade products having the same function (Elavarasan, 2014). The important functional properties of FPH include solubility, emulsifying properties, foaming properties and fat absorption capacity (Motoki and Kumazawa, 2000).

Lipid oxidation is of great concern to the food industry and consumers, as it leads to the development of undesirable off-flavors, off-odors, dark colors, taste deterioration and formation of potentially toxic reaction products (Noguchi and Niki, 1999; Lin and Liang, 2002; Niki, 2010; Lin et al., 2010). Furthermore diseases like cancer, coronary heart problems and Alzheimer's are also reported to be caused partially by oxidation or free radical reactions in the body (Diaz et al., 1997; Bougatef et al., 2010; Ngo et al., 2010). Lipid oxidation in food products can be controlled by reducing metal ions and minimizing exposure to light and oxygen using appropriate packaging methods as well as by incorporation of antioxidants. Antioxidants are substances used to prolong the shelf life and maintain the nutritional

quality of lipid-containing foods (Rajaram and Nazeer, 2010). They also assist to modulate the consequences of oxidative damage in the human body (Munoz et al., 2010). Many synthetic antioxidants such as BHT, BHA, TBHQ and propyl gallate (PG) are used in the food and pharmaceutical industries to retard lipid oxidation (Bernardini et al., 2016). However the use of synthetic antioxidants result in potential health issues (Byun et al., 2009; Bougatef et al., 2010) and hence there is growing interest to identify alternative natural and safe sources of food antioxidants for replacing these synthetic ones (Sarmadi and Ismail, 2010; Bernardini et al., 2016). Fish protein hydrolysate is well established for its antioxidant properties on account for the bioactive peptides they possess. They usually vary from 2-20 amino acid residues with the molecular mass of less than 6000 Da (Jun et al., 2004; Wang et al., 2008; Bougatef et al., 2010). These peptides are inactive within the sequence of parent protein and are released upon enzymatic cleavage.

Fish protein hydrolysates have potential application as functional ingredients in different foods on account of the numerous important and unique properties that they possess viz., functional as well as bioactive properties (Chalamaiah et al., 2010). They are also a source of specific amino acids for dietic formulations (Sumaya-Martinez et al., 2005) which are easily absorbed and utilized for various metabolic activities (Nesse et al., 2011). They have been successfully tested as emulsifiers, foaming agents, dispersants, antioxidants etc. for incorporation into different food systems such as cereal products, fish and meat products, desserts and crackers for providing desirable characteristics to the product as well as to improve their storage stability (Yu and Tan, 1990; Kristinsson and Rasco, 2000; Pacheco-Aguilar et al., 2008; Zhang et al., 2013). Hence with increasing knowledge of these advantages of fish protein hydrolysates, more researches are being focused on the development of fish-derived functional and nutraceutical foods.

Numerous investigations have been carried out on various aspects of fish protein hydrolysates. Of these, attempts for arriving at an optimum degree of hydrolysis (DH) using response surface methodology (RSM) have been made extensively on various seafood substrates considering nitrogen recovery (Ogonda et al., 2017), bioactive properties (Wangtueai et al., 2016; Wang et al., 2017) and functional properties (Jamil et al., 2016), as process responses. However, it is well understood that the properties of hydrolysates depend to a large extent on the nature of polypeptide fragments formed, rather than the DH achieved during the hydrolytic process. It is quite obvious that peptides from the same source having the same DH exhibit significant variations in their properties. The extent to which these properties may alter is less explored, so far. Many times, a combined optimization for entirely different spectrum of properties such as bioactive and functional properties may be of less significance, when the hydrolysate is intended for a specific application. This essentially means that, separate optimization designs are required for extracting functional/surface-active and bioactive hydrolysates, considering the process responses specific to the intended property, so as to arrive at more accurate and technically viable parameters for the particular hydrolysis process. Hence, a study was proposed with the aim of standardization of enzymatic hydrolytic conditions to obtain protein hydrolysate from yellowfin tuna (*Thunnus albacares*) red meat with specific properties for their potential applications. Further, characterization and storage stability of the derived tuna protein hydrolysate and their performance evaluation in the incorporated food systems were determined.

Scope of the study

Numerous studies have been carried out for arriving at an optimum processing condition giving hydrolysates exhibiting either functional or bioactive properties. However, no comprehensive studies have been reported offering separate

sets of optimised process parameters for the same substrate yielding hydrolysates with either functional or antioxidative properties. Hence, the current investigation was intended towards optimizing the effect of hydrolysis variables viz.,enzyme-substrate ratio (E/S) and hydrolysis duration, using appropriate statistical tools, for separate extraction of functional/surface-active and antioxidant rich hydrolysates from the cannery waste; cooked meat of yellowfin tuna red meat with thrust to maximum protein recovery. Moreover, a variability range of degree of hydrolysis values with respect to individual properties exhibited by hydrolysates under each hydrolytic condition was derived by statistical means. Further, these derived peptides were comprehensively characterized, assessed for its storage stability, economic feasibility and their performance evaluation in different selected food systems were determined.

Objectives

- Optimization of enzymatic hydrolytic conditions of fish protein for their functional applications and nutraceutical applications using appropriate statistical tool.
- Characterization of the optimized fish protein hydrolysates for its functional and bioactive properties.
- Evaluation of storage stability and economic feasibility of the optimized fish protein hydrolysates.
- Assessment of the optimized fish protein hydrolysate incorporated food systems for their functional performance and bioactive properties.

Chapter 2
Review of Literature

2.1 Tuna red meat as a source of protein

Over the past few years, utilization of fish wastes has been of increasing interest on account of the superior quality as well as safety aspects. This biomass provides proteins with high nutritional properties and a good pattern of essential amino acids. Tuna and tuna products are extensively utilized in many parts of the world on account of their delicacy as well as nutritional properties viz., richness in proteins. Moreover, the tuna waste constitutes a biomass of particular interest to upgrade because of their global economic importance and their international trade for canning. As only the white meat of tuna is used in canning or sashimi, the tuna industry generates a large amount of waste or by-products. The solid wastes generated from the processing industry constitute as much as 70 % of the original material of which dark tuna muscle accounts for about 12 % of raw tuna butchered for canning (Guerard et al., 2002; Chalamaiah et al., 2012; Saidi et al., 2014).

Fish processing discards including tuna wastes are commonly considered as low-value resources with negligible market value (Arvanitoyannis and Kassaveti, 2008) and are currently used to produce fish oil, fishmeal, fertilizer, pet food, and fish silage (Kim and Mendis, 2006; Herpandi et al., 2011). However, the recognition of limited biological resources and increasing environmental pollution has emphasized the need for better utilization of these by-products.

Several authors (Plascencia et al., 2002; Kim and Mendis, 2006; Nishioka et al., 2007) have reported that similar to fish meat, their wastes are also valuable sources of compounds such as proteins, lipids, minerals etc. Characterization of the chemical composition of the discarded waste from many fish species showed that the protein content is generally over 50 % on dry weight basis (Bechtel, 2003; Sathivel et al., 2003). Kim and Mendis (2006) reported a number of bioactive compounds from fish by-product proteins. These by-products are very important bio-resources that can be utilized for applications in food, health-care products, and pharmaceuticals or as specialty feeds for fish and other animals. Proper utilization of these protein rich fish processing discards could be achieved by enzymatic conversion of these sources into protein hydrolysates which has immense application scope in food and pharmaceutical areas (Chalamaiah et al., 2012; He et al., 2013).

2.2 Fish protein hydrolysate

The benefits of hydrolyzing food proteins to make functional protein ingredients and nutritional supplements are a more recent technology, with the first commercially available protein hydrolysates appearing only around the late 1940s. Protein hydrolysate is defined as proteins that are broken down into peptides of various sizes either chemically (using acids or bases) or biologically (using enzymes) (Rustad, 2003; Pasupuleti and Braun, 2010). Fish protein hydrolysates (FPH) possess many desirable properties such as health promoting bioactivities, making them eligible ingredients in nutraceuticals and functional foods (Kristinsson, 2007; Harnedy and FitzGerald, 2012).Use of different fish species/ substrate, proteolytic enzymes and adequate control of the process parameters such as temperature, pH, time and enzyme-substrate ratio facilitate the optimized production of FPH with desirable molecular structures and bioactive properties with therapeutic or nutritional interest (Guerard et al., 2002; Chabeaud et al., 2009).

2.3 Enzymatic hydrolysis

Enzymatic hydrolysis is the most promising one compared to other processes as it results in products with high functionality and organoleptic characteristics of the food in relation to its nutritive value and intestinal absorption characteristics (Kristinsson and Rasco, 2000; Yoshie-Stark et al., 2006; Pasupuleti and Braun, 2010; Wisuthiphaet et al., 2015). This method also requires relatively small amount of enzymes that can be easily deactivated and mild conditions of hydrolysis. Moreover, the use of enzymes does not destroy amino acids and resulting mixtures of peptides can be purified easily (Herpandi et al., 2011). An added benefit of the use of enzymes particularly proteolytic enzymes is their ability to increase protein recovery and also aid in deriving bioactive compounds from complex raw materials (Rubio-Rodriguez et al., 2010). The enzymatic hydrolysis of fish muscle proteins is characterized by an initial rapid phase, during which a large number of peptide bonds are hydrolysed, and further the rate of enzymatic hydrolysis decreases and reaches a stationary phase where no apparent hydrolysis takes place (Shahidi et al., 1995; Ren et al., 2008a). This rate change is associated with enzyme inactivation, hydrolysis product inhibition, low Km value of soluble peptides that act as effective substrate competitors to the unhydrolysed fish protein, and possibly auto digestion of the enzyme.

Several proteolytic enzymes can be used for the hydrolysis of fish processing waste (Simpson et al.,1989). A wide variety of proteolytic enzymes are commercially available from animal, plant and microbial sources. The most commonly used enzymes for protein hydrolysates from animal sources are pancreatin, trypsin and pepsin; plant sources are papain and bromelain and from fermentation sources are bacterial and fungal proteases (Pasupuleti and Braun, 2010; Hou et al., 2017). The general application criterion for these enzymes is that they should be of food grade

and if from microbial origin, the producing organism has to be of non-pathogenic nature (Bhaskar and Mahendrakar, 2008).

Table 2.1 Proteases used for hydrolysis

Type of Proteases	Common Names	pH range	Preferential Specificity [a]
Aspartic protease	Pepsin, Pepsin A, Pepsin I, Pepsin II	1-4	Aromatic-COOH and $-NH_2$, Leu-, Asp-,Glu-COOH
Serine Protease	Trypsin	7-9	Lys-, Arg-COOH
Serine Protease	Chymotrypsin	8-9	Phe-, Tyr-, Trp-COOH
Mix. trypsin, chymo-trypsin, elastaseand carboxypeptidase A/B	Pancreatin	7-9	Very broad specificity
Cysteine Protease (Papaya fruit)	Papain pure	5-7	Lys-, Arg-, Phe-X-COOH
Mixture of papain, chymopapain and Lysozyme	Papain crude	5-9	Broad specificity
Cysteine Protease	Ficin	5-8	Phe-, Tyr-COOH
Cysteine Protease	Bromelain	5-8	Lys-, Arg-, Phe-, Tyr-COOH
Metalloprotease	Neutrase	6-8	Leu-, Phe-NH and others
Serine protease	Alcalase	7-12	Broad specificity
Mixture of aspartic protease, metalloprotease, serine protease, and carboxypeptidase	Takadiastase, Fungal Protease, Sumyzyme LP, Veron P, Panazyme, Prozyme, Biozyme A, Sanzyme	4-8	Very broad specificity

Source: Adler-Nissen, 1986

[a] Referring to the carbonyl terminal end after cleaving

Among the different proteases, papain is one of the most common enzymes of choice (Hoyle and Merritt, 1994; Shahidi et al., 1995; Wisuthiphaet et al., 2015) due to its low cost, easy availability, optimum activity at near neutral pH and outstanding value for liberating several B-vitamins from their bound forms. Papain is a cysteine protease extracted from latex of papaya (*Carica papaya*). It is an endoprotease with a single polypeptide of 212 amino acids with a molecular weight of 23,350 Daltons (Kristinsson, 2006). It comprises of three disulfide bond and is heat stable at neutral pH (Konno et al., 2004). The optimum pH for papain (including crude papain) is 5.0-9.0 and is stable up to 80 °C in presence of substrates. The use of papain is more extensive in the brewing industry (75 % of the papain production) followed by meat (10 %), fish (5 %) and others (11 %) (Kim et al., 2004).

2.4 Factors influencing enzymatic hydrolysis

Enzymatic hydrolysis of proteins is influenced by various factors such as the protein source, the type of enzyme used, the physicochemical conditions of the reaction media including enzyme / substrate ratio, time, temperature, and pH (Ren et al., 2008a; Benjakul et al., 2010; He et al., 2012). Generally the substrate preferred as protein source for hydrolysate preparation should be of lean variety with minimum fat content. Use of fatty fishes for FPH calls for additional treatments to remove excess fat (Ritchie and Mackie, 1982) and hence increases the cost of production. The type of enzyme used in enzymatic protein hydrolysis is very important because it dictates the cleavage patterns of the peptide bonds (Shahidi and Zhong, 2008). Proteases from different sources viz., animal, plant, and microbial are commonly used to obtain a more selective hydrolysis as they are specific for peptide bonds adjacent to certain amino acid residues (Korhonen and Pihlanto, 2003). The physicochemical condition of the reaction determines the degree of hydrolysis as well as the molecular weight of the peptides which are contributors to the bioactive property exhibited by the peptides (Ren et al., 2008b)

Table 2.2 Different conditions for enzymatic hydrolysis of fish proteins

Substrate	Enzymes	Hydrolysis conditions	Reference
Yellowfin tuna waste	Alcalase	Temperature: 50°C pH: 8.0 Time: 30-300 min E/S ratio: 0.2-3%	Guerard et al., 2001
	Umamizyme	Temperature: 45°C pH: 7.0 Time: 4 h E/S ratio: 1.5%	Guerard et al., 2002
Herring by-products	Alcalase	Temperature: 50°C pH: 8.0 Time: Variation E/S ratio: 0.5%	Sathivel et al., 2003
Threadfin bream	Alcalase	Temperature: 60°C pH: 8.5 Time: 120 min E/S ratio: 2%	Normah et al., 2005
Grass carp skin	Alcalase	Temperature: 59, 58 and 60°C pH: 8.0, 8.0 and 9.0 Time: 75, 110, 120 min E/S ratio: 0.12, 0.57 and 1.08%	Wassawa et al., 2007
Yellow stripe trevally muscle proteins	Alcalase	Temperature: 60°C pH: 8.5 Time: 20 min E/S ratio: 0.25 to 10%	Klompong et al., 2007
	Flavourzyme	Temperature: 50°C pH: 7.0 Time: 20 min E/S ratio: 0.25 to 10%	
Catla visceral waste proteins	Alcalase	Temperature: 50°C pH: 8.5 Time: 135 min E/S ratio: 1.5%	Bhaskar et al., 2008
Persian sturgeon viscera	Alcalase	Temperature: 35, 45 and 55°C pH: 8.5 Time: 205 min E/S ratio: 0.1 AU/g	Ovissipour et al., 2009a

Substrate	Enzymes	Hydrolysis conditions	Reference
Small croaker proteins	Flavourzyme Protamex	Temperature: 25°C pH: 7.0 Time: 120 min E/S ratio: 0.75% Temperature: 25°C pH: 7.0 Time: 120 min E/S ratio: 1%	Choi et al., 2009
Ornate threadfin bream muscle	Skipjack tuna pepsin extract	Temperature: 50°C pH: 2.0 Time: 60 min E/S ratio: 0.026%	Nalinanon et al., 2011
Striped catfish frame protein	Papain Bromelain	Temperature: 60°C pH: 7.0 Time: 90 min E/S ratio: 0.5% Temperature: 55°C pH: 7.0 Time: 90 min E/S ratio: 0.5%	Tanuja et al., 2012
Cobia frame	Alcalase	Temperature: 40, 60 and 60°C pH: 8.5, 9.5, 10.5 Time: 120, 180 and 300 min E/S ratio: 1.5, 2 and 20%	Amiza et al., 2012
Skipjack tuna dark flesh	Alcalase Protamex Neutrase Flavourzyme	Temperature: 55°C pH: 8.0 Time: 240 min E/S ratio: 0.5-2% Temperature: 50°C pH: 7.5 Time: 240 min E/S ratio: 0.5-2% Temperature: 45°C pH: 7.0 Time: 240 min E/S ratio: 0.5-2% Temperature: 50°C pH: 7.5 Time: 240 min E/S ratio: 0.5-2%	Herpandi et al., 2012

Substrate	Enzymes	Hydrolysis conditions	Reference
Toothed pony fish muscle	Hybrid catfish visceral enzymes	Temperature: 50°C pH: 9.0 Time: 15 min E/S ratio: 0.01%	Klomklao et al., 2013
Protein isolate from Nile tilapia and broad head catfish mince	Alcalase	Temperature: 50***°C*** pH: 8.0 Time: 120 min E/S ratio: 1.1-1.3% Temperature: 50***°C*** pH: 8.0 Time: 120 min E/S ratio: 3.8-4.3%	Yarnpakdee et al., 2014
Nile tilapia by product	Alcalase	Temperature: 60°C pH: 7.5 Time: 120 min E/S ratio: 2.5% Temperature: 45°C Time: 240 min E/S ratio: 0.5%	Roslan et al., 2014 Silva et al., 2014
Spanish Mackerel	Alcalase, trypsin, protemax flavourzyme	Temperature: 55, 37, 50 and 50°C, respectively pH: 8.5, 8.5, 7.0 and 7.0, respectively Time: 120 min E/S ratio: 400 U/g	Kong et al., 2015
Ponyfish, Yellow stripe travally and Mackerel	Papain	Temperature: 40°C Time: 5, 10, 15 h E/S ratio: 2, 4, 6%	Wisuthiphaet et al., 2015
Nile tilapia	Alcalase	Temperature: 50°C pH: 6.0 Time: 240 min E/S ratio: 0.2%	Bernardi et al., 2016
Monk fish	Alcalase Bromelain	Temperature: 45, 55, 65 °C pH 7.0, 7.5, 8.0, 8.5 Enzyme conc: 0.1 AU/g protein (Alcalase) 200 GDU/g protein (Bromelain)	Greyling, 2017

Substrate	Enzymes	Hydrolysis conditions	Reference
Dagaa (*Rastrineobola argentea*)	Alcalase	Temperature: 50 - 58°C pH: 7-11 Time: 120 min Solvent ratio: 0-3% (v/w)	Ogonda et al., 2017
Tilapia	Papain	Temperature: 27, 30, 50 and 70°C pH: 4.0, 6.5, 7.0 and 9.0 Time: 30, 60, 90 and 120 min E/S ratio: 0.5, 1.0, 1.5 and 2%	Srikanya et al., 2018
Yellowfin tuna red meat	Papain	Temperature: 60°C pH: 6.5 Time: 30-240 min E/S ratio: 0.25-1.5 %	Parvathy et al., 2018a
Mackerel viscera	Trypsin, papain, neutrase, acid protease and flavourzyme	Temperature: 30-60°C pH: 5-8 Time: 4-8 h Enzyme conc: 800-1800 U/g Water/substrate ratio: 1.0-25 (v/w)	Wang et al., 2018

2.5 Proximate composition of fish protein hydrolysates

Chemical composition of fish protein hydrolysates is important in nutrition perspective of human health. Protein is the major component of interest in fish protein hydrolysate and many researchers reported protein content of fish protein hydrolysates between 60 % to 90 % of total composition (Choi et al., 2009; Khantaphant et al., 2011; Parvathy et al., 2016). The high protein content for fish protein hydrolysates was due to solubilization of proteins during hydrolysis and removal of insoluble solid matter by centrifugation (Liceaga-Gesualdo and Li-Chan, 1999; Chalamaiah et al., 2010). High protein content of fish protein hydrolysates demonstrated its potential use as protein supplements for human nutrition. Majority of the studies reported a fat content of below 5 % for different fish protein hydrolysates (Abdul-Hamid et al., 2002; Thiansilakul et al., 2007a; Wasswa et al., 2007; Bhaskar et al., 2008; Ovissipour et al., 2009a). The low fat content of fish protein hydrolysates was because of removal of lipids with insoluble protein fractions by centrifugation. Most of the studies demonstrated that protein hydrolysates from various fish proteins contained moisture below 10 % (Gbogouri et al., 2004; Chalamaiah et al., 2010; Foh et al., 2011; Parvathy et al., 2016; Bhingarde, 2017). The low moisture content of protein hydrolysates was related to the type of sample and to the higher temperatures employed during the process of evaporation and spray drying. Low moisture content facilitated better handling as well as storage stability to the hydrolysates. The ash content of fish protein hydrolysates reported in many studies ranged between 0.45 % to 27 % of total composition (Choi et al., 2009; Yin et al., 2010; Mazorra-Manzano et al., 2012). Several authors have reported that the usage of added acid or base for pH adjustment of medium leads to high ash content of fish protein hydrolysates (Gbogouri et al., 2004; Dong et al., 2005; Choi et al., 2009).

Several studies have been reported on the proximate composition of protein hydrolysate from different species viz., herring (*Clupea harengus*) (Liceaga-Gesualdo and Li-Chan, 1999; Sathivel et al., 2003); Atlantic salmon (*Salmo salar*) (Kristinsson and Rasco, 2000); black tilapia (*Oreochromis mossambicus*) (Abdul-Hamid et al., 2002); salmon (*Salmo salar*) (Gbogouri et al., 2004); slender lizard fish (*Saurida elongate*) (Dong et al., 2005); tuna (Nilsang, 2005); red salmon (*Oncorhynchus nerka*) (Sathivel et al., 2005); sardinelle (*Sardinella aurita*) (Souissi et al., 2007); round scad (*Decapterus maruadsi*) (Thiansilakul et al., 2007a); grass carp (*Ctenopharyngodon idella*) (Wasswa et al., 2007); catla (*Catla catla*) (Bhaskar et al., 2008); Pacific whiting (*Merluccius productus*) (Pacheco-Aguilar et al., 2008; Mazorra-Manzano et al., 2011); croaker (*Pennahia argentata*) (Choi et al., 2009); Persian sturgeon (*Acipenser persicus*) (Ovissipour et al., 2009a); channel catfish (*Ictalurus punctatus*) (Yin et al., 2010); brownstripe red snapper (*Lutjanus vita*) (Khantaphant et al., 2011); king fish (*Scomberomorus commersonii*) (Abdulazeez et al., 2013); tilapia (*Oreochromis niloticus*) (Roslan et al., 2014; Silva et al., 2014); pony fish (*Eubleekeria splendens*), yellow stripe travally (*Selaroides leptolipis*), mackerel (*Decapterus maruadsi*) (Wisuthiphaet et al., 2015), pony fish (*Leiognathus bindus*) (Johnrose et al., 2016); tuna (*Thunnus albacares*) (Parvathy et al., 2016); cuttle fish (*Sepia pharaonis*) (Raftani Amiri et al., 2016), silver catfish (*Arius thalassinus*) (Abraha et al., 2017), Malabar sole fish (*Cynoglossus macrostomus*) (Bhingarde, 2017); monkfish (*Lophius vomerinus*) (Greyling, 2017); rainbow trout (*Oncorhynchus mykiss*) (Rajabzadeh et al., 2017); mackerel tuna (*Euthynnus affinis*) (Parvathy et al., 2018b); tilapia fish (*Oreochromis niloticus*) (Srikanya et al., 2018) etc.

2.6 Amino acid composition of fish protein hydrolysates

Hydrolysis of protein results in hydrolysates composed of a mixture of free amino acids and short chain peptides exhibiting many advantages as nutraceuticals or functional foods because of their amino acid profile. Santos et al. (2011) opined that amino acid composition of fish protein hydrolysates is important because of the nutritional value and the influence on the functional properties. Several authors have described the amino acid composition of protein hydrolysates produced from processing waste proteins of different fish species. Among different body parts of fish, muscle protein is the most extensively studied and reported source (Klompong et al., 2009a; Nakajima et al., 2009; Ghassem et al., 2011).

Fish protein hydrolysates have been reported to exhibit variation in their amino acid composition which is mainly due to factors such as raw material, enzyme source, and hydrolysis conditions (Klompong et al., 2009a; Chalamaiah et al., 2012). Among all the amino acids, aspartic acid and glutamic acid were found to be higher in most of the reported fish protein hydrolysates (Klompong et al., 2009a; Yin et al., 2010; Ghassem et al., 2011; Parvathy et al., 2016; Wisuthiphaet et al., 2016). Similar to fish muscle hydrolysates, other body parts like head, skin and visceral hydrolysates were reported to contain all the essential and non-essential amino acids (Sathivel et al., 2005; Bhaskar et al., 2008; Gimenez et al., 2009; Ovissipour et al., 2009a; Yin et al., 2010).

Studies on the amino acid composition of various fish species reported include those from herring (*Clupea harengus*) (Liceaga-Gesualdo and Li-Chan, 1999; Sathivel et al., 2003); Icelandic scallop (*Chlamys islandica*) (Mukhin et al., 2001); black tilapia (*Oreochromis mossambicus*) (Abdul-Hamid et al., 2002); salmon (*Salmo salar*) (Gbogouri et al., 2004); slender lizard fish (*Saurida elongate*) (Dong et al., 2005); tuna (Nilsang et al., 2005); red salmon (*Oncorhynchus nerka*)

(Sathivel et al., 2005a); round scad (*Decapterus maruadsi*) (Thiansilakul et al., 2007a); grass carp (*Ctenopharyngodon idella*) (Wasswa et al., 2007); silver carp (*Hypophthalmichthys molitrix*) (Dong et al., 2008); catla (*Catla catla*) (Bhaskar et al., 2008); sole and squid (Gimenez et al., 2009); yellow stripe trevally (*Selaroides leptolepis*) (Klompong et al., 2009a); Atlantic salmon, coho salmon, Alaska pollack and southern blue whiting (Nakajima et al., 2009); Persian sturgeon (*Acipenser persicus*) (Ovissipour et al., 2009a); cat fish (*Ictalurus punctatus*) (Yin et al., 2010); bluewing searobin (*Prionotus punctatus*) (Santos et al., 2011); loach (*Misgurnus anguilliacaudatus*) (You et al., 2011); tilapia (*Oreochromis niloticus*) (Roslan et al., 2014; Silva et al., 2014; Yarnpakdee et al., 2015); skip jack tuna (*Katsuwonus pelamis*) (Liu et al., 2015); cod (Godinho et al., 2016); pony fish (*Leiognathus bindus*) (Johnrose et al., 2016); tuna (*Thunnus albacares*) (Parvathy et al., 2016); cuttle fish (*Sepia pharaonis*) (Raftani Amiri et al., 2016); grey triggerfish (*Balistes capriscus*) (Siala et al., 2016); monk fish (*Lophius vomerinus*) (Greyling, 2017); rainbow trout (*Oncorhynchus mykiss*) (Rajabzadeh et al., 2018); tilapia (Tejpal et al., 2017); salmon (Idowu et al., 2019) etc.

2.7 Functional properties of fish protein hydrolysates

Functional properties are those physicochemical properties, which affect the behaviour of proteins in food systems during storage, processing, preparation and consumption (Kinsella, 1982; Hall and Ahmad, 1992; Phillips et al., 1994). It is these characteristics, which influence the 'quality' and organoleptic attributes in food. Hence these attributes are important particularly if they are used as ingredients in food products. Functional properties are related to protein structure viz., the sequence and composition of amino acids, molecular weights, conformation and the net charge distributed on the molecule (Damodaran, 1996; Casarin et al., 2008).

Enzymatic hydrolysis generates a mixture of free amino acids, di-, tri- and

oligopeptides, increases the number of polar groups and hydrolysate solubilities thereby modifying the functionalities and bioavailability (Adler-Nissen, 1986; Kristinsson and Rasco, 2000). Functional properties are important when the fish protein hydrolysates interact with other components of food such as oil and water. Reports suggested the functional properties of peptides to be linked to the concentration in the diet and to the protein source (Elaziz et al., 2017). Studies have indicated fish-derived bioactive peptides to have superior functional properties in comparison to other sources (Taheri et al., 2013). There are extensive studies on the functional properties of fish protein hydrolysates from different fish species where in they exhibited enhanced properties, when compared with un-hydrolysed fish protein, or other commercial food-grade products having the same function (Liu et al., 2015; Parvathy et al., 2018a; Sripokar et al., 2019). The important functional properties of FPH include solubility, emulsifying properties, foaming properties and fat absorption capacity (Motoki and Kumazawa, 2000).Hydrolytic conditions like E/S ratio, nature of enzymes, time, pH influenced the functional properties of fish protein hydrolysates (Tanuja et al., 2012; Naqash and Nazeer, 2012). Previous studies (Klompong et al., 2007) have reported decreased interfacial activities and increased solubility with increasing DH in fish protein hydrolysates. Similarly Nalinanon et al. (2011) also reported that functional properties like emulsion and foaming properties are governed by their DH and hydrolysate concentrations.

2.7.1 Solubility

Solubility is the amount of protein that goes into the solution under specified conditions. It is regarded as the most important functional property as many of the other functional properties like emulsifying and foaming properties are influenced by this parameter (Wilding et al., 1984). Hence, solubility can be considered as an excellent indicator of protein functionalities and its potential

applications (Mahmoud, 1994). Intact fish myofibrillar proteins have the problem of the lack of solubility in water over a wide range of pH and enzymatic hydrolysis is very important in increasing the solubility of these proteins (Spinelli et al., 1972; Venugopal and Shahidi, 1994). Hydrophobic and ionic interactions are the major factors that influence the solubility characteristics of proteins. Hydrophobic interactions promote protein-protein interactions and result in decreased solubility whereas ionic interactions promote protein-water interactions and result in increased solubility (Adler-Nissen, 1986). Geirsdottir et al. (2011) opined that the soluble nature of FPH in a wide range of ionic strengths and pH values makes it possible to use them effectively in seafood products for improved functional properties like water-binding capacity. Longer processing times for FPH production leads to a high degree of hydrolysis which results in protein solutions with smaller molecular weights having higher solubility (Shahidi et al.,1995). It was hypothesized that there is an increase in hydrophilic polar groups leading to an increase in their water-solubility (Kristinsson and Rasco, 2000).

Solubility of protein hydrolysates from different species were studied. Species viz., salmon (Gbogouri et al., 2004); blue whiting (Geirsdottir et al., 2011); skip jack tuna (Liu et al., 2015); common carp (Saputra and Nurhayati, 2016); Alaska Pollock (Liu et al., 2018); yellowfin tuna (Parvathy et al., 2018a); mackerel tuna (Parvathy et al., 2018b); etc. are a few among them.

2.7.2 Fat absorption capacity

The capacity of hydrolysate to absorb fat / oil is an important attribute that influences the taste of the product. It is an important functional characteristic required especially for the meat and confectionery industry. Fat absorption capacity correlates with surface hydrophobicity and protein hydrolysates develops this hydrophobicity on account of the hydrolysis which cleaves the protein chain

resulting in the exposure of more internal hydrophobic groups (Kristinsson and Rasco, 2000). The mechanism of fat absorption was attributed mostly to physical entrapment of the oil and thus, the higher bulk density of the protein more is the fat absorption. Generally, hydrolysates are mixed with a specified amount of excess fat for a particular time and then centrifuged at a low centrifugal force and the fat absorption is expressed as g of fat absorbed / g of protein (Shahidi et al., 1995).

A few among the studies reported on the fat absorption capacity of hydrolysate from different fish species include those from red salmon (Sathivel et al., 2005a); cod (Slizyte et al., 2005); grass carp (Wasswa et al., 2007); tilapia (Foh et al., 2011; 2012); blue whiting (Geirsdottir et al., 2011); yellowtail king fish (He et al., 2015); tuna (Parvathy et al. 2016); cuttle fish (Raftani Amiri et al., 2016); Alaska pollock (Liu et al., 2018) etc.

2.7.3 Emulsifying properties

An emulsion is a system containing two immiscible liquid phases, one of which is dispersed in the other as droplets varying between 0.1 and 50 μm in diameter. The phase present in the form of droplets is called the dispersed phase while the matrix in which the droplets are dispersed is called the continuous phase (Nawar, 1985). Emulsions are thermodynamically unstable systems as a result of the large positive energy at the interface of the two liquids (Comas et al., 2006). Emulsifiers are able to form a protective coating around the oil droplets leading to prevention of coalescence phenomenon (Kasapis et al., 2009). The emulsifying properties of FPH are directly connected to their surface properties. Fish protein hydrolysates are good emulsifiers due to their improved amphiphilic nature, as they expose more hydrophilic and hydrophobic groups that enable orientation at the oil–water interface for more effective adsorption (Klompong et al., 2007). Kristinsson and Rasco (2000) reported that protein hydrolysates should consist of at least 20

residues to possess good emulsifying capacity. Desirable surface active proteins and protein hydrolysates have three major attributes such as ability to rapidly adsorb to an interface, ability to rapidly unfold and reorient at an interface and ability to interact with the neighbouring molecules at interface and form a strong cohesive, viscoelastic film that can withstand thermal and mechanical motions (Philips, 1981; Damodaran, 1996). However, the extent of hydrolysis has to be carefully controlled, as excessive hydrolysis can decrease the emulsifying capacity of protein hydrolysates (Kristinsson and Rasco, 2000; Gbogouri et al., 2004; Klompong et al., 2007).

Emulsifying properties of proteins are measured as emulsion activity index (EAI) and emulsion stability index (ESI). EAI measures the area of oil-water interface stabilized by a unit weight of protein and ESI measures an emulsion's ability to resist breakdown (Wu et al., 1998). Mutilangi et al. (1996) reported that higher content of larger molecular weight peptides or more hydrophobic peptides contribute to the stability of the emulsion. Small peptides and amino acids were less efficient in reducing the interfacial tension due to the lack of unfolding and reorientation at the interface as the large peptides do (Gbogouri et al., 2004).

Extensive studies were reported on emulsifying properties from various seafood sources like cod (Slizyte et al., 2005); yellow stripe trevally (Klompong et al., 2007); Pacific whiting (Pacheco-Aguilar et al., 2008); tilapia (Foh et al., 2012); skip jack tuna (Liu et al., 2015); sardine and small-spotted catshark (Garcia-Moreno et al., 2016); pony fish (Johnrose et al., 2016); cuttle fish (Raftani Amiri et al., 2016); tilapia (Tejpal et al., 2017); starry triggerfish (Sripokar et al., 2019) etc.

2.7.4 Foaming properties

Food foams consist of air droplets dispersed in and enveloped by a continuous liquid containing a soluble surfactant lowering the surface and interfacial tension of the liquid (Kinsella and Melachouris, 1976). Similar to emulsifying properties, foaming properties also rely on the surface properties of protein. Townsend and Nakai (1983) showed that total hydrophobicity, or the hydrophobicity of exposed or unfolded protein, have a significant correlation to foaming formation.Similar to other functional properties, foaming properties of protein hydrolysate was related to the degree of hydrolysis (Kuehler and Stine, 1974).

Foaming properties are usually expressed as foam formation / foaming capacity and foam stability. Foaming capacity is referred to as the ability of protein to form foams is described as overrun. Overrun is the percentage of excess volume produced by whipping a protein containing liquid compared with the initial volume of the liquid (Phillips et al., 1987). Foam stability is measured by whipping the protein solution and measuring the decrease in the volume in a specific period.

Previous literature reported foaming properties from different fish species viz., herring (Liceaga-Gesualdo and Li-Chan, 1999); yellow stripe trevally (Klompong et al., 2007); rainbow trout (Taheri et al., 2013); pony fish (Johnrose et al., 2016); tuna (Parvathy et al., 2016); cuttle fish (Raftani Amiri et al., 2016); Alaska Pollock (Liu et al., 2018); starry triggerfish (Sripokar et al., 2019) etc. indicating their application potential in various food systems.

2.7.5 Sensory properties

Sensory evaluation is a scientific discipline used to evoke, measure, analyze, and interpret reactions to those characteristics of foods and materials as they are perceived by the senses of sight, smell, taste, touch, and hearing (Stone et al., 2012).

Sensory properties of a product are extremely important for the successful adaptation and acceptance by the food industry and the consumer (Kristinnson and Rasco, 2000). Although enzymatic hydrolysis of proteins develops desirable functional properties, it has the disadvantage of generating bitterness which is identified as a major hindrance regarding utilization and commercialization of bioactive FPH (Dauksas et al., 2004; Kim and Wijesekara, 2010). The mechanism of bitterness is not very clear, but it has been documented to be associated with the presence of bitter peptides mainly comprising hydrophobic amino acids. In addition to hydrophobicity of peptides, peptide length, amino acid sequence and spatial structure also influences the perception of bitter taste (Kim and Li-Chan, 2006).Hydrolysis of protein results in exposing buried hydrophobic peptides, which readily interact with the taste buds resulting in detection of bitter taste. Two functional groups are necessary to produce bitterness, such as a pair of hydrophobic groups or a hydrophobic or a basic group (Tamura et al., 1990). An extensive hydrolysis to free amino acids, however, decreases the bitterness of these bitter peptides because hydrophobic peptides are far more bitter compared with a mixture of free amino acids.

The sensory properties of protein hydrolysate from species like cod (Liaset et al., 2000); salmon (Kouakou et al., 2014); Nile tilapia (Yarnpakdee et al., 2015); Atlantic salmon (Aspevik et al., 2016); yellowfin tuna (Parvathy et al., 2018a); mackerel tuna (Parvathy et al., 2018b) have been previously reported.

Many techniques have been suggested to reduce or mask bitterness in hydrolysates, but few of them was applied to FPH (Adler-Nissen, 1986; Saha and Hayashi, 2001; Leksrisompong et al., 2012).Strict control of any hydrolysis experiment and termination at low degree of hydrolysis isa common and desirable method to prevent the development of a bitter taste and the retention of functional properties (Hou et al., 2011). As enzymes have different preferences for amino

acids, choosing the most appropriate enzyme is the most widespread methodology for reducing bitterness (Kristinsson, 2006). Enzymes with a high preference for hydrophobic amino acids such as alcalase are often preferred and frequently yield products of low bitterness (Adler-Nissen, 1986). The use of exopeptidases, as opposed to endoproteinases, is the most frequent and economic method adopted in overcoming the bitterness in fish protein hydrolysates, particularly exopeptidases that split off hydrophobic amino acids from bitter peptides (Nilsang et al., 2005; Cheung et al., 2015; Fu et al., 2019). Many studies have shown that proteolytic preparations containing exopeptidases and endoproteinases produce less bitter peptides than single proteases (Vegarud and Langsrud, 1989; Moll, 1990). It was suggested that oxidation products play a part in the development of bitter taste (Liu et al., 2000). A few suggested methods for bitterness reduction include treating hydrolysates with activated carbon that partly removes bitter peptides with absorption (Shahidi et al., 1995; Suh et al., 2000; Bansal and Goyal, 2005; Aspevik, 2016), extracting bitter peptides with solvents (Lalasidis, 1978; Dauksas et al., 2004) and by plastein reaction which is the formation of a gel-like proteinaceous substance from a concentrated protein hydrolysate (Gong et al., 2015). Masking can be performed by adding additives or molecules, e.g. cyclodextrin, to the hydrolysate to mask the bitter taste (Tamura et al., 1990; Aspevik et al., 2016). Masking additives promotes conformational alterations of the peptides and introduction of sweet tastes that cover the bitterness (Linde et al., 2009).

Bitterness evaluation in protein hydrolysates can be carried out by traditional sensory analysis by trained sensory panel. Though sensory evaluation by trained panellists is the most direct way, it is quite time-consuming due to extensive training required to obtain reliable results (Newman et al., 2014). Other modifications in the method include taste dilution analysis (Seo et al., 2008), category scaling

(Lovsin-Kukman et al., 1996), line scaling (Aaslyng and Frøst, 2010) and caffeine equivalency (Kodera et al., 2006). Limitations in sensory analysis have created an increasing interest for alternative methods like objective evaluation. Of these, application of electronic tongue, has great potential for addressing the challenges associated with sensory evaluation. Electronic tongue is a device equipped with numerous sensors connected to the central chemometric processing unit that can measure and compare diverse tastes (Newman et al., 2014).The sensors serve as taste receptors similar to human tongue and the obtained data are subsequently analysed using chemometrics or artificial intelligence to identify taste (Podrazka et al., 2018; Fu et al., 2019). A number of investigations have been carried out recently employing electronic tongue to evaluate bitterness of protein hydrolysates (Cheung and Li-Chan, 2014; Newman et al., 2014).

2.8 Antioxidative activity of fish protein hydrolysates

Lipid oxidation is of great concern to the food industry and consumers because it leads to the development of undesirable off-flavors, off-odors, dark colors, taste deterioration and formation of potentially toxic reaction products (Noguchi and Niki, 1999; Lin and Liang, 2002; Niki, 2010; Lin et al., 2010). Furthermore diseases like cancer, coronary heart problems and Alzheimer's were also reported to be caused partially by oxidation or free radical reactions in the body (Diaz et al., 1997; Bougatef et al., 2010; Ngo et al., 2010). Lipid oxidation in food products can be controlled by reducing metal ions and minimizing exposure to light and oxygen using packaging as well as by incorporation of antioxidants. Antioxidants are substances used to prolong the shelf life and maintain the nutritional quality of lipid-containing foods (Rajaram and Nazeer, 2010) and to modulate the consequences of oxidative damage in the human body (Munoz et al., 2010). Halliwell and Gutteridge (2007) defined an antioxidant as any substance that when present at low concentration compared with those of an oxidizable substrate significantly delays

or prevents oxidation of that substrate. Many synthetic antioxidants such as BHT, BHA, TBHQ and propyl gallate (PG) were used in the food and pharmaceutical industries to retard lipid oxidation (Bernardini et al., 2016). The use of synthetic antioxidants result in potential health issues (Byun et al., 2009; Bougatef et al., 2010) and hence there is growing interest to identify alternative natural and safe sources of food antioxidants for replacing these synthetic ones (Bernardini et al., 2016). These identified antioxidative activities have a potential to develop safe and nonhazardous natural antioxidants for the complications arose from oxidation of biomolecules.

Fish protein hydrolysate is well established for its antioxidant properties on account for the bioactive peptides they possess. The antioxidant activity of peptides has been attributed to their chain length and hydrophobicity as well as to certain amino acids. They usually vary from 2-20 amino acid residues with the molecular mass of less than 6000 Da (Jun et al., 2004; Wang et al., 2008; Bougatef et al., 2010). These peptides are inactive within the sequence of parent protein and released upon enzymatic cleavage. The first known scientific study reported on the antioxidant activity of fish protein hydrolysates was in 1995 by Shahidi et al. Since then a number of studies reported the antioxidative activities of various fish protein hydrolysates (Klompong et al., 2007; Thiansilakul et al., 2007b; Yang et al., 2008; Phanturat et al., 2010; Zhong et al., 2011; Elavarasan et al., 2014; Dey and Dora, 2014). Davalos et al. (2004) in their studies, reported that several amino acids, such as Trp, Tyr, and Met, followed by Cys, His, and Phe showed highest antioxidant activity. It is well known that aromatic amino acid residues can easily donate protons to electron-deficient radicals and participate directly in radical scavenging activity. Further, the antioxidant activity of histidine-containing peptides has been reported and attributed to the hydrogen donating, lipid peroxyl radical trapping, and/or metal ion-chelating ability (Rajapakse et al., 2005).

Table 2.3 Antioxidative properties of fish protein hydrolysates

Substrate	Enzymes	Antioxidative properties	Reference
Limanda aspera frame waste	Various enzymes (Mackerel Intestine crude enzyme, Alcalase etc.)	Linoleic acid peroxidation	Jun et al., 2004
Theragra chalcogramma frame waste	Crude proteinase from mackerel intestine	Linoleic acid peroxidation inhibition activity; Hydroxyl radical scavenging	Je et al., 2005
Big head carp muscle	Alcalase	DPPH radical scavenging activity	Li et al., 2006
Johnius belengerii frame	Various enzymes viz., Pepsin, Trypsin etc.	DPPH radical scavenging activity, hydroxyl radical scavenging activity, peroxyl radicalscavenging activity, superoxide radical scavenging activity and lipid peroxidation inhibition activity	Kim et al., 2007
Sardinella aurita head and viscera	Alcalase	DPPH radical scavenging activity and linoleic acid peroxidation	Souissi et al., 2007
Sardinelle heads and viscera	Alcalase Sardine crude enzyme	DPPH radical scavenging activity reducing power assay and linoleic acid autoxidation inhibition activity	Bougatef et al., 2008
Pacific hake mince	Validase and Flavourzyme	DPPH, FRAP, ABTS, ORAC, Metal chelating activity, linoleic acid peroxidation	Samaranayaka and Li-Chan, 2008

Substrate	Enzymes	Antioxidative properties	Reference
Catfish protein isolate	Protamex	metal chelating ability, DPPH radical scavenging ability, FRAP, oxygen radical absorbance capacity (ORAC)	Theodore et al., 2008
Gadus morhua backbones	Protamex	DPPH radical scavenging activity and iron mediated liposomes oxidation reducing activity	Slizyte et al., 2009
Sardinella aurita head and viscera	Alcalase Enzyme extracts from sardinelle, cuttlefish and smooth hound	DPPH radical scavenging activity, Ferric (Fe^{3+}) reducing antioxidant activity, ß-carotene bleaching inhibition activity	Barkia et al., 2010
Sardinella aurita heads and viscera	Alcalase Crude enzyme preparations from *Aspergillus clavatus, B. licheniformis* and viscera of *Sardina pilchardus*	DPPH radical scavenging activity, linoleic acid autoxidation inhibition activity and reducing power activity	Bougatef et al., 2010
Argentine croaker (*Umbrina canosai*)	Flavourzyme, Chymotrypsin and Trypsin	Peroxidation of linoleic acid, hydroxyl radical scavenging, DPPH free radical scavenging, ABTS free radical scavenging and reducing power	Centenaro et al., 2011
Lutjanus vitta muscle	Alcalase, Flavourzyme and Pyloric caeca protease	DPPH radical scavenging activity, ABTS radical scavenging activity, Ferric reducing antioxidant power (FRAP), Ferrous ion chelating activity, ß –carotene linoleic acid emulsion model system and Lecithin liposome model	Khantaphant et al., 2011

Substrate	Enzymes	Antioxidative properties	Reference
Nemipterus hexodon muscle	Skipjack tuna pepsin extract	DPPH radical scavenging activity, ABTS radical scavenging activity and Ferrous ion chelating activity	Nalinanon et al., 2011
Silver carp processing by-products	Alcalase Flavourzyme Neutrase Protamex Papain Pepsin and Trypsin	DPPH radical scavenging activity, hydroxyl radical scavenging activity, Superoxide anion radical scavenging activity and linoleic acid autoxidation inhibition activity	Zhong et al., 2011
Goby muscle proteins	Alcalase	DPPH radical scavenging activity and reducing power assay	Nasri et al., 2012
Nemipterus japonicus muscle	Papain, Pepsin Trypsin	DPPH radical scavenging activity, Ferric (Fe^{3+}) reducing antioxidant power, Fe^{2+} chelating activity and Lipid peroxidation inhibition activity	Naqash and Nazeer, 2012
Striped catfish frame meat	Papain Bromelain	DPPH radical scavenging activity (90%), ferric reducing antioxidant power assay	Tanuja et al., 2012
Salmon	Pepsin	DPPH radical scavenging activity	Girgih et al., 2013
Mackerel backbone	Pepsin Papain	DPPH radical scavenging activity	Sheriff et al., 2013
Red tilapia	Alcalase Thermolysin	DPPH radical scavenging activity, ABTS and Reducing Power	Daud et al., 2015
Spanish Mackerel frame waste	Alcalase, Trypsin, Protemax Flavourzyme	DPPH radical scavenging activity	Kong et al., 2015

Substrate	Enzymes	Antioxidative properties	Reference
Nile tilapia	Alcalase, Flavourzyme Protamex Papain	DPPH radical scavenging activity, ABTS, FRAP and Metal Chelating activity	Yarnpakdee et al., 2015
Nile tilapia by product	Alcalase	Oxygen Radical Absorbance Capacity, FRAP, ABTS	Bernardi et al., 2016
Tuna waste	Bromelain	DPPH radical scavenging activity	Parvathy et al., 2016
Grey triggerfish (*Balistes capriscus*) muscle	Crude enzyme from Zebra Benny, Sardinelle and *Bacillus mojavensis* A21	1,1-diphenyl-2-picrylhydrazyl (DPPH.) radical method, reducing power assay, chelating activity, β-carotene bleaching and DNA nicking assay	Siala et al., 2016
Common carp roe	Alcalase	DPPH scavenging activity, metal ion chelating activity	Ghelichi et al., 2018
Alaska pollock protein isolate	Neutrase	DPPH, superoxide, and hydroxyl free radical-scavenging activities	Liu et al., 2018
Pacific white shrimp *(Litopenaeus vannamei)* cephalothorax	Alcalase	Ferrous ion chelating activity, ABTS radical scavenging activity, ORAC value, ferric reducing antioxidant activity and DPPH radical scavenging activity	Sinthusamran et al., 2018
Tilapia waste (*Oreochromis niloticus*)	Papain	DPPH, FRAP, Reducing Power and Metal chelating activity	Srikanya et al., 2018
Starry triggerfish *(Abalistes stellaris)* muscle	Trypsin	DPPH radical scavenging activity, ABTS radical scavenging activity, FRAP and metal chelating activity	Sripokar et al., 2019

2.9 Applications of fish protein hydrolysates

In addition to the nutritional properties of food proteins, their role as a valuable source of peptides with multifunctional activities is well demonstrated (Dhaval et al., 2016). This nutritional richness of proteins and their hydrolysates is essentially associated with their essential amino acids content while the nature of bioactive peptides determines the biofunctionalities as well as bioactivities (Saadi et al., 2015). Compared to the parent protein, their biopeptides offer a lot of advantages viz., relatively superior bioactivity and a wide spectrum of therapeutic action. Further they are considered to be milder and safer for the consumers with protein hydrolysates and peptides of low molecular weights to be less allergenic than their parent proteins.

Fish protein hydrolysates have potential application as functional ingredients in different foods on account of the numerous important and unique properties that they possess viz., water holding capacity, oil absorption capacity, protein solubility, gelling activity, foaming capacity and emulsification ability (Chalamaiah et al., 2010). Food application of fish protein hydrolysate accounts for nutritional enrichment as well as functional stabilization and studies have been reported in food systems like beverages and snack products (Sinha et al., 2007; Pacheco-Aguilar et al., 2008; Leksrisompong et al., 2012; Ismail and Sahibon, 2018). The water binding capability of hydrolysate results in hydrogen binding with food components facilitating water entrapment and finds suitability in foods like meat, sausages, breads, cakes, boiled foods etc (Ibarra et al., 2013). Incorporation of protein hydrolysate in soups and gravies on account of their viscous behaviour have been reported by Zhang et al. (2013). Similarly the emulsifying property of protein hydrolysate facilitates formation and stabilization of fat emulsions in various products viz., sausages, bologna, soups, cakes, protein spreads and mayonnaise (Sathivel et al., 2005; Cavalheiro et al., 2014; Intarasirisawat et al., 2014).

Incorporation of hydrolysate in various products viz., meats, sausages, doughnuts, spreads, crackers, deep fried products exploring their fat absorption property were reported (Yu and Tan, 1990; He et al., 2015a). The antioxidant property of fish protein hydrolysate has also been well demonstrated in various products viz., fish muscle (Dekkers et al., 2011); fish fillets (Sathivel et al., 2008; Dey and Dora, 2014); fish sausage (Intarasirisawat et al., 2014); hamburgers (Bernardi et al., 2016); fish oil (Morales-Medina et al., 2016); dressed fish (Parvathy et al., 2018c) etc. A study conducted by Cheung et al. (2009) on protein hydrolysates from Pacific hake (*Merluccius productus*) provided strong evidence to support development of FPH as a new generation cryoprotectant to maintain quality of frozen fish. Similar studies were reported by Kittiphattanabawon (2012) in fishery product to reduce protein denaturation. Studies by Yarnpakdee et al. (2012a) have confirmed pre-treatment of raw material to be effective in reducing the total lipid content and hence lipid oxidation and fishy odour and taste. This further facilitated in successful fortification of milk with fish protein hydrolysate at 0.2 % level.

They are also a source of specific amino acids for dietic formulations (Sumaya-Martinez et al., 2005) which are easily absorbed and utilized for various metabolic activities (Nesse et al., 2011). They are used for maintaining the nutritional status of individuals with nutritional or physiological needs that are not provided by conventional foods (Clemente, 2000). Protein hydrolysates are also used in sport nutrition as high-energy supplement to maximize muscle protein anabolism in healthy athletes (Manninen, 2009).

Fish protein hydrolysates (FPHs) have been used in aquaculture feeds in order to enhance the growth performance and immunological status of fish and shell fish (Refstie et al., 2004; Hevroy et al., 2005; Kotzamanis et al.,2007; Hermannsdottir et al., 2009; Nguyen et al., 2012; Silva et al., 2017).FPHs have also

found application as an excellent source of nitrogen for maintaining the growth of different microorganisms (Klompong et al., 2009b; Hsu, 2010). Ghorbel et al. (2005) used various fish protein hydrolysates (FPHs) from sardinelle (*Sardinella aurita*) as nitrogen sources for the production of extracellular lipase by the filamentous fungus *Rhizopus oryzae*. In another study by Safari et al. (2012) the hydrolysates generated from yellowfin tuna (*Thunnus albacares*) head waste was shown to be effective in promoting the growth of lactic acid bacteria better than the commercial MRS media. Studies have also been reported on the antimicrobial property of protein hydrolysate from various seafood sources (Di Bernardini et al., 2011; Ghelichi et al., 2018). Studies have also been reported on the role of fish protein hydrolysates to alter the lipid metabolism in experimental animals revealing their suitability as a cardioprotective nutrient (Wergedahl et al., 2004; Khaled et al., 2012).

Chapter 3

Quality assessment of peptides from white and red meat of yellowfin tuna *(Thunnus albacares)*

3.1 Introduction

Seafood and seafood products are one of the mainly used protein sources for human consumption. This marine biomass is considered to be safe as well as superior food commodity with regard to their nutritional properties especially protein with desirable essential amino acid pattern. However, a major share of this source is not utilized to its best and goes for the production of low-priced by-products such as fish flour, fish oil, animal feed etc. or remains unutilized as waste. This scenario calls for an urgent requirement for efficient solutions for their effective utilization to counter for human nutritional requirement as well as increasing environmental pollution issues (Petrova et al., 2018).

Tuna and tuna products have a widespread consumer demand globally on account of their delicacy and richness in protein and are widely used for the production of thermally processed products. Hence tuna waste constitutes a biomass of particular interest to upgrade on account of this global economic importance and their role in international trade for canning. Tuna processing produces 30 to 35 % products, 20 to 35 % solid waste and 20 to 35 % liquid wastes (Wongsakul et al., 2003). Globally, tuna canning industry is reported to generate to the tune of

around 4,50,000 t of byproducts per annum (Sultanbawa and Aksnes, 2006) which constitutes as much as 70 % of the original material. Of this, tuna red meat accounts for about 12 % of raw tuna used (Guerard et al., 2002). Fish processing discards including tuna wastes are commonly considered as low-value resources with insignificant market demand and are currently used to produce fish oil, fishmeal, fertilizer, pet food and fish silage (Kim and Mendis, 2006). Reports suggested that seafood by products are also valuable sources of compounds such as proteins, lipids, minerals etc and a number of bioactive compounds have been identified from them (Kim and Mendis, 2006).Enzymatic conversion of these sources into protein hydrolysates is an effective utilization method, which has immense application scope in food and pharmaceutical areas (Chalamaiah et al., 2012; He et al., 2013).

Protein hydrolysates are bioactive peptides obtained by the breakdown of proteins by hydrolysis either chemically or enzymatically. This process facilitates recovery of essential nutrients viz., amino acids as well as has immense scope in food, nutraceutical and pharmaceutical industry on account of the excellent physicochemical, functional as well as bioactive properties they possess (He et al., 2013; Halim et al., 2016). Previous studies have indicated variations with respect to the properties in red and white meat of tuna hydrolysate which may be on account of species, seasonal variations etc (Sanchez-Zapata et al., 2011; Parvathy et al., 2018b). The present study was focused on comparative evaluation of the properties of hydrolysates derived from white and red meat of yellowfin tuna (*Thunnus albacares*), a high demand species in the domestic as well as international market. Protein hydrolysate was prepared under similar hydrolytic conditions viz., 60 min hydrolysis time at 60^{o}C and physiological pH (6.5), employing 1 % (w/w) papain to obtain spray dried tuna white meat (TWPH) and tuna red meat protein hydrolysates (TRPH) which were further evaluated for nutritional, functional and antioxidant properties for further suitable food and pharmaceutical applications.

3.2 Materials and methods

3.2.1 Fish, enzyme and chemicals

Fresh yellowfin tuna (*Thunnus albacares*) was procured from local fish landing centre and brought immediately to the laboratory in iced condition (Fig. 3.1). Tuna was cleaned and dressed to separate its white and red meat which was further used as raw material (Fig. 3.2) for the preparation of tuna white meat protein hydrolysate (TWPH) and tuna red meat protein hydrolysate (TRPH), respectively. Papain (Hi Media, India) obtained from papaya latex was employed for carrying out hydrolysis. Analytical grade reagents were used for the whole study.

Fig. 3.1 Yellowfin tuna (*Thunnus albacares*)

Fig. 3.2 Red and white meat of yellowfin tuna

3.2.2 Preparation of tuna protein hydrolysate

Tuna white and red meat were comminuted separately and thoroughly using a blender and to this double the amount of water was added to get fine slurry

of meat. This slurry was further subjected to a high temperature of 80 - 90°C for 30 min for complete arrest of endogenous enzyme activity. This was followed by hydrolysis process using papain as the enzyme, which was performed in a shaking water bath (Shaking bath, Neolab Instruments, Mumbai, India) maintained at 60°C and a physiological pH of 6.5 was adopted. Conditions viz., enzyme:substrate (E/S) ratio and duration of hydrolysis were maintained at 1.0 % and 60 min, respectively based on previous studies conducted. Hydrolysis was terminated by raising the process temperature to 80-90°C for 15-20 min and the resultant solution was course filtered and centrifuged (K-24A, Remi Instruments, Mumbai) at 8000 g at 10°C for 20 min to obtain protein hydrolysate solution (Fig. 3.3) which was further spray dried (Hemaraj Enterprises, Mumbai) for quality evaluation studies.

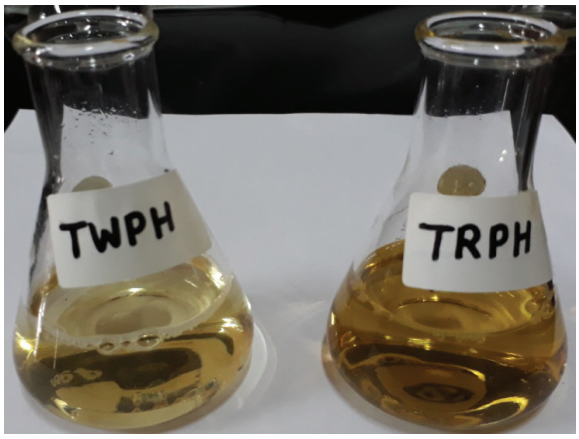


Fig. 3.3 Tuna white meat and red meat protein hydrolysate solution

3.2.3 Protein content and protein recovery

Protein content of tuna meat and hydrolysates were analysed as per AOAC (2012) adopting micro-Kjeldahl method. Known quantity of the sample (1 g tuna meat and 0.1 g tuna protein hydrolysate) was weighed accurately and digested with 10 ml of concentrated sulphuric acid and a pinch of digestion mixture in digestion flask. A few glass beads were added to the digestion flask to avoid bumping. The digestion was continued till the solution became clear and heating was stopped. The digested solution was cooled and made up to a known volume with distilled water.

An aliquot of 2 ml was transferred to Kjeldahl distillation unit with 10 ml of 40 % sodium hydroxide. 10 ml of 2 % boric acid with 2-3 drops of mixed indicator was taken in a conical flask in which liberated ammonia was absorbed. The absorption of ammonia was indicated by change in colour of boric acid with indicator from pink to green. Back washing was followed for two times after distillation of each sample. The boric acid was titrated against N / 50 H_2SO_4 till the pink color was developed. Crude protein (%) of samples was calculated by multiplying the nitrogen value obtained by a factor of 6.25.

The percentage of protein recovery from the fish was defined as the ratio of protein yield obtained from the extraction process to the amount of total protein estimated by Kjeldahl and was calculated as follows:

$$\text{Recovered protein (\%)} = \frac{\text{Protein in hydrolysate sample x volume of hydrolysate}}{\text{Weight of raw material taken x protein in raw material}} \times 100$$

3.2.4 Yield

Yield of fish protein hydrolysate was calculated as the quantity of hydrolysate powder obtained after spray drying from the amount of raw material used for hydrolysis and was expressed as:

$$\text{Yield (\%)} = \frac{\text{Amount of hydrolysate powder obtained}}{\text{Total amount of raw material used}} \times 100$$

3.2.5 Degree of hydrolysis and proteolytic activity

Formal titration method as described by Taylor (1957) was used to determine the α-amino nitrogen content of tuna protein hydrolysate. Aliquot of protein hydrolysate solution (2 ml) was mixed with equi-volume of formaldehyde solution (30 %) and double distilled water. To this mixture, two to three drops of phenolphthalein indicator was added. This solution was titrated using standard sodium hydroxide solution (0.1 N) till the pink color appeared. The alpha amino nitrogen content was calculated as:

$$\alpha \text{ - amino nitrogen} = \frac{14\,(TVs - TVb) \times N \times TV}{SV}$$

where, 14- atomic weight of nitrogen; TVs- Volume of NaOH used for titration in sample; TVb- Volume of NaOH used for titration in blank; N-Normality of NaOH used; TV-Total volume used for titration; SV-Volume of hydrolysate sample taken for titration.

Degree of hydrolysis was determined (Cao et al., 2008) as the percentage of α - amino nitrogen in hydrolysate, to the total nitrogen content in the raw material (AOAC, 2012) as follows:

$$DH\ (\%) = \frac{AAN \times TWH \times 100}{TN \times TWM}$$

Where, AAN is α-amino nitrogen (mg/ml of hydrolysates); TWH: total amount of hydrolysates obtained (ml); TN: total nitrogen content in raw material (mg/g of protein); TWM: total amount of raw material taken.

Proteolytic activity of the sample was projected from the tyrosine content of the protein hydrolysate which measured the extent of hydrolysis under given conditions. Absorbance of a known measure of diluted liquid hydrolysate solution (≈ 50 µl made upto 3 ml with double distilled water) was measured at 280 nm (Lambda 25 UV/Vis, Perkin Elmer Life and Analytical Sciences, Singapore). Standard curve of L-tyrosine in 0.2 M HCl was used to determine the tyrosine content of sample using a concentration ranging from 0.025 mg / ml to 0.2 mg / ml of tyrosine. The analysis was carried out in triplicate and average value was used for plotting. Tyrosine content of unknown sample obtained from standard graph was expressed in µ mole of tyrosine liberated/mg of protein (Gajanan, 2014).

3.2.6 Colour and browning intensity

Hydrolysate solution (1.0 %) was analysed for its colour characteristics viz., L* (the degree of lightness), a* (redness (+)/greenness (-)) and b* (yellowness

(+) or blueness (-)) using Hunter Lab colorimeter (Colorflex EZ 45/0, Hunter Associates Lab inc., Reston, Virginia, USA).

The browning intensity of hydrolysate samples were determined by measuring the absorbance of filtered samples (80 mg/ml) using UV-VIS spectrophotometer (Lambda 25 UV/Vis, Perkin Elmer Life and Analytical Sciences, Singapore) at 420 nm (Elavarasan and Shamasundar, 2016).

3.2.7 Ultraviolet absorption spectra

UV absorption spectra of the diluted hydrolysate samples (2 mg/ml) were determined as per the methodology reported by Elavarasan and Shamasundar (2016). UV-VIS spectrophotometer (Lambda 25 UV/Vis, Perkin Elmer Life and Analytical Sciences, Singapore) under the conditions viz., wavelength range of 200-330 nm and scan speed of 2 nm/sec was employed for the spectra.

3.2.8 Functional properties

3.2.8.1 Protein solubility

Protein solubility of the samples was calculated as per the methodology of Morr et al. (1985). Protein hydrolysate samples (10 mg/ml, 20 ml) were well dispersed in distilled water at ambient temperature for 30 min. This was followed by centrifuging at 7500 g for 15 min (K-24A, Remi Instruments, Mumbai) to collect the supernatant solution. Protein solubility was calculated as percentage of total protein in supernatant to the total protein in sample.

$$\text{Protein Solubility (\%)} = \frac{\text{Total Protein content in Supernatant}}{\text{Total Protein content in Sample}} \times 100$$

3.2.8.2 Foaming properties

The methodology of Sathe and Salunkhe (1981) was adopted for determining the foaming properties of protein solution. A known volume of protein solution (1.0 %) was homogenized (230 VAC T-25 digital Ultra-turrax, IKA, India) at 16,000

rpm for 2 min at ambient temperature and the whipped sample was immediately transferred to a measuring cylinder and the volume read immediately and after three minutes to calculate the properties as:

$$\text{Foaming capacity/stability \%} = \frac{(A-B)}{B} \times 100$$

where A is the volume after whipping to determine foaming capacity; volume after standing (foam stability), B is the volume before whipping.

3.2.8.3 Emulsifying properties

Evaluation of emulsifying properties was done as per the methodology of Pearce and Kinsella (1978). 10 ml vegetable oil and 30 ml of 1 % protein hydrolysate solution were thoroughly homogenized (230 VAC T- 25 digital Ultra-turrax, IKA, India) for 1 min @ 20,000 rpm. An aliquot of the emulsion (50 µl) pipetted from the bottom of the container at 0 and 10 min were mixed with 0.1 % sodium dodecyl sulphate solution (5 ml) to read the absorbance at 500 nm (Lambda 25 UV/Vis, Perkin Elmer Life and Analytical Sciences, Singapore) immediately (A_0) and 10 min (A_{10}) after emulsion formations:

$$\text{EAI(Emulsion Activity Index) } (m^2/g) = \frac{2 \times 2.303 \times A_0}{0.25 \times \text{wt of protein}}$$

$$\text{ESI (Emulsion Stability Index) (min)} = \frac{A_{10} \times \Delta t}{\Delta A}$$

3.2.8.4 Oil absorption capacity

Oil absorption capacity (OAC) of protein samples was determined following the methodology of Shahidi et al. (1995). Hydrolysate sample and sunflower oil, in the ratio of 1: 20 (w/v) was taken in a pre-weighed centrifuge tube and vortexed (Expo Hitech, India) for 30 sec followed by centrifugation (K-24A, Remi Instruments, Mumbai) at 2800 g for 25 min and the supernatant/free oil was decanted. OAC was

denoted as the weight of oil held per gram of the sample (g oil/g sample).

3.2.8.5 Sensory properties

The sample acceptability was assessed as per the methodology by Normah et al. (2013). Rawa porridge, used as carrier for the study, was mixed with 1 % (5:1 (w/v)) hydrolysate solutions and evaluated for attributes viz., color, odour, taste and overall acceptability using nine point hedonic scale (Annexure 1). The sample bitterness was scored using 10 point scale designed with 01 as 'no bitterness' to 10 indicating 'extreme bitterness' (Nilsang et al., 2005). The scale of bitterness was anchored using standard caffeine solution as the reference (Annexure 2).

3.2.9 Antioxidative properties

3.2.9.1 DPPH radical scavenging activity

DPPH radical scavenging activity was determined as proposed by Shimada et al.(1992). A known concentration of protein hydrolysate solution (2.0 mg protein/ ml) was mixed with equal volume of 0.1 mM DPPH dissolved in 95% ethanol. The mixture was further incubated in dark for 30 min. The absorbance of the resultant solution was recorded at 517 nm (Lambda 25 UV/Vis, Perkin Elmer Life and Analytical Sciences, Singapore). Sample solution and ethanol (1:1 v/v) was considered as sample blank and DPPH with distilled water (1:1v/v) was used as control:

$$\text{DPPH (\%)} = \frac{\text{Abs control} - (\text{Abs sample} - \text{Abs sample blank}) \times 100}{\text{Abs control}}$$

3.2.9.2 Reducing power

Reducing power (RP) of sample was assessed following the methodology of Oyaiza (1986). About 0.5 ml of 1 % protein solution mixed with 0.2 M phosphate buffer (pH 6.6) and 1 % potassium ferricyanide (2.5 ml each) was incubated at 50^{o}C for 20 min. Further an aliquot (2.5 ml) of 10 % trichoroacetic acid was added to the

mixture, centrifuged at 3000 rpm (K-24A, Remi Instruments, Mumbai) for 10 min and 2.5 ml of supernatant was mixed with equal volume of distilled water and 0.1 % ferric chloride and absorbance read at 700 nm (Lambda 25 UV/Vis, Perkin Elmer Life and Analytical Sciences, Singapore) (Oyaiza, 1986). Increased absorbance of the reaction mixture indicates increasing reducing power.

3.2.9.3 Ferric reducing antioxidant power

FRAP was assayed according to the method described by Benzie and Strain (1996). FRAP solution was prepared freshly by incubating a premix containing 300 mM acetate buffer (pH 3.6) (25 ml), 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) solution (2.5 ml) in 40 mM HCl and 2.5 ml of 20 mM $FeCl_3$. $6H_2O$ solution at 37 °C for 30 min. 2850 µl of this FRAP solution and 150 µl of 10 mg/ml sample was mixed and incubated in dark for 30 min to form ferrous tripyridyl triazine complex which was measured for the absorbance at 593 nm (Lambda 25 UV/Vis, Perkin Elmer Life and Analytical Sciences, Singapore). Ascorbic Acid standard curve for FRAP was derived to measure the FRAP activity in terms of mM Ascorbic acid equivalents.

3.2.9.4 Metal chelating activity

The chelating activity on Fe2+ was determined, using the method of Decker and Welch (1990). One ml of known concentration of sample solution was mixed with 3.7 ml of distilled water. To this diluted solution, 2 mM $FeCl_2$ (0.1 ml) and 5 mM 3-(2-pyridyl)-5,6- bis(4-phenyl-sulfonic acid)-1,2,4-triazine (ferrozine) (0.2 ml) was added, incubated at ambient conditions for 20 min and the absorbance read at 562 nm (Lambda 25 UV/Vis, Perkin Elmer Life and Analytical Sciences, Singapore). Control was prepared in the same manner with distilled water instead of sample. Metal chelating activity was calculated as:

$$MC\ (\%) = 1 - \frac{\text{(Absorbance of sample)}}{\text{(Absorbance of control)}} \times 100$$

3.2.9.5 ABTS radical (2,20 -azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) scavenging activity

The modified method of Re et al. (1999) was followed to determine ABTS radical scavenging activity of the sample. The stock solution of ABTS radical stock solution consisting of 7 mM ABTS in 2.45 mM potassium persulfate, was pre-incubated in dark for 16 h at ambient temperature. The working solution of ABTS radical with absorbance of 0.70 ± 0.02 at 734 nm was prepared and mixed with 20 µl of sample (10 mg/ml) or distilled water (in control) and the reduction in absorbance was noted (Lambda 25 UV/Vis, Perkin Elmer Life and Analytical Sciences, Singapore) after incubation at 37^{o}C for 10 min in dark.

$$\text{ABTS radical scavenging activity} = \left(1 - \frac{\text{Abs sample}}{\text{Abs control}}\right) \times 100$$

3.2.10 Statistical interpretation

All analysis done in triplicate was subjected to analysis of variance (ANOVA) and the differences between means were evaluated by Duncan's multiple range test. SPSS statistic programme (SPSS 16.0 for Windows, SPSS Inc., Chicago, IL,) was used for data interpretation.

3.3 Results and discussion

3.3.1 Protein content and protein recovery

Assessment regarding the nutrient intake from food consumption requires reliable data on the food composition. Knowledge regarding the fundamentals of food-based dietary guidelines containing the essential information on food sources for different nutrients is essential for healthy nutrition. Furthermore, food composition tables can provide information on chemical forms of nutrients and the presence and amounts of interacting components, and thus provide information on their bioavailability. Further knowledge regarding the nutritional status of food helps in streamlining the processing possibilities as well as for exploring their utility for various applications in food and pharmaceutical sectors. Fish by-products like tuna red meat have nutritional composition comparable to that of the main product viz., white meat which facilitates its diversion to high value end products with enormous application potentials (Herpandi et al., 2011). Tuna is known for its richness in protein and is regarded as one of the richest source of seafood protein. The protein content in tuna white meat and red meat of yellowfin tuna revealed a value of 25.99 ± 0.24 % and 24.03±0.11 %, respectively which got concentrated to 84.4 ± 2.35 % and 85.87 ± 0.91 %, on conversion to their respective protein hydrolysates viz., TWPH and TRPH (Fig. 3.4). Results indicated comparable protein content between white and red meat of yellowfin tuna. Sanchez-Zapata et al. (2011) in their studies reported a protein content of 26.92 ± 0.27 % for yellowfin tuna dark meat. During enzymatic hydrolysis, parent protein is subjected to breakage, facilitating their selective extraction by proper solubilization to yield higher protein content in the derived hydrolysate. Various studies on hydrolysate derived from different seafood substrates, revealed a protein content ranging from 60 - 90 % of total composition (Choi et al., 2009; Khantaphant et al., 2011). The high protein content in fish protein

hydrolysates explores its potential use as protein supplements for human nutrition as well as offers numerous technological applications due to their intrinsic functional properties.

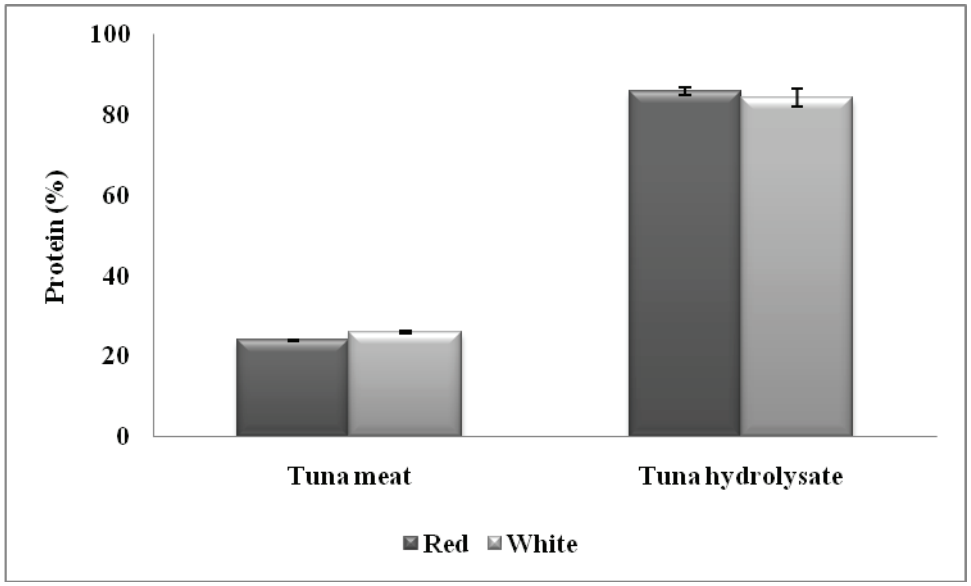


Fig. 3.4 Protein content of white meat and red meat of yellowfin tuna and their respective hydrolysates

A major determinant factor that determines the hydrolysis efficiency is the recovery of protein from the parent substrate to its final hydrolysate. The present study indicated a recovery of 50.34 % and 44.67 %, respectively in TWPH and TRPH from their parent substrates on hydrolysis (Fig. 3.5). Higher protein recovery was observed from tuna white meat than their counterparts, to their respective hydrolysates. He et al. (2013) has reported that the extent of hydrolysis, influenced by factors like protein substrate used, type and amount of enzymes used, hydrolysis period etc, to have a major influence on the recovery of protein. Studies have indicated higher protein content in hydrolysates that have undergone greater extent of hydrolysis. During hydrolysis, more breakage of peptide bonds occur which facilitate the release of low molecular weight protein hydrolysates which are more water soluble thereby increasing the protein content in the resultant hydrolysate solution. Serial enzymatic hydrolysis of parent protein is also an effective method by which the protein present in the substrate is well extracted (Binsi et al., 2016).

3.3.2 Yield

The sustainability as well as economic viability of a process is determined by the product yield obtained during the process. Several factors influence this variable which includes degree of hydrolysis, drying method adopted etc. The present study revealed a comparable yield of 6.1 and 6.0 %, for spray dried TWPH and TRPH, respectively from their solution (Fig. 3.5). However the values indicated a lower yield which matches with the general yield reports for spray-dried hydrolysates. This is due to the fact that only the soluble fractions were dried and also on account of the solid losses that occured in the apparatus during drying operation. Drying operations carried out under batch mode generally offer a lower yield while higher operations on continuous mode are reported to give better yield. The current drying operation was also done on a small scale batch mode, which must have resulted in lower yield. Reports have indicated a yield of about 6.6 % for freeze dried herring fish protein hydrolysate (Liceaga-Gesualdo and Li-Chan, 1999). Gajanan et al. (2016) reported a hydrolysate yield ranging from 4.6 to 9.7 % from threadfin bream frame waste for degree of hydrolysis ranging from 5 -15 %. In general, reports suggested a yield ranging between 10-15 % based on the substrate used as well as hydrolytic conditions and further drying methods adopted (Quaglia and Orban, 1990).

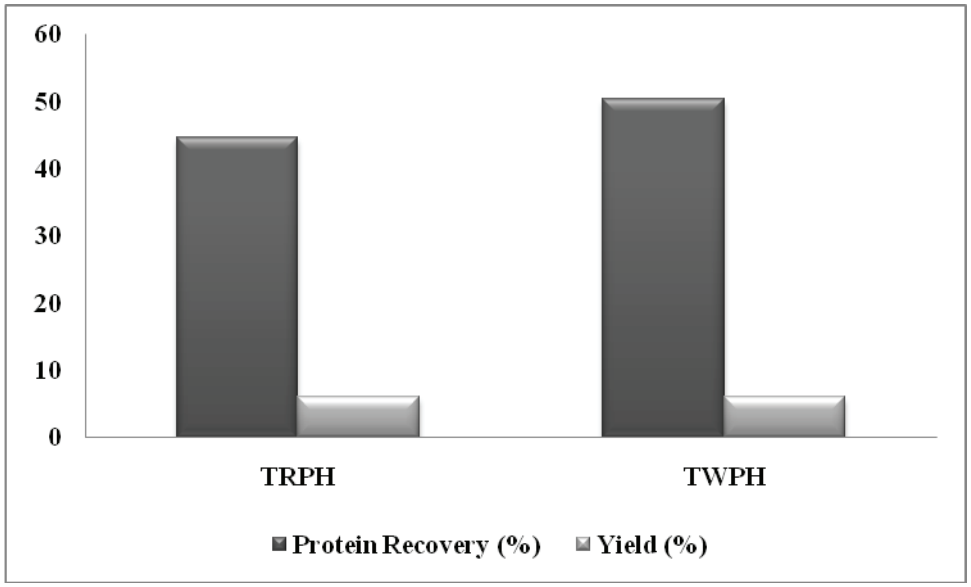


Fig. 3.5 Protein recovery and yield of white meat and red meat hydrolysates from yellowfin tuna

3.3.3 Degree of hydrolysis and proteolytic activity

Determination of degree of hydrolysis (DH) is one of the most viable method followed to evaluate the extent of hydrolysis undergone by a protein substrate. This in turn is evidently one of the most significant variable which influences the other attributes viz., functional and bioactive ones exhibited by the protein hydrolysates (Himonides et al., 2011). Degree of hydrolysis is influenced by various factors like protein source, enzyme used, hydrolytic conditions viz., type of enzyme, enzyme-substrate ratio, hydrolysis time etc. In the present study similar process conditions were adopted, except for variations in substrate used for tuna protein hydrolysis. Results indicated a DH of 15.48 ± 0.17 % and 15.69 ± 0.21 % for TWPH and TRPH, respectively after 60 min hydrolysis time, which was not significantly different. The amount of tyrosine liberated during hydrolysis on account of the peptide breakage is indicative of the extent of hydrolysis and it was observed that the proteolytic activity was in well proportion with the DH values indicating 0.274 ± 0.011 and 0.274 ± 0.007 for TWPH and TRPH, respectively. Gajanan (2014) in her studies

reported the proteolyic activity to be increasing with hydrolysis in threadfin bream frame waste hydrolysate. Similar observations were made by Elavarasan (2014) in catla protein hydrolysate where a progressive increase in the tyrosine liberated was observed with increase in time of hydrolysis.

3.3.4 Colour and browning intensity

Among the sensory attributes, colour and appearance of a product seems to be the preliminary aesthetic attributes which have an influencial role in determining the consumer acceptability. These variables are in turn influenced by other factors including substrate type, hydrolysis conditions and the drying methods adopted etc. The L*, a*, b* value of tuna white meat were 37.54 ± 0.11, 9.71 ± 0.16, 16.85 ± 0.37 and for red meat 22.46 ± 0.25, 10.37 ± 0.05, 14.82 ± 0.09, respectively (Fig. 3.6) indicating significant difference ($p < 0.05$). The current work indicated that TRPH was darker in comparison to their counterpart which was mainly on account of the raw material compositional variations. These variations ($p < 0.05$) were noticeable in the derived hydrolysate solutions too with L, a*, b* values of 2.58 ± 0.05, -0.1 ± 0.04, -0.03 ± 0.01, respectively for TWPH (20 ml). Similarly TRPH indicated a value of 2.69 ± 0.06, 0.83 ± 0.03 and 2.14 ± 0.15, respectively on instrumental analysis (Fig. 3.7). Parvathy et al. (2016) reported these colour variations in hydrolysate being due to the compositional variation of the raw material used. Similar to the colour values which indicated a higher redness and yellowness in TRPH solutions, browning intensity values also substantiated a higher value of 0.35 ± 0.12 for hydrolysate derived from tuna red meat ($p < 0.05$) than their counterpart which exhibited a value of 0.115 ± 0.001. As recommended by Jemil et al. (2014), the presence of pigments like myoglobin and melanin in the red meat in comparison to white meat and their oxidation must have resulted in this variation in the derived hydrolysates.

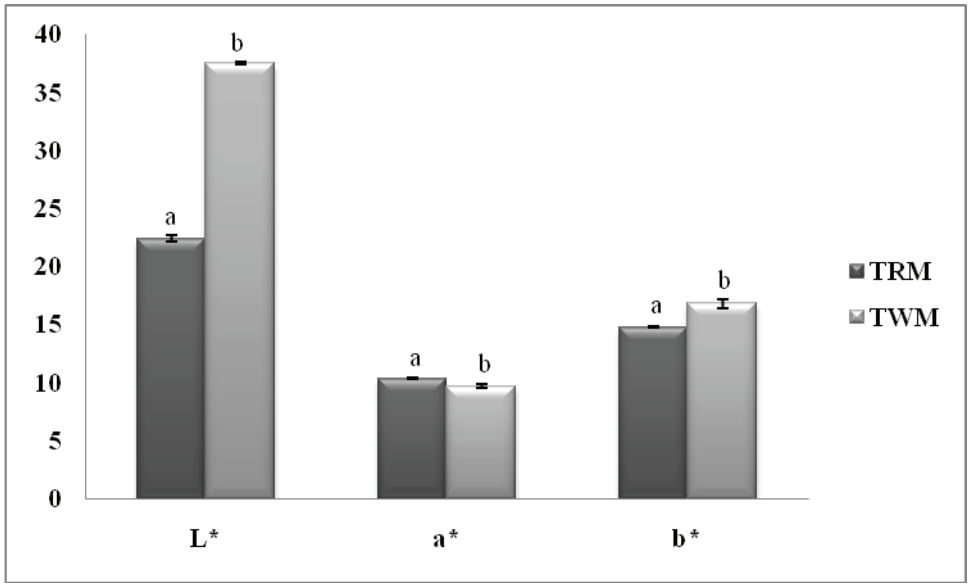


Fig. 3.6 Colour characteristics of white meat and red meat of yellowfin tuna

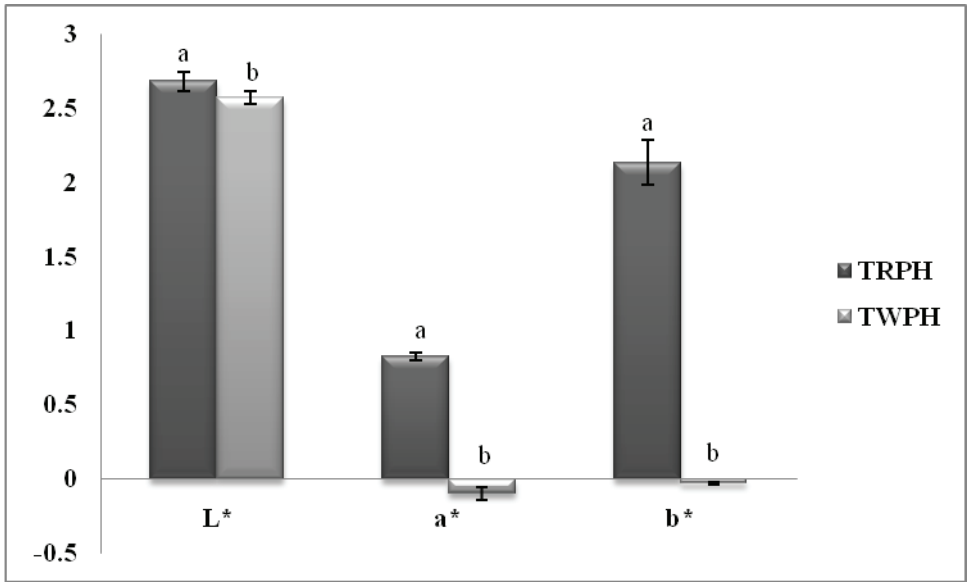


Fig. 3.7 Colour characteristics of white meat and red meat hydrolysates from yellowfin tuna

3.3.5 UV absorption spectra

UV spectrophotometry is a potential technique that helps in the qualitative analysis of protein hydrolysates. During enzymatic hydrolysis of protein molecules, the peptide bond gets cleaved thus producing a more prominent effect on the hydrolysate spectra. Silvestre (1997) have reported this method to be efficient in

identifying some variations in hydrolysis with respect to the employment of trypsin and pancreatin. The absorption spectra of tuna protein hydrolysates in the UV region (Fig. 3.8) indicated nearly identical absorption pattern with maximum absorbance at 200 - 230 nm. A rapid decrease in the absorption was observed from 230 to 240 nm which was further followed by a gradual decrease in wavelength ranging from 240-300 nm. Reports suggest peptides to absorb wavelength in spectra ranging from 180-230 nm and aromatic side chains of tyrosine, tryptophan and phenyl alanine to have absorption affinity in range of 270 - 280 nm (Elavarasan and Shamasundar, 2016). The present study indicated absence of aromatic side chains in the derived peptides. Further there were only slight variations in the spectral properties of the hydrolysate with tuna white meat hydrolysate indicating a comparatively higher UV absorption pattern in the wavelength range of 230 - 280 nm than red meat hydrolysate which must be on account of the raw material compositional variations.

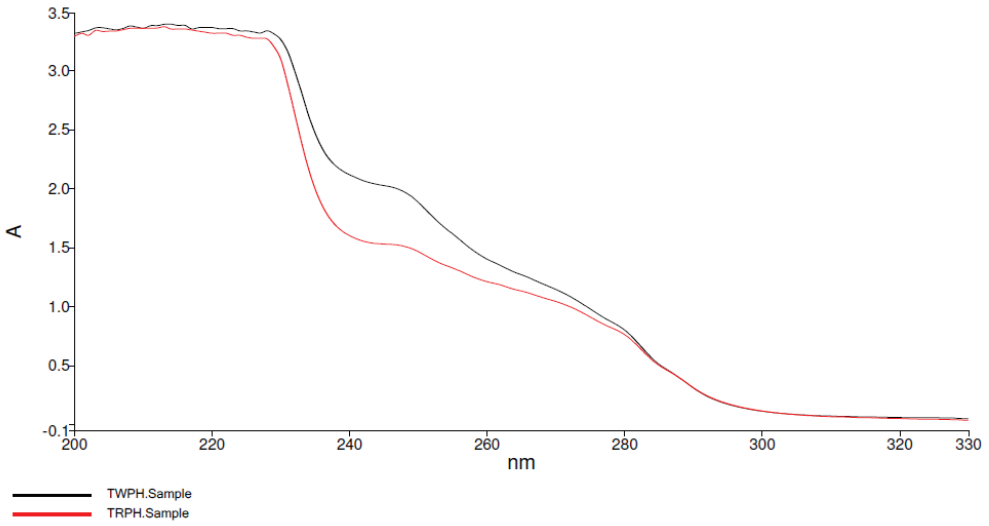


Fig. 3.8 Ultraviolet absorption spectra of protein hydrolysate from white and red meat of yellowfin tuna

3.3.6 Functional properties

3.3.6.1 Protein solubility

Solubility is regarded as the most significant and generally the first physico-chemical property considered for the development of innovative protein ingredients as it influences the other functional properties exhibited by the product (Jemil et al., 2014). Protein solubility is often referred to as functional property or simply functionality. It is the amount of total protein that enters the solution, but does not sediment due to centrifugation. High solubility of fish protein hydrolysates is often due to cleavage of proteins resulting in low molecular weight peptides with increased number of polar groups available for hydrogen bonding with water dipoles. Proper balance between hydrophilic and hydrophobic elements in the peptides is also a factor influencing solubility. The present study revealed a fairly high protein solubility of 86.53 ± 0.73 % and 88.74 ± 0.53 %, respectively for TWPH and TRPH (Fig. 3.9). Geirsdottir et al. (2011) observed a drastic increase in the solubility pattern, indicating a solubility of 15 % for unhydrolysed fish protein to 70 % in hydrolysed fish protein from Blue whiting. Enzymatic hydrolysis of proteins facilitate an increased exposure of hydrophilic polar groups thereby releasing more water soluble peptides into the solution which facilitate increased solubility in comparison to the intact protein.

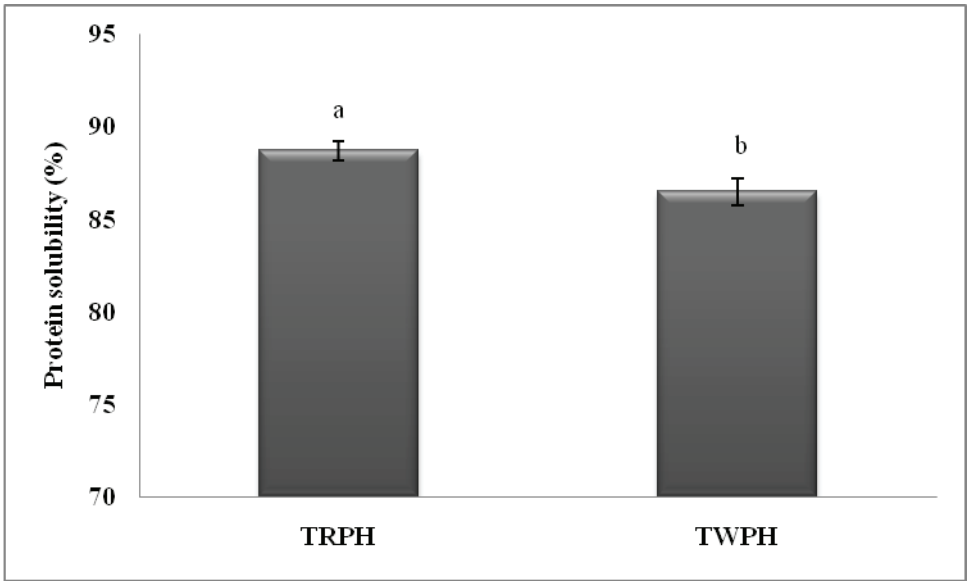


Fig. 3.9 Protein solubility of white meat and red meat hydrolysates from yellowfin tuna

3.3.6.2 Foaming properties

Proteins in dispersions cause a lowering of the surface tension at the water/air interface, thus creating foam. Foaming is of great interest in the food industry as it provides desirable and unique texture to a range of aerated foods and beverages. Similarly, like foaming its stability is also of importance for consumer acceptability as perception of quality is highly influenced by its appearance (McCarthy et al., 2013). Protein hydrolysis results in the exposure of more of hydrophobic residues facilitating enhanced foaming properties. Foaming properties viz., foaming capacity and foam stability of tuna protein hydrolysates were determined (Fig. 3.10). Foaming capacity of TWPH and TRPH were 126.7 ± 5.8 % and 150 ± 10 %, respectively indicating significant difference ($p < 0.05$) whereas comparable foaming stability after 3 minutes was observed, being 40 ± 10 % and 36.7 ± 5.8 %, respectively. The properties exhibited by the hydrolysate can't be explained with respect to the extent of hydrolysis as the raw materials used were different viz., tuna red and white meat,

which also have influence on the resultant hydrolysate functionalities. Sanchez-Zapata et al. (2011) have reported variations to occur in functionality on account of variations in the raw material composition, especially the type of protein used for hydrolysate preparation.

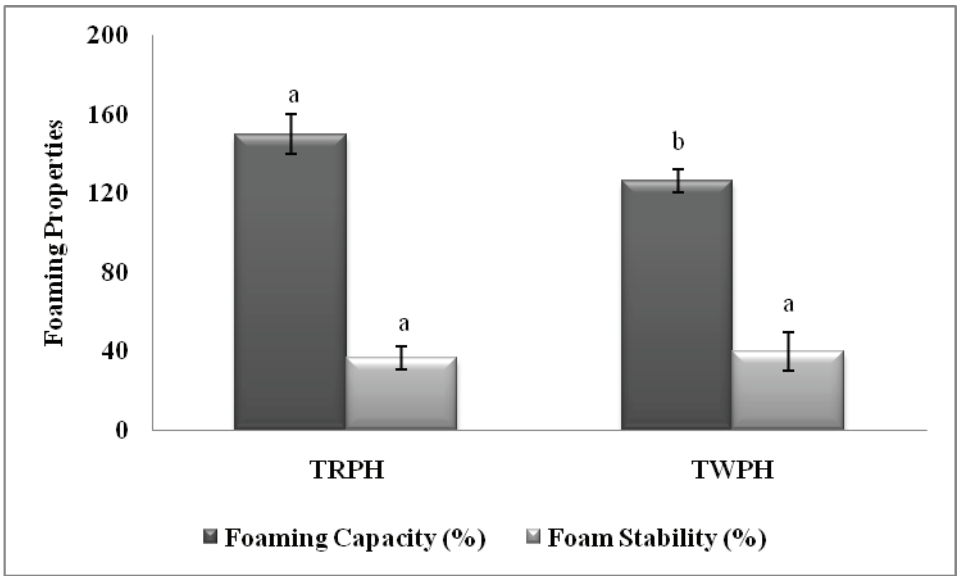


Fig. 3.10 Foaming properties of white meat and red meat hydrolysates from yellowfin tuna

3.3.6.3 Emulsifying properties

The capability of proteins to interact with lipids and form stable emulsions is essential to yield a stable food product. Emulsifying properties of hydrolysed proteins are directly related to surface properties with influence on the degree of hydrolysis which effectively reduces interfacial tension between hydrophilic as well as hydrophobic components in food system (dos Santos et al., 2011) Emulsifying properties (Fig. 3.11) viz., emulsifying activity index and emulsion stability index were observed to be 13.85 ±0.36 m^2/g and 31.39 ± 0.32 min, respectively for TWPH and 15.04 ± 0.36 m^2/g and 38.71 ± 2.51 min, respectively for TRPH. Results indicated a significantly higher ($p < 0.05$) emulsifying properties for TRPH compared to

TWPH. Taheri et al. (2013) in their study reported the peptide sequence as well as its amphiphilic nature to be important factors influencing emulsion properties than peptide length or extent of hydrolysis. Hence though similar range of DH was observed for the hydrolysate samples in the present study, the properties exhibited by them varied. Jemil et al. (2014) reported an EAI in the range of 21.31 - 47.58 m^2/g and an ESI ranging from 22.64 – 47.75 min in hydrolysates derived from different sources viz., Sardinella, Zebra blenny, Goby and Ray muscle. Generally reports suggest that limited hydrolysis improves the emulsification properties of proteins by exposing hydrophobic amino acid residues which interact with oil, while the hydrophilic residues interact with water (Mccarthy et al., 2013).

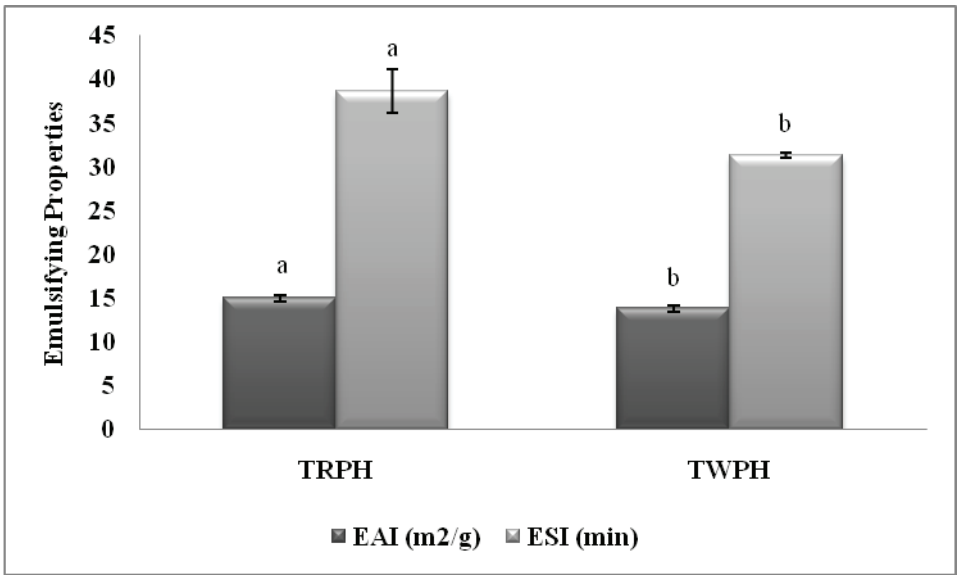


Fig. 3.11 Emulsifying properties of white meat and red meat hydrolysates from yellowfin tuna

3.3.6.4 Oil absorption capacity

The ability of peptides to bind fat influences the palatability of food product and thereby its applicability in food Industry (Tanuja et al., 2012). The present study indicated an OAC of 1.49 ± 0.03 g/g and 1.35 ± 0.02 g/g, respectively for

TWPH and TRPH ($p < 0.05$) (Fig. 3.12). Oil absorption mechanism is a combined attribute of physical entrapment of oil together with sample hydrophobicity and reports suggest excessive hydrolyzation to compromise for the molecule's structural integrity resulting in its degradation and resultant capacity to entrap oil (He et al., 2013). Present study revealed fairly good OAC for the hydrolysates on account of the limited hydrolysis. Authors like Foh et al. (2011) and Geirsdottir et al. (2011) reported comparatively superior oil binding capacity for hydrolysates from species like tilapia and blue whiting, to that of commercial food-grade oil binders proving their potential to be utilized as commercial oil binders in food industry.

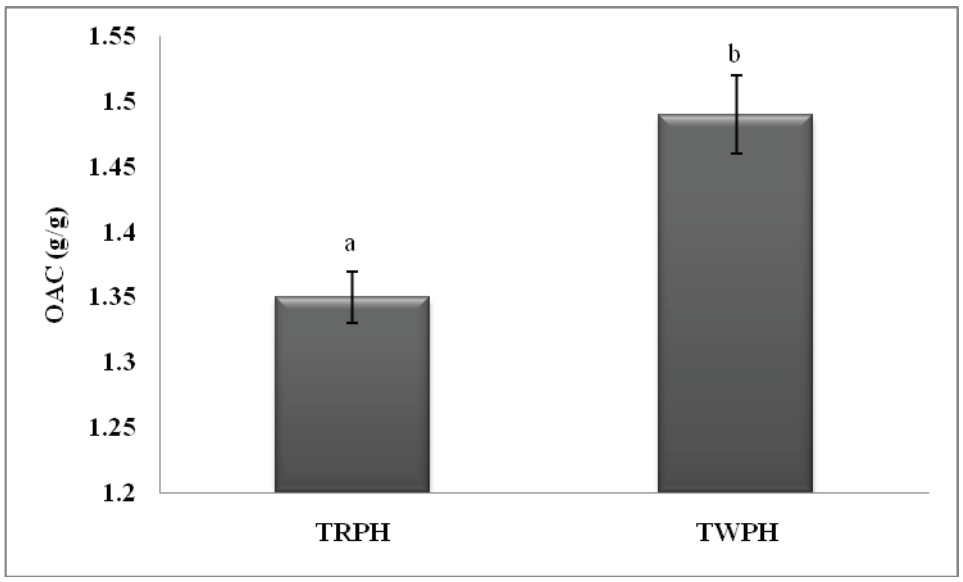


Fig. 3.12 Oil absorption capacity of white meat and red meat hydrolysates from yellowfin tuna

3.3.6.5 Sensory properties

The degree of bitterness that develops during hydrolysis is associated with the level of hydrophobic amino acids and the release of bitter tasting peptides (Nilsang et al., 2005). Factors viz., type of substrate, nature of enzymes as well as the hydrolysis conditions play effective roles in determining the final physico-chemical properties of hydrolysate especially sensory acceptability with regard to

the bitterness generated (Normah et al., 2013). Effective application of a product in food system demands a combination of enhanced functionality along with sensory acceptability. In the current study, the samples exhibited very slight bitterness (2.3 ± 0.5) in TRPH while hardly any bitterness (1.5 ± 0.7) was observed in TWPH when incorporated in porridge at 0.2 %. Similarly the acceptability studies indicated a sensory score of 6.7 ± 0.5 for TRPH whereas it was 7.4 ± 0.5 for TWPH ($p < 0.05$) (Fig. 3.13). This variation in the sensory properties may be on account of the variation in nature of substrate used for the study. However the observations indicates its suitability to be incorporated in food system.

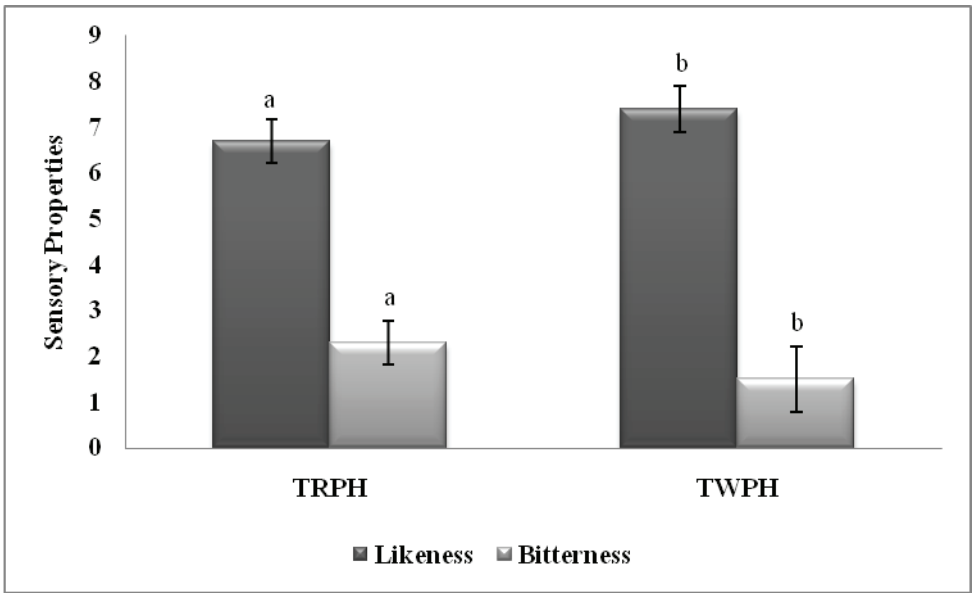


Fig. 3.13 Sensory properties of white meat and red meat hydrolysates from yellowfin tuna

3.3.7 Antioxidative properties

3.3.7.1 DPPH radical scavenging activity

Numerous methods are used to evaluate antioxidant activities of natural compounds in foods or biological systems. Among them one of the most common free radical method employed to assess antioxidant activity in vitro is the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method. The ability

of fish peptides in exerting potent antioxidative activities in different oxidative systems is well reported (Rajapakse et al., 2005). Hydrolysis of protein results in its structure unfolding to facilitate the exposure of more of hydrophobic amino acids which in turn leads to improved antioxidative activity compared to the intact protein (Sarmadi and Ismail, 2010). Depending on the assay system, an antioxidant may exhibit variations in their potential based on the antioxidative mechanism being measured as well as the reaction conditions used in the various assays (Najafian and Babji, 2012). Hence in the present study different antioxidant assays were carried out to comparatively analyse the properties exhibited by the tuna hydrolysates. DPPH radical-scavenging activity determines the hydrogen-donating ability of protein hydrolysates which assists in breaking of the radical chain reaction (Yarnpakdee et al., 2015). The comparison of this antioxidant assay in 0.2% solutions of TWPH and TRPH indicated a significant difference ($p < 0.05$) revealing higher potential for TWPH than TRPH (Fig. 3.14). Yarnpakdee et al. (2015) observed differences in the DPPH radical scavenging activity in Nile tilapia hydrolysate to be associated with the extent of hydrolysis with an increase upto 30 % beyond which it decreased. However the better antioxidant potential of TWPH in comparison to TRPH in the present study can't be related to the extent of hydrolysis undergone by the sample. Similar reports were suggested by Slizyte et al. (2016) mentioning that degree of hydrolysis and size of produced peptides can not alone predict the DPPH scavenging ability.

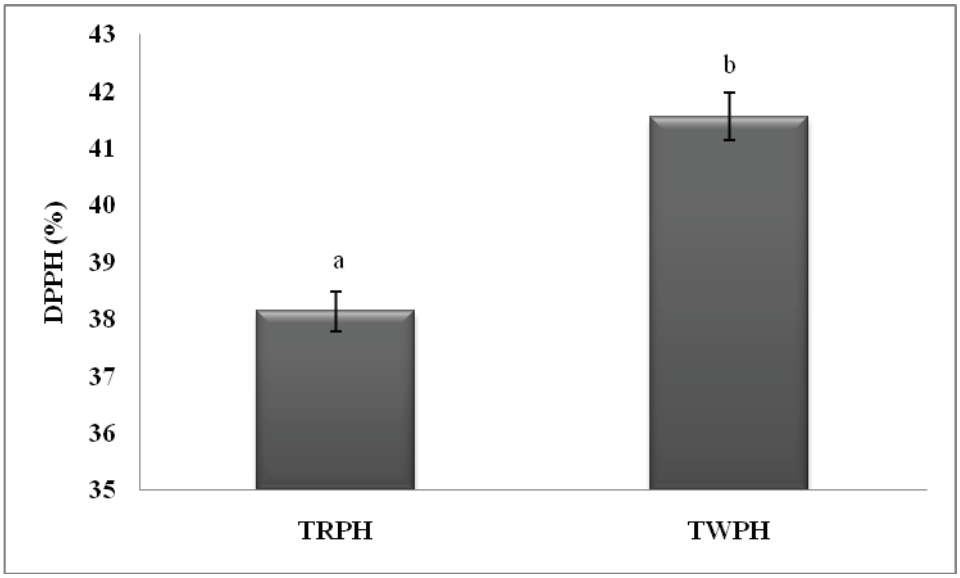


Fig. 3.14 DPPH radical scavenging activity of white meat and red meat hydrolysates from yellowfin tuna

3.3.7.2 Reducing power and Ferric reducing antioxidant power (FRAP)

Protein hydrolysates possess the ability to donate electron/hydrogen and free radicals facilitating oxidation stable substances by interrupting the free radical chain reactions or prevent their formation (You et al., 2010a). The present study reported a reducing power of 0.470 ± 0.011 and 0.341 ± 0.016, respectively for TWPH and TRPH with significant difference ($p < 0.05$) (Fig. 3.15). However the FRAP of the samples were comparable indicating a value of 33.09 ± 0.49 for TWPH and 32.92 ± 0.38 for TRPH (Fig. 3.16). Choonpicharn et al. (2015) have reported good FRAP activity in hydrolysates from tilapia skin. Bougatef et al. (2010) suggested antioxidative properties of fish peptides to be related to their sequence, composition as well as hydrophobicity. Therefore though the DH remained comparable between the hydrolysates, the different pattern of peptides derived from them must have resulted in diverse reducing activity.

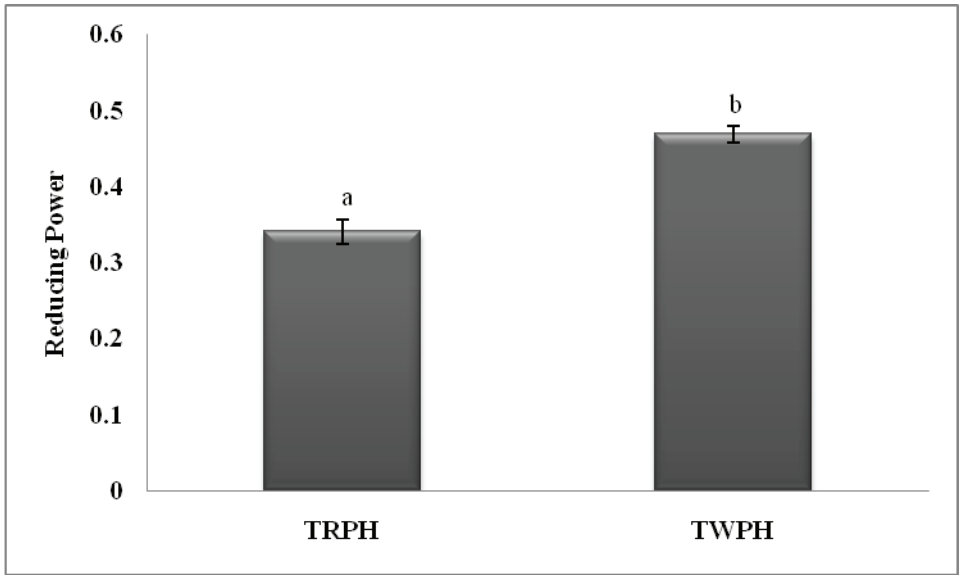


Fig. 3.15 Reducing power of white meat and red meat hydrolysates from yellowfin tuna

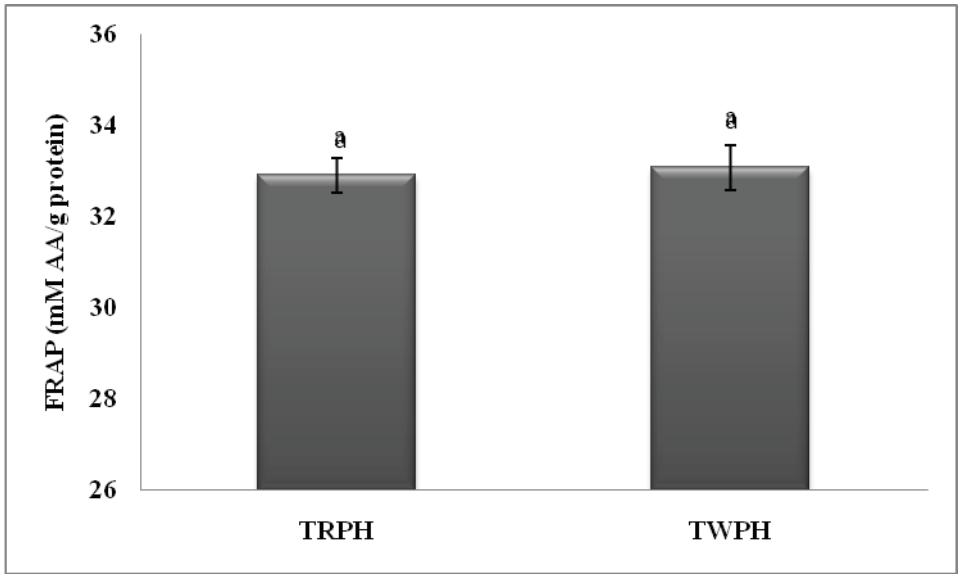


Fig. 3.16 FRAP of white meat and red meat hydrolysates from yellowfin tuna

3.3.7.3 Metal chelating ability

Different mechanisms of actions are adopted by antioxidant peptides to terminate free radical scavenging activity of which sequestration of prooxidative metals facilitate effective retardation of oxidation (Yarnpakde et al., 2015).

Antioxidants chelate the transition metal ions in foods thereby retarding the oxidation reaction by disturbing the autoxidation rate as well as collapse of hydroperoxide to volatile compounds. The present study revealed a significantly ($p < 0.05$) higher metal chelating activity of 16.53 ± 0.96 % for TWPH whereas it was 4.73 ± 0.58 % for TRPH (Fig. 3.17). Similar to the present study, Tanuja et al. (2012) reported a lower metal chelating activity (< 20 %) for papain and bromelain derived hydrolysates from frame meat of striped cat fish.

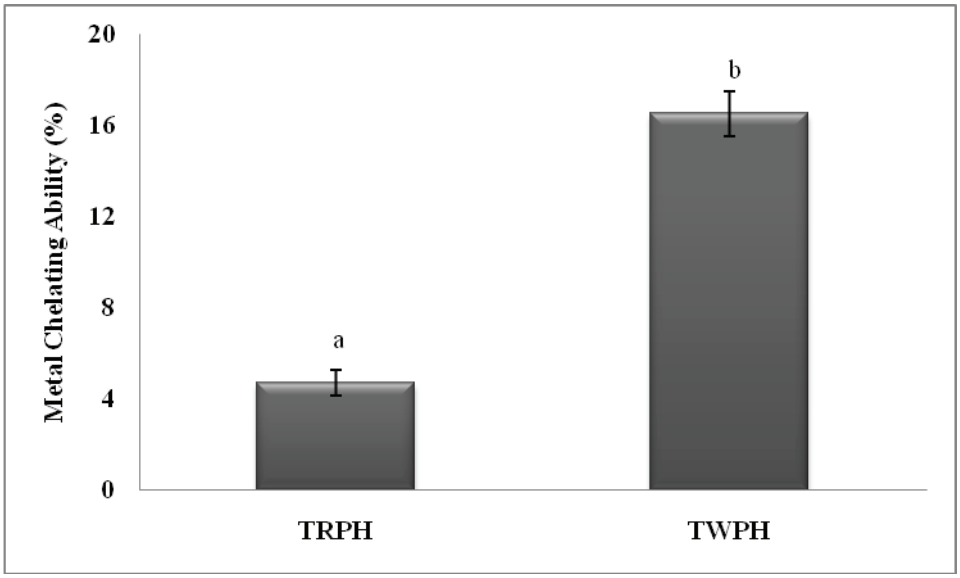


Fig. 3.17 Metal chelating ability of white meat and red meat hydrolysates from yellowfin tuna

3.3.7.4 ABTS radical scavenging activity

The ABTS assay measures the relative ability of antioxidants to scavenge the ABTS generated in aqueous phase, as compared with a standard. This method is rapid and can be used over a wide range of pH values, in both aqueous and organic solvent systems. Further on account of its good repeatability and simplicity, it is widely reported (Ratnavathi and Komala, 2016). In the present study, TWPH and TRPH reported significantly different ($p < 0.05$) ABTS values of 64.57 ± 0.16 %

and 56.83 ± 0.35 %, respectively (Fig. 3.18). Earlier studies conducted by Bernardi et al. (2016) in hydrolysates from Nile tilapia by-products also indicated superior ABTS activity. The amino acid constituents and the sequence of the peptides are determinant factors for their antioxidant activity which are dependent on substrate type, selection of appropriate proteolytic enzymes, the physico-chemical conditions of hydrolysis etc (Samaranayaka and Li-chan, 2011). Enzymes like papain exhibits specific substrate preferences, primarily for bulky hydrophobic or aromatic residues which must have resulted in variations in the nature of peptides formed as well as resultant properties (Tavano, 2013).

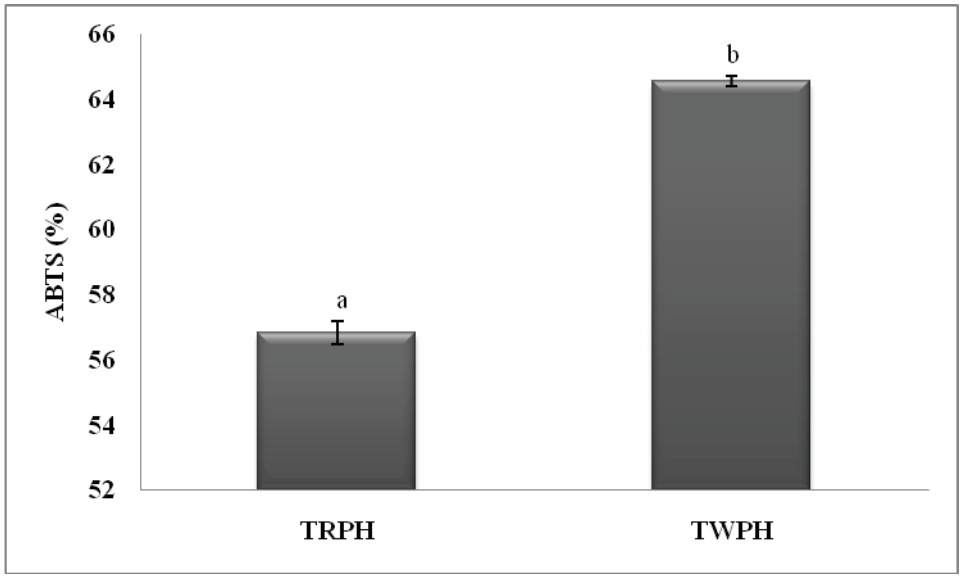


Fig. 3.18 ABTS radical scavenging activity of white meat and red meat hydrolysates from yellowfin tuna

3.4 Conclusion

Tuna red meat, generally discarded as a by-product in canning industry is potential source of high quality proteins which can be effectively utilized for the conversion to economically demanding hydrolysate which has immense application potential in food and pharmaceutical industry. The present study revealed its application potential by comparison of the hydrolysates from red meat with a reference source like tuna white meat. The extent of hydrolysis undergone was similar but the functional and bioactive properties indicated variations as they were dependent mainly on the nature of peptides formed during hydrolysis rather than the peptide chain length. The current investigation paves possibilities for further exploration of this protein rich substrate for its application.

Chapter 4

Process optimisation for the selective extraction of functional and antioxidative hydrolysates from cooked tuna red meat (*Thunnus albacares*) using RSM

4.1 Introduction

Tuna resources, which includes tuna (*Thunnus* spp.) as well as tuna-like species are significant sources of food and hence play a vital role in the economy of many countries. More than 48 species are distributed globally in the Atlantic, Indian, Pacific Oceans as well as the Mediterranean Sea. The annual global production of tuna has undergone a marked increase from less than 0.6 million metric tons in 1950 to nearly 5 million metric tons of major commercial tuna catch by 2016. Globally, tuna resources have high commercial value on account of its demand for thermally processed delicacies. Canned as well as other shelf-stable tuna products provide ample and inexpensive protein to markets around the world, while smaller amounts of high-quality tuna steaks and sashimi make their way to well off markets in Asia, Europe, and North America. As previously mentioned, during tuna canning process, only about one-third of the whole fish is used and the process discards generated during these operations including the red meat is enormous and accounts to about 50 to 70 % of whole fish (Saidi et al., 2014).

Recovery and conversion of these proteinaceous discards to functional and bioactive hydrolysates is a promisive option for the industry. These hydrolysates containing peptides with distinct range of molecular weight, exhibit superior functional and bioactive properties compared to that of parent protein (He et al., 2013; Binsi et al., 2016). Based on the extent of hydrolysis that the parent protein undergoes, the properties exhibited by the hydrolysates vary considerably.

Response Surface Methodology (RSM) is regarded as a constructive statistical method widely adopted for the investigation of complex processes. It helps to define effect of the independent variables, alone or in combinations, on the process to generate an accurate mathematical model that explains the overall process adopting a significant estimation (Shankar et al., 2010). Reports by Wu et al. (2007) also suggested this statistical technique to be significantly advantageous in reducing the number of experiments required to assess multiple variables and their interactions; thus being less laborious and time-consuming than other approaches.

Numerous optimization studies have been carried out globally by researchers in hydrolysates from different sources for maximizing the protein recovery (Awuor et al., 2017) antioxidative activity (Guerard et al., 2007; Wangtueai et al., 2016; Wang et al., 2017) and functional properties simultaneously with yield (Jamil et al., 2016) as process responses using RSM. In these protocols, antioxidant or functional attributes were considered as response variables to generate process conditions for deriving hydrolysates optimized for antioxidant or functional properties alone. However, it is well understood that the properties of hydrolysates depend to a large extent on the nature of polypeptide fragments formed, rather than the DH achieved during the hydrolytic process. It is quite obvious that peptides from the same source having the same DH exhibit significant variations in their properties. The extent to which these properties may alter is less explored, so far. Many a times, a combined optimisation for entirely different spectrum of properties such

as bioactive, surface-active and functional properties may be of less significance, when the hydrolysate is intended for a specific application. This essentially means that, separate optimisation designs are required for extracting surface-active and bioactive hydrolysates, considering the process responses specific to the intended property, so as to arrive at more accurate and technically viable parameters for the particular hydrolysis process.

At present, the red meat generated during tuna canning operations is mainly converted to low value by-products like animal feed, fertilizers etc. As mentioned earlier, numerous studies have been carried out for arriving at an optimum processing condition giving hydrolysates exhibiting either functional or bioactive properties. However no comprehensive studies have been reported offering separate sets of optimised process parameters for the same substrate yielding hydrolysates with either functional or antioxidant properties. Hence, novelty of the current analysis lies in offering separate sets of process conditions to derive hydrolysates with functional or antioxidant properties from tuna red meat, a tuna cannery by-product with emphasis to maximum protein recovery. Hence, the objective was to optimize the effect of hydrolysis variables viz., E/S and hydrolysis duration, using an RSM based central composite design, for separate extraction of surface-active and antioxidant rich hydrolysates from the cannery waste; cooked meat of yellowfin tuna red meat with thrust to maximum protein recovery. As food application demands sensory acceptability, for optimization of peptides with functional properties, minimization of bitterness was also emphasized. Moreover, a variability range of DH values with respect to individual properties exhibited by hydrolysates under each hydrolytic conditions was derived by statistical means.

4.2 Materials and methods

4.2.1 Raw material and chemicals

Tuna red meat was collected as by-product after canning and retorting from Forstar Frozen Foods Pvt. Ltd., Taloja, Navi Mumbai, which had undergone prior heat treatment at 121 ^{o}C for 1 hr (Fig. 4.1). It was initially washed with boiled water (1:4 (w/v)) for five minutes, pressed and further subjected to washing with cold 0.2 % (w/v) sodium bicarbonate solution (1:4 (w/v)) for two min and pressed to remove excess moisture. This washed meat was used as the starting material for the preparation of protein hydrolysates. Papain enzyme (Hi Media, India) from papaya latex was used for hydrolysis. All other chemicals used for the study were of analytical grade.

Fig. 4.1 Tuna red meat

4.2.2 Preparation of protein hydrolysate

The washed red meat was comminuted thoroughly using an electric blender, weighed and added with twice the amount of water for each run. A preliminary trial was carried out with respect to the optimum temperature of hydrolysis, ranging from 40-80^{o}C at 5^{o}C interval considering degree of hydrolysis, protein recovery and proteolytic activity as process variables. The other major parameters viz. pH, enzyme: substrate ratio (E/S) and hydrolysis time were kept constant at 6.5, 0.5 %

and 30 min, respectively. Further experiments based on the statistical design were performed in a shaking water bath (Shaking bath, Neolab Instruments, Mumbai, India) with continuous agitation at this constant optimised temperature. On completion of the process, the hydrolysis was terminated by heating the solution to 85-90°C for 15 - 20 min. The resultant solution was cooled and centrifuged at 8000 g at 10°C for 20 min (K-24A, Remi Instruments, Mumbai) to obtain supernatant which was further spray dried (Lab 2 Advanced Laboratory type, Hemraj, Mumbai) and used for analysis.

Fig. 4.2 Tuna protein hydrolysate solutions

4.2.3 Experimental design

Response Surface Methodology with a central composite design (CCD) with two independent variables at three levels was chosen based on the results of preliminary experiments. The input factors were enzyme-substrate ratio (E/S) (X_1) and hydrolysis time (X_2). pH was maintained constant and optimized hydrolysis temperature was adopted. Single and combined effects of the variables on the responses were studied by formulating thirteen experimental runs. The responses included protein recovery, foaming, emulsifying, oil absorption and sensory properties for functionality optimization. Similarly variables viz., protein recovery,

DPPH radical scavenging activity, FRAP, metal chelating activity and ABTS radical scavenging activity were considered for optimization of antioxidative properties. Contour plots and response surface graphs were generated by the predictive model to envisage the critical points and the effectiveness of each factor. Desirability score was computed for multi response optimization of response variables for functionality of hydrolysate and antioxidative properties with emphasis to protein recovery and the optimum combination of enzyme substrate ratio and hydrolysis time was selected.

4.2.4 Determination of proximate composition

Proximate composition of tuna red meat before and after treatment was estimated as per AOAC (2012). The moisture was determined by oven drying method. The percentage weight loss of food on account of evaporation of water from them by drying was made use. A known quantity of sample was placed in thermostatically controlled hot air oven and the reduction in weight was checked by repeated weighing and cooling of the sample in desiccator till the weight become constant.

The estimation of crude fat content was done by adopting soxhlet extraction method using petroleum ether as the extraction solvent. Known quantity of moisture free sample was accurately weighed into an extraction thimble and was placed in an extractor which was connected to a pre-weighed dry receiving flask and water condenser. The solvent in the receiving flask was evaporated completely and weighed for the fat content. The result was expressed as amount of crude fat per 100 g sample.

The ash content was determined using muffle furnace by incineration method. Known quantity of moisture free sample was taken in a pre-weighed clean dry silica crucible and charred at low heat, followed by incineration in a muffle

furnace at 550° C to get white ash. Silica crucibles were finally cooled in desiccator and weighed and ash content was expressed as amount of ash per 100 g sample.

Protein content of tuna red meat and hydrolysates were estimated by kjelhdahl method (detailed in chapter 3; section 3.2.3).

4.2.5 Determination of degree of hydrolysis and proteolytic activity

Degree of hydrolysis was estimated as per the methodology described by Hoyle and Merritt (1994). To the supernatant, one volume of 20 % trichloroacetic acid (TCA) was added, followed by centrifugation at 2560 g at for 15 min to collect the 10 % TCA-soluble materials. Briefly, degree of hydrolysis was computed as

$$\% \text{ DH} = \frac{\text{10 \% TCA soluble } N_2 \text{ in the sample}}{\text{Total } N_2 \text{ in the sample}} \times 100$$

Proteolytic activity of the sample was projected from the tyrosine content of the protein hydrolysate which measured the extent of hydrolysis under given conditions (detailed in chapter 3; section 3.2.5).

4.2.6 Determination of protein recovery

Protein recovery in hydrolysate was defined as the ratio of protein yield obtained from the extraction process to the amount of total protein estimated by Kjeldahl and was calculated as follows:

$$\text{Recovered protein (\%)} = \frac{\text{Protein in hydrolysate sample x volume of hydrolysate}}{\text{Weight of raw material taken x percentage of protein in raw material}} \times 100$$

4.2.7 Determination of functional and antioxidative properties

Functional properties of the hydrolysates viz., foaming properties (Sathe and Salunkhe, 1981); emulsifying properties (Pearce and Kinsella, 1978); oil absorption capacity (Shahidi et al.,1995) of the hydrolysates were determined. Ten trained panellists were assigned for the sensory analysis for bitterness adopting the methodology of Nilsang et al. (2005) with modifications. Antioxidative

properties determined included DPPH radical-scavenging activity (Shimada et al., 1992); ferric reducing antioxidant power (FRAP) (Benzie and Strain, 1996); metal chelating ability (Decker and Welch, 1990) and ABTS radical (2,20 -azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) scavenging activity (Re et al., 1999) (detailed in chapter 3; section 3.2.8 and 3.2.9).

4.2.8 Statistical model development

The CCD in the experimental design consisted of 13 experimental points conducted in random order (5 factorial points, 5 axial points and 3 center points) (Table 4.1). Second order/ quadratic and third order/cubic regression models were fitted to the response variables as a function of input variables using the polynomial equation:

Second order regression: $Y = \beta_0 + \beta_i X_i + \beta_{ij} X_i X_j + \beta_{ii} X_i^2$, $i \neq j = 1,2$

Third order regression: $Y = \beta_0 + \beta_i X_i + \beta_{ij} X_i X_j + \beta_{ii} X_i^2 + \beta_{iij} X_i^2 X_j$, $i \neq j = 1,2$

Y being the response; β_0:the offset term; $\beta_{i,}$ β_{ij}, β_{ii} and β_{iij}being the regression coefficients and Xi and Xj, the levels of the independent variables. The performance of the fitted model was assessed by Coefficient of determination (R^2) and mean square error (MSE). Significance of the regression coefficients was determined at 5% level of significance ($p<0.05$). A software Design expert 7.0 was used to fit the models.

Table 4.1 Experimental design and responses of the dependent variables to the hydrolysis conditions

Design point[a]	X_1	X_2	DH	PR	FC	FS	EAI	ESI	OAC	Bitterness	DPPH	FRAP	MC	ABTS
1	0.25	30	12.42	32.66	160	130	182.4	37.01	1.23	3	69.60	28.87	54.67	48.11
2	0.88	240	30.78	43.44	130	25	75.95	23.41	1.32	9	78.13	45.58	38.25	56.75
3	1.5	240	38.10	47.13	130	30	70.54	25.36	1.25	10	82.08	48.56	25.83	54.08
4	1.5	30	24.65	49.89	180	25	97.34	33.92	1.38	8	72.08	37.16	30.83	53.69
5	1.5	135	31.18	48.45	162	30	74.01	28.48	1.34	9	81.14	42.32	22.73	53.85
6	0.25	135	18.54	35.26	167	105	107.39	20.33	1.23	5	65.50	28.77	56.44	47.11
7	0.25	30	12.43	32.90	177	145	176.99	33.98	1.27	4	67.79	29.44	49.31	47.35
8	1.5	30	24.66	46.24	198	20	90.74	29.15	1.35	8	72.60	37.08	33.26	54.01
9	1.5	240	39.12	50.11	130	20	67.43	28.84	1.28	10	81.98	47.92	25.12	54.27
10	0.88	30	20.98	42.63	205	48	92.98	32.65	1.22	7	78.52	38.71	20.29	57.92
11	0.25	240	20.17	33.95	154	20	101.73	34.43	1.27	6.5	73.37	37.11	79.42	52.48
12	0.88	240	30.92	43.14	122	20	75.95	23.42	1.37	9.5	79.13	44.49	44.85	56.51
13	0.25	240	20.58	34.42	133	10	108.24	31.68	1.23	6	73.42	36.79	81.07	54.41

[a]Experiments were run at random, X_1: Enzyme-substrate ratio (%), X_2: Hydrolysis time, DH: Degree of hydrolysis (%), PR: Protein recovery (%), FC: Foaming Capacity (%), FS: Foam Stability (%), EAI: Emulsifying Activity Index (m^2/g), ESI: Emulsion Stability Index (min), OAC: Oil Absorption Capacity (g/g), DPPH radical scavenging activity (%), FRAP (mM Ascorbic Acid/g protein), MC: Metal Chelating activity (mg EDTA/g protein), ABTS radical scavenging activity (%)

4.3 Results and discussion

4.3.1 Proximate composition of raw material mince

The proximate composition of tuna red meat mince before and after water washing was assessed. Tuna red meat was initially washed with boiled water and subsequently with 0.2 % cold sodium bicarbonate solution to remove excess of fat and pigments. Previously, Bhaskar et al. (2008) suggested a prior hot water washing (85^{o} C for 20 min) for catla visceral waste followed by centrifugation to increase the stability of the hydrolysates towards lipid oxidation. Elavarasan (2014) has reported water washing process to be ideal as it facilitates the removal of unwanted components from the raw material. The washing process increased the moisture content of mince by 3.38 % (67.11 ± 0.02 to 70.49 ± 0.48 %), with a proportional decrease in the protein content from 28.19 ± 0.62 to 24.98 ± 0.24 %. This was also accompanied by a significant ($p < 0.05$) reduction in the fat (2.22 ± 0.02 % to 1.4 ± 0.17 %) and ash content (1.43 ± 0.06 to 0.64 ± 0.01 %) of the mince to almost half of its initial value. The higher moisture content observed in the washed mince might be either due to the hydration of myofibrillar proteins or may be a relative increase associated with the loss of water soluble proteins, fat and mineral during the leaching process.

4.3.2 Optimization of hydrolysis parameters

The optimisation of hydrolytic parameters was carried out in two steps. Initially, the temperature for the hydrolysis was optimised keeping E/S, pH and time as constant, followed by the optimisation of E/S (X_1) and hydrolysis time (X_2) under optimum conditions of temperature and pH. The response variables considered for optimising the process temperature were degree of hydrolysis, protein recovery and proteolytic activity (Fig. 4.3). All the variables were found to be increasing upto 60^{o}C, and thereafter showed a decreasing trend. Hence, the subsequent hydrolysis

experiment was carried out at a single temperature of 60°C. Similarly, the pH opted for the hydrolysis process was the initial pH of the substrate (washed mince), ie pH 6.5, since it falls within the optimal pH range of papain enzyme as indicated by the manufacturer (pH 6-7).

4.3.3 RSM based optimisation of process variables

The influence of E/S (X_1) and hydrolysis time (X_2) under optimum conditions of temperature and pH by papain on cooked tuna red meat protein was determined using central composite design. The response variables considered for optimising the derivation of surface-active hydrolysis were foaming, emulsifying and oil absorption properties together with sensory property. Similarly, DPPH free radical scavenging, FRAP, metal chelating and ABTS radical scavenging activities were considered as response variables for optimising the conditions for extracting antioxidant hydrolysates. For both the optimizations, protein recovery was also given special emphasis. A multiple regression analysis technique was performed to determine all the coefficients of linear (X_1, X_2), quadratic (X_1^2, X_2^2) and interaction (X_1X_2) terms to fit a full response surface model for the responses (Table 4.2).

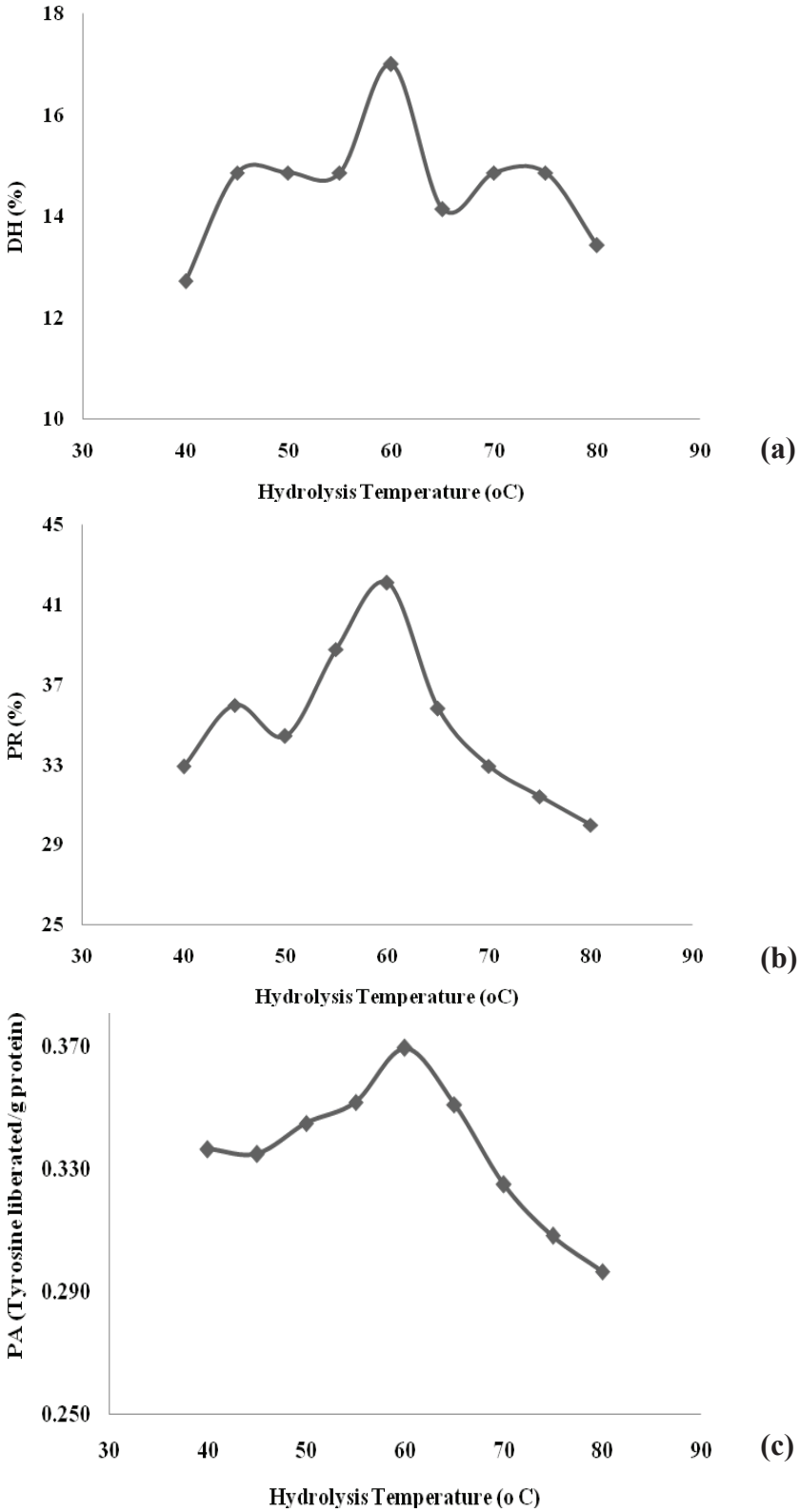


Fig. 4.3 Temperature optimization with **a.** degree of hydrolysis **b.** protein recovery and **c.** proteolytic activity as response variables

Table 4.2 Regression coefficient of fitted models with R^2 values

Response	Fitted Model	X_1	X_2	$X_1 * X_2$	X_1^2	X_2^2	R^2
PR	Quadratic	+20.78791*	+0.030116	-3.23891E-003	-4.98737	-8.51669E-005	0.9775
FC	Quadratic	+33.04668	+0.035246*	-0.13009	-8.02579	-6.12245E-004	0.8598
FS	Quadratic	-155.97747*	-0.23379*	+0.47671*	+26.29818	-1.58730E-003	0.9603
EAI	Quadratic	-173.62993*	-0.88444*	+0.18966*	+59.28886*	+1.92528E-003*	0.9463
Bitterness	Quadratic	+8.84451*	+0.015197*	-2.85900E-003	-2.95917*	-5.66893E-006	0.9879
DPPH	Quadratic	+19.73935*	-0.010204*	+0.018901	-9.06230	+7.21088E-005	0.8265
FRAP	Quadratic	+22.62689*	-0.026598*	+0.012619	-9.14333*	+2.10544E-004	0.9787
MC	Quadratic	-78.85435*	-0.012196*	-0.13251*	+38.53727*	+7.12358E-004*	0.9794
ABTS	Quadratic	+25.86574*	-0.017272	-0.020599*	-11.32258*	+1.65079E-004	0.8931

X_1: Enzyme-substrate ratio (%), X_2: Hydrolysis time, DH: Degree of hydrolysis (%), PR: Protein recovery, FC: Foaming Capacity (%), FS: Foam Stability (%), EAI: Emulsifying Activity Index (m^2/g), DPPH radical scavenging activity (%), FRAP (mM Ascorbic Acid/g protein), MC: Metal Chelating activity (mg EDTA/g protein), ABTS radical scavenging activity (%); * significant at 5 % level ($p < 0.05$).

4.3.4 Variations in degree of hydrolysis

Previously, several authors have suggested degree of hydrolysis as the major contributor to the specific properties exhibited by the peptides (Ren et al., 2008a; Taheri et al., 2013). However, peptides derived from the same source having similar DH values quite often vary in their properties. Amarowicz (2008) suggested that the presence of specific peptides liberated from protein as well as and the amount of free amino acids to be important in determination of bioactive properties of protein hydrolysate. Hence, in the present study, DH was not included in the RSM analysis matrix. However the DH values were independently determined for each hydrolytic conditions mentioned in the matrix. Further, the changes in the individual properties were discussed in relation to the changes in DH values. The DH varied in direct proportion with the variations in the independent variables viz., X_1 and X_2 as indicated by the correlation coefficients of 0.936 and 0.998, respectively (Fig. 4.4a and b). Similar observations were reported by Ovissipour et al. (2012) during enzymatic hydrolysis of proteins from yellow fin tuna head where DH increased with increasing hydrolysis time. However, a gradual reduction in the rate of increase was observed above E/S ratio of 0.88 (Fig.4.4a), which might be on account of the unavailability of substrate for the hydrolysis. The changes in DH indicated no evidence of feedback inhibition within the range of 30 -240 min as indicated by a constant rate of increase in DH values as the hydrolysis progressed (Fig. 4.4b).

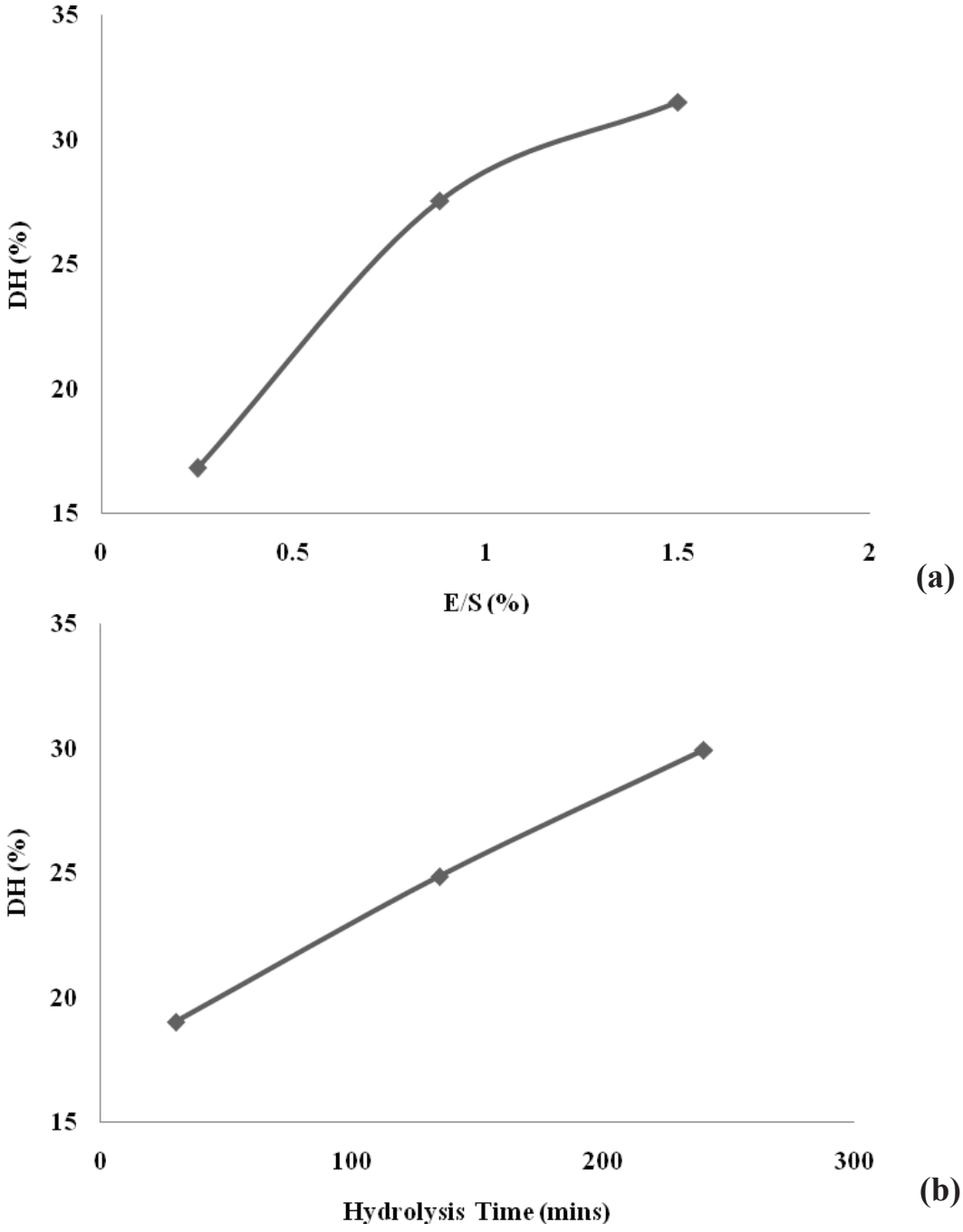
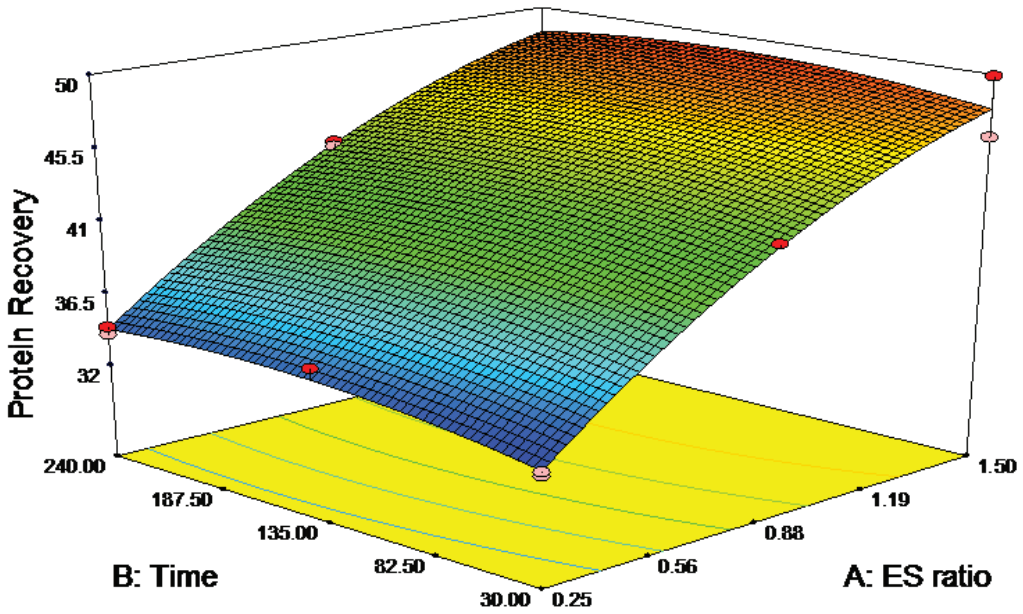


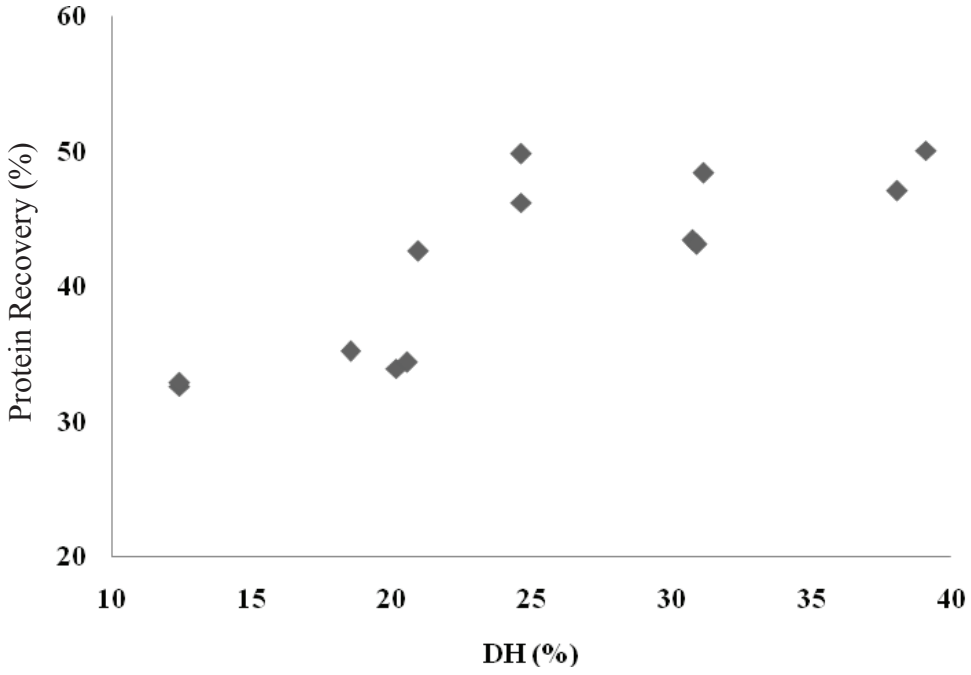
Fig. 4.4 Variations in degree of hydrolysis with **a.** E/S ratio and **b.** hydrolysis time

4.3.5 Variations in protein recovery

The protein recovery is considered as a response variable for both the optimisation designs, as it adds to the economics of the hydrolysis operation. The variations in this response during the hydrolytic process was best explained by second order response surface model ($p < 0.05$) (Table 4.2; Fig. 4.5a) with an R^2 value of 0.98 and MSE of 1.78. The adjusted R^2 of the fitted model was 0.96 and analysis of lack of fit was found to be insignificant ($p > 0.05$) indicating the suitability of the model. The precision measures of S/N (signal to noise ratio) was greater than 4 (17.797) indicating adequate model discrimination (Myers et al., 2009). The linear effect of X_1 on nitrogen recovery was found to have a statistically significant effect ($p < 0.05$) with high regression coefficient value of 20.79, whereas that of X_2 was marginal. The quadratic effects of both the variables showed negative values indicating that the protein recovery reached a threshold level at certain value of E/S and time, thereafter showing a reduction in the rate of increase. It is generally agreed that the high E/S ratio and longer hydrolysis period favours higher protein recovery (Mendonca Diniz and Martin, 1998; Liaset et al., 2002). In the present study, protein recovery varied directly with the degree of hydrolysis ($R^2 = 0.67$) of up to 25 % and thereafter showed a slightly decreasing or more or less similar values (Fig. 4.5b). However for similar DH, variations were observed in this response ranging on an average from 5 – 9 %. It was also noticed that as indicated in quadratic equation, E/S had more influence than time with higher E/S giving more recovery of protein from substrate than the period of hydrolysis. From the regression coefficients of statically fitted models observed in the present study, it may be inferred that increasing the concentration of enzyme is more beneficial in getting higher protein recovery than increasing the duration of hydrolysis beyond a DH value of 25 %.



(a)



(b)

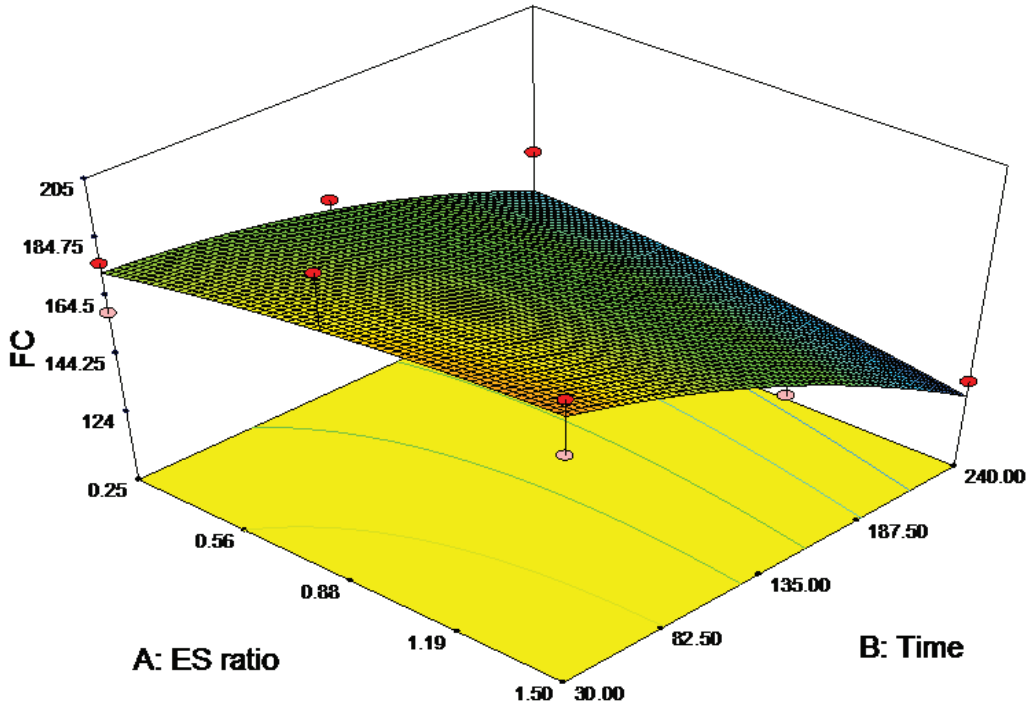
Fig. 4.5 Variations in protein recovery (%) **a.** in response to enzyme-substrate ratio and hydrolysis time; **b.** in relation to DH

4.3.6 Variations in functional properties

4.3.6.1 Foaming properties

Foaming properties are usually expressed in terms of foaming capacity and foam stability. Second order regression model with an R^2 value of 0.86 explained the changes in foaming capacity of hydrolysate under different hydrolytic conditions. The regression coefficient values of fitted quadratic model for foaming properties are shown in Table 4.2. The FC values showed strong positive linearity with X_1 (Fig. 4.6a), however the quadratic effect showed negative values. In the case of X_2, the linear effect on protein recovery was minimum, however was found to be statistically significant ($p < 0.05$).This essentially means that, FC values increased initially with increase in E/S ratio to reach a threshold value, and thereafter decreased with every unit of increase in E/S ratio. Foaming capacity was higher at lower DH (Fig. 4.6b) and it ranged from 122 – 205 % under different conditions of E/S ratio and hydrolysis time which in turn influenced the DH. For similar DH it exhibited wide variations in the property ranging from 35 - 60 %, on an average. Similar to protein recovery, E/S had higher influential role than time and hence for similar DH, the hydrolytic condition with higher E/S gave better foaming capacity for the derived hydrolysate.

Variations in the foam stability were explained by quadratic regression model with an R^2 of 0.96. The Foam stability values showed strong negative linear effect with X_1 while giving a positive quadratic effect, whereas both linear and quadratic effect was found to be marginal for X_2. Similarly, the interaction effects of both X_1 and X_2 on foaming properties were also minimum (Table 4.2; Fig. 4.7a). The results suggested a drastic reduction in FS with increase in the concentration of enzyme upto certain degree of hydrolysis, thereafter showing constant values (Fig. 4.7b). There was a general trend of decrease in the foaming properties with increase in DH (Table 4.1). However, it was observed that for similar range of DH, distinctly different response were exhibited, which further suggest that the nature of peptides formed under different hydrolytic conditions play a major role in determining the final properties of hydrolysate.



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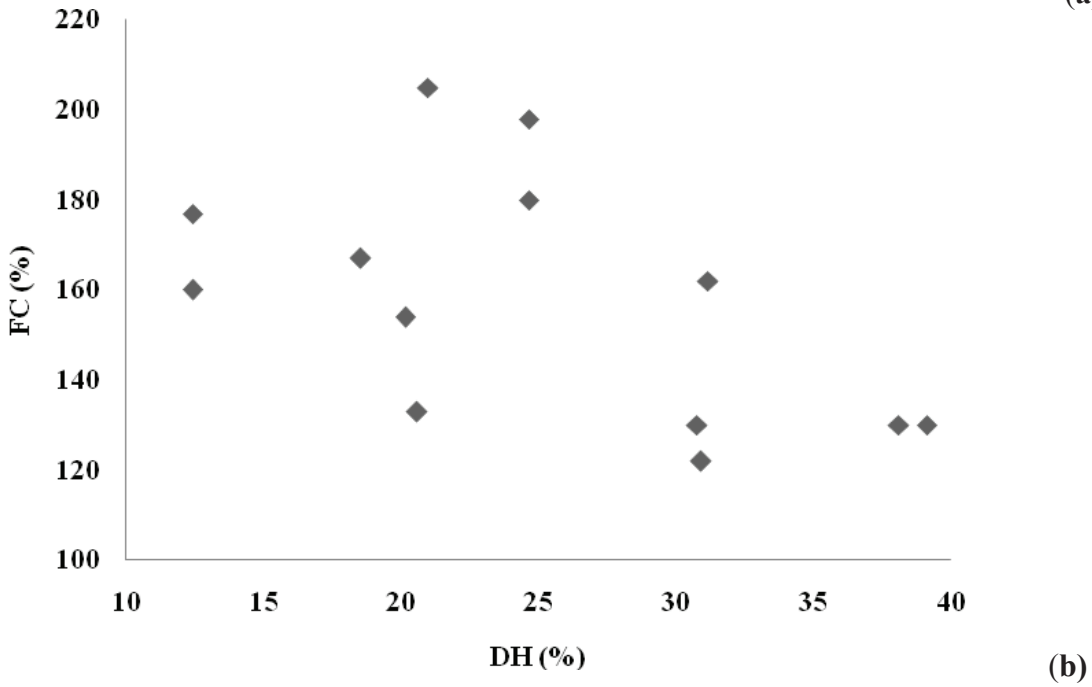
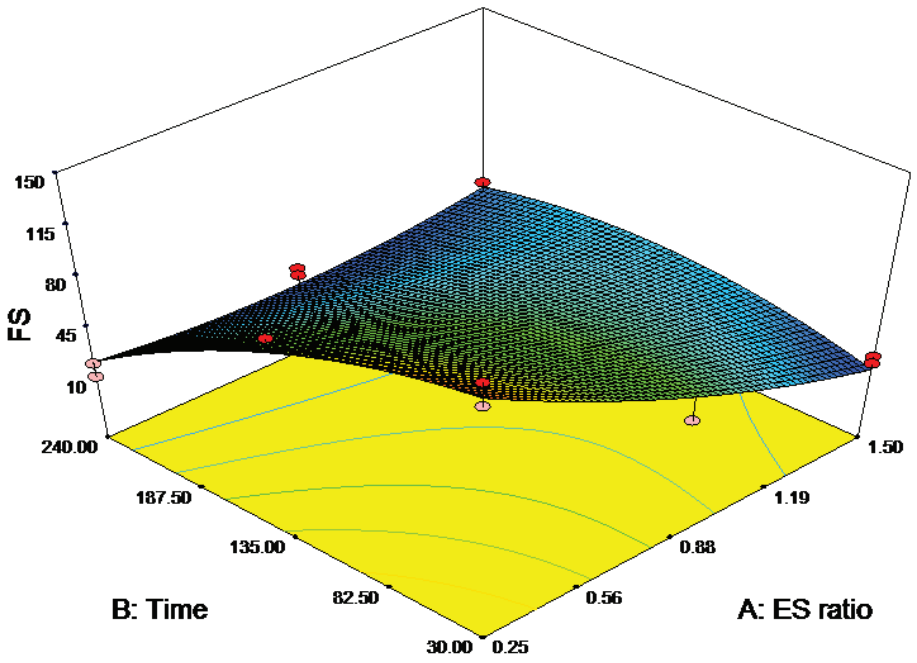


(b)

Fig. 4.6 Variations in foaming capacity (%) **a.** in response to enzyme-substrate ratio and hydrolysis time; **b.** in relation to DH



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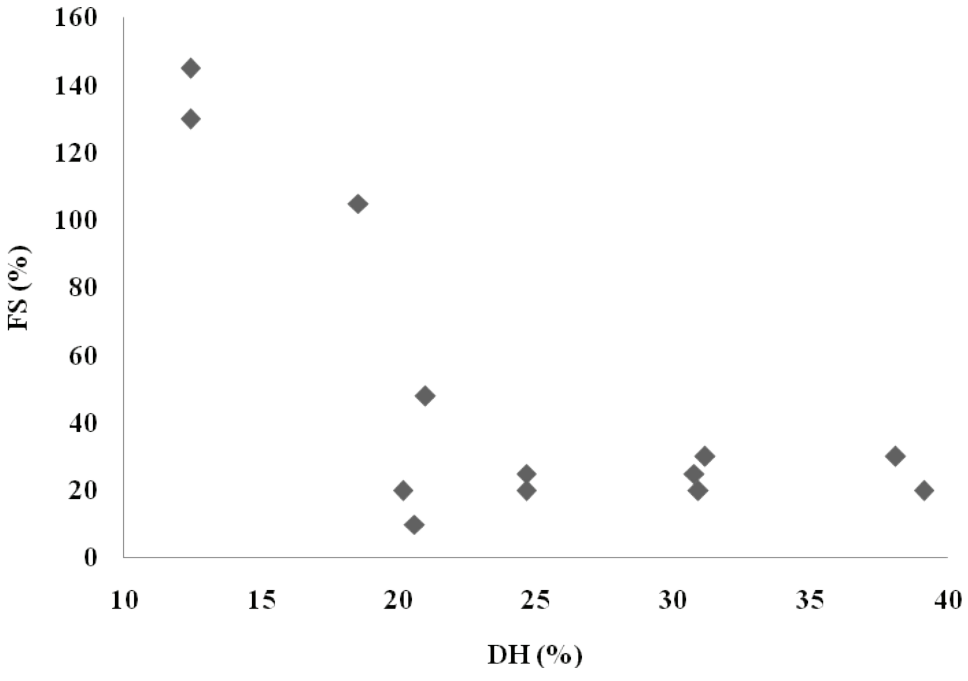


(b)

Fig. 4.7 Variations in foam stability (%) **a.** in response to enzyme-substrate ratio and hydrolysis time; **b.** in relation to DH

4.3.6.2 Emulsifying properties

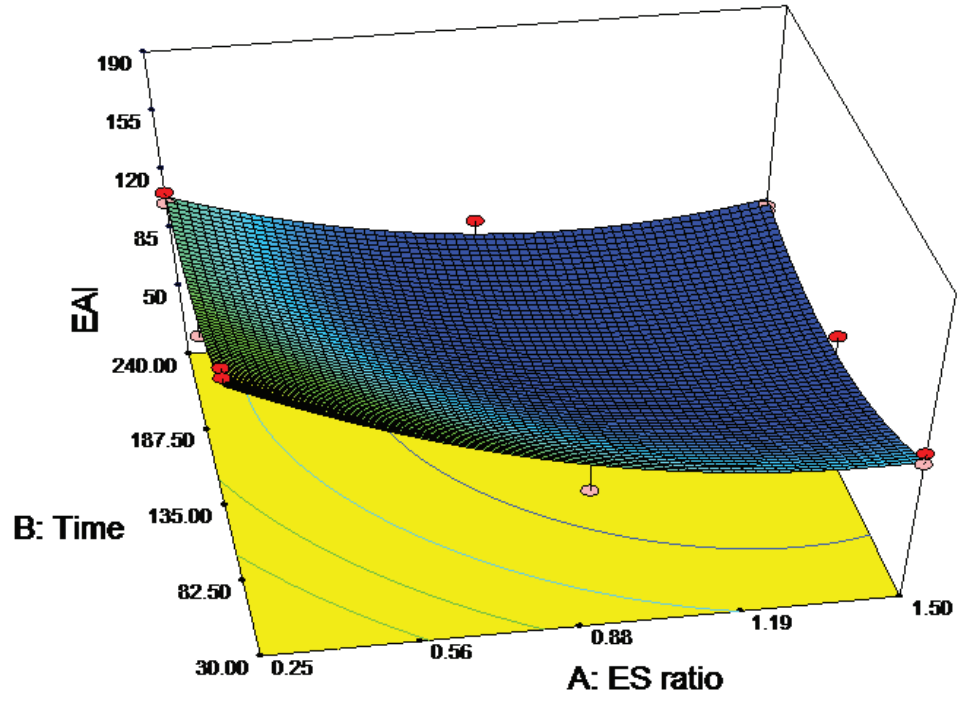
Second order regression model was best fitted for explaining the changes in EAI. The high coefficient of determination ($R^2 = 0.95$) indicated that the model as fitted can explain 94.6 % of the EAI variability (Table 4.2; Fig. 4.8a). Linear, second order and interaction of both X_1 and X_2 were found to be significant ($p < 0.05$) in influencing the variations of this response. Both the factors were inversely related to the changes in response as indicated by negative regression coefficient. However linear as well as quadratic terms of $X_1$was observed to be more important for response variations indicating a regression coefficient of 173.63 and 59.29, respectively while X_2 showed only a marginal influence on EAI.

ESI was explained using cubic model with an R^2 of 0.92. An adequate precision of 8.510, indicating the signal to noise ratio was observed and as the value was greater than the desired value of 4, the present model indicated its fitness for explaining the variations in ESI (Fig. 4.9a). The linear terms of $X_1$and X_2 as well as second order of X_2 and interaction of linear terms X_1 and second order of X_2 were the significant terms ($p < 0.05$). X_1 and X_2 were equally influential for the variations in this response however, X_1 was directly related while X_2 was inversely related to ESI in hydrolysate.

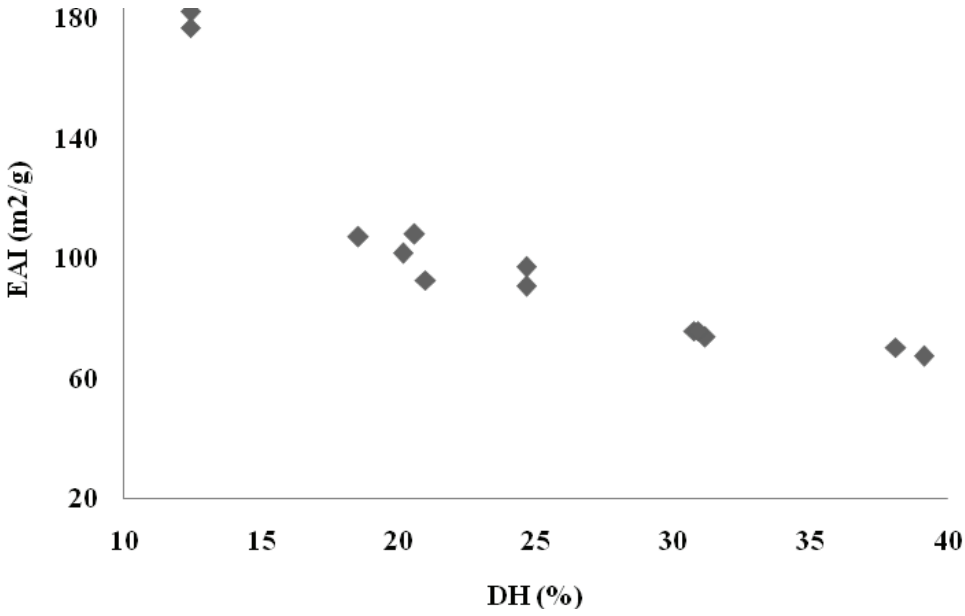
The equation explaining the variations in ESI in terms of coded factors was:

$$ESI = 20.66 + 4.08\ X_1^* - 4.62\ X_2^* - 0.50\ X_1X_2 + 3.75\ X_1^2 + 7.39\ X_2^{2*} + 2.90\ X_1^2 X_2 - 6.55\ X_1\ X_2^{2*}$$

Both EAI and ESI indicated significant reduction in values with progress of hydrolysis, with the highest values at a DH of 12.42 % which is the lowest DH obtained during the study, corresponding to X_1 of 0.25 % and X_2 of 30 min (Table 4.1, Fig. 4.8b, 4.9b). In general, the emulsifying properties showed marked decrease in the values above DH value of 20 %. EAI exhibited an inverse relation with DH ($R^2 = 0.75$) and variations of up to 12 m^2/g were observed for similar DH generated through different combinations of X_1 and X_2. However the variations in ESI for similar DH ranged from 0.5 min - 5 min (Fig. 4.9b).Kristinsson and Rasco (2000) stated to have exceptional emulsifying activity and stability for hydrolysate produced under limited degree of hydrolysis. Similar reports of greater emulsifying capacity and emulsion stability were noticeable when DH was low for salmon byproduct hydrolysate (Gbogouri et al., 2004) and sardine hydrolysate (Quaglia and Orban, 1990).



(a)



(b)

Fig. 4.8 Variations in emulsifying activity index (m^2/g) **a.** in response to enzyme-substrate ratio and hydrolysis time; **b.** in relation to DH

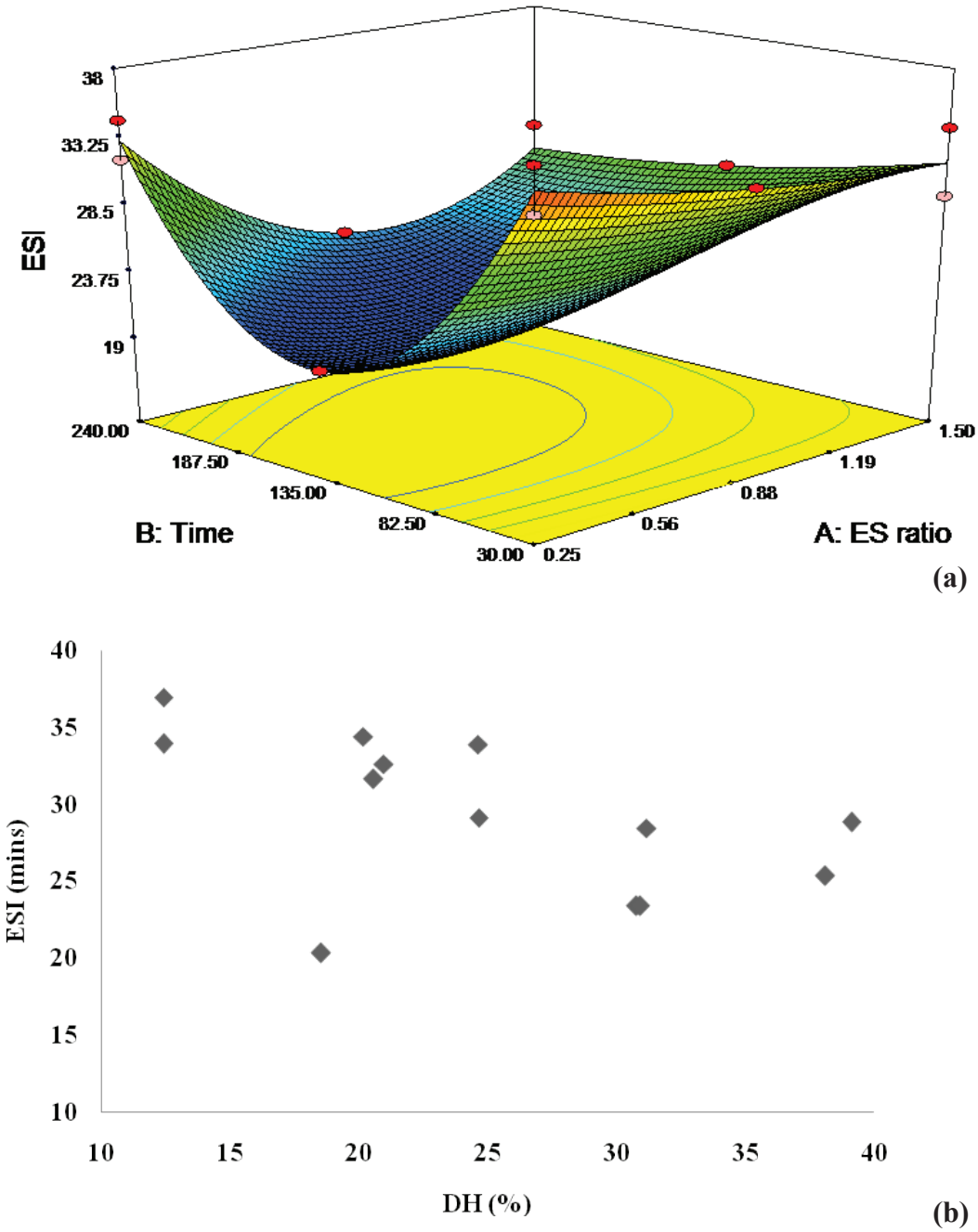


Fig. 4.9 Variations in emulsion stability index (min) **a.** in response to enzyme-substrate ratio and hydrolysis time; **b.** in relation to DH

4.3.6.3 Oil absorption capacity

OAC is related to the surface hydrophobicity in hydrolysates which is facilitated by the hydrolysis process. Cubic model with an R^2 of 0.88 best explained the variations in this response (Table 4.2; Fig. 4.10a). All the influencing variables X_1 and X_2 had a marginal effect with positive correlation. However linear terms of X_2 as well as interaction effect of second order terms of E/S and linear terms of hydrolysis time ($X1^2X_2$) were significant ($p < 0.05$). The model was fitted using the equation in terms of coded factor as:

$$OAC = 1.28 + 0.056\ X_1 + 0.063\ X_2{*} - 0.023\ X_1X_2 + 4.731\ E{-}003\ X_1^2 - 2.388\ E\text{-}003\ X_2^2 - 0.084\ X_1^2\ X_2{*} - 0.019\ X_1\ X_2^2$$

During hydrolysis, the variation in OAC was minimum ranging between 1.23 – 1.38 g/g with the changes in factors viz., X_1 or X_2 (Table 4.1). Unlike the foaming and emulsifying properties, higher OAC value was observed at a slightly higher DH value of 24.65 %, however further decreased above DH value of 30 %.The correlation studies of OAC with DH also substantiated varying response with no definite increase or decrease (Fig. 4.10b). Variations in the hydrophobicity of the polypeptide fragment formed during hydrolysis might have resulted in wide variations in OAC exhibited by the hydrolysate derived under similar degree of hydrolysis. Similar to this, DH ranging between 2.4 – 2.8 ml/g was reported in cobia frame hydrolysates by Amiza et al. (2012) and an OAC ranging between 0.9 – 1.4 g/g was reported in hake by-product hydrolysates by Pires et al. (2012).

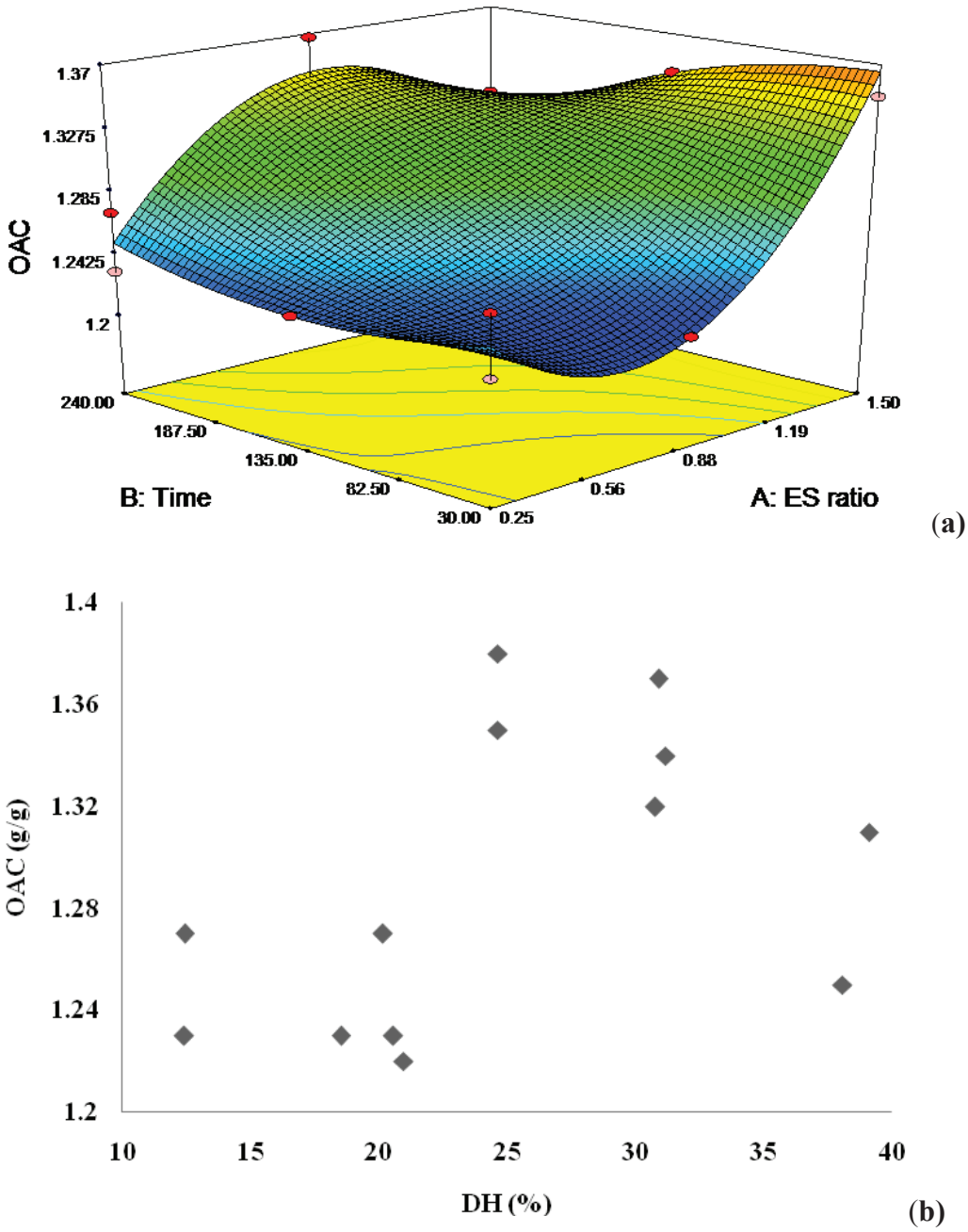
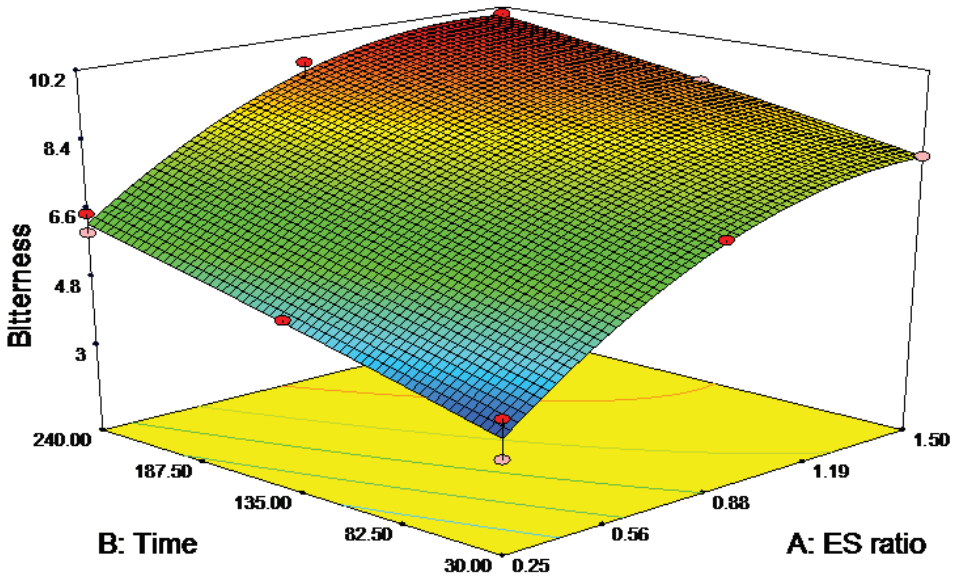


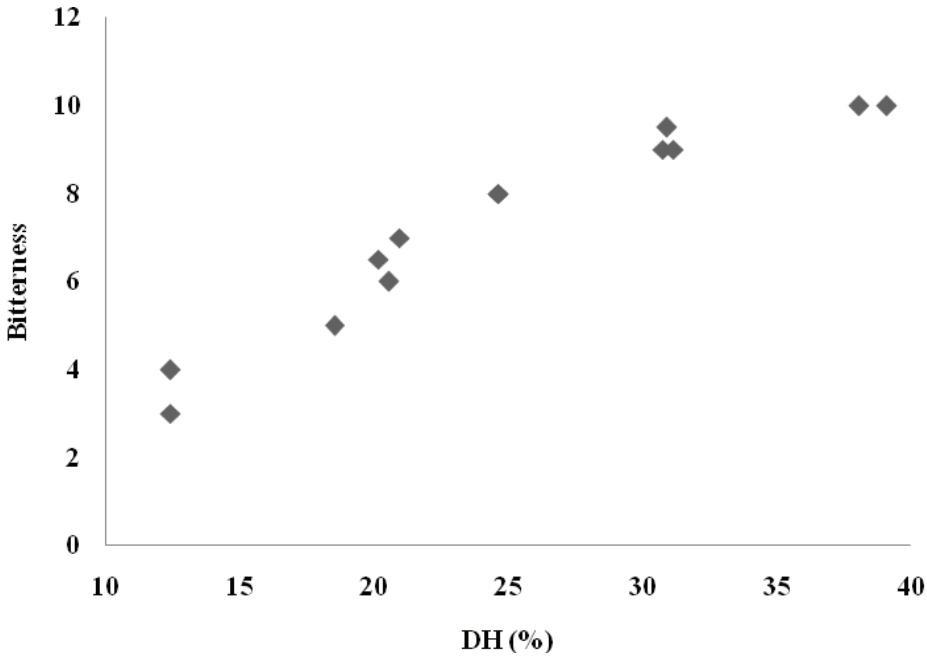
Fig. 4.10 Variations in oil absorption capacity (g/g) **a.** in response to enzyme substrate ratio and hydrolysis time; **b.** in relation to DH

4.3.6.4 Sensory property

Although enzymatic hydrolysis of protein develops desirable functional properties, it has the disadvantage of generating bitterness which is identified as a major hindrance in theutilization and commercialization of bioactive FPH (Kim and Wijesekara, 2010). The mechanism of bitterness is not very clear, but it is widely accepted that hydrophobic amino acids are one of the major contributors. Of the many techniques suggested to reduce or mask bitterness in hydrolysates, strict control of any hydrolysis experiment and termination at low degree of hydrolysis is desirable to prevent the development of a bitter taste and retention of functional properties(Adler-Nissen, 1986; Saha and Hayashi, 2001).Quadratic regression model ($p < 0.05$) with a high determination coefficient ($R^2 = 0.99$) and an MSE of 0.11 was fitted to predict the trends of bitterness of hydrolysate generated with various combinations of X_1 and X_2. The adjusted R^2 value of 0.98 and predicted R^2 value of 0.96 which were in reasonable agreement, further confirms the high significance of the fitted model. Linear terms of both X_1 and X_2 as well as second order term of X_1 were significant terms ($p < 0.05$) with X_1 being more influential in determining the variations of bitterness while hydrolysis time (X_2) had a marginal influence for the same extent of hydrolysis (Table 4.2). However beyond an extended limit, X_1 had minimum influence in bitterness generation in the hydrolysates as indicated by a negative coefficient for quadratic regression terms. Response surface graphs generated by the predictive model clearly indicated the trends in the bitterness with X_1 and X_2 (Fig. 4.11a).The observed trend was well in agreement with the changes in DH, as indicated by the R^2 (0.915) value of correlation graph where bitterness increased almost linearly with DH up to a value of 30 % and thereafter showed a stagnating trend (Fig. 4.11b). The variations between the samples having same DH obtained through different combinations of X_1 and X_2 were minimum with a slightly higher influence for E/S in the generation of bitterness than period of hydrolysis which was substantiated from regression equation for this response.



(a)



(b)

Fig. 4.11 Variations in bitterness **a.** in response to enzyme-substrate ratio and hydrolysis time; **b.** in relation to DH

4.3.7 Variations in antioxidative properties

4.3.7.1 DPPH radical scavenging activity

DPPH is a stable free radical with an absorbance maximum at 517 nm in ethanol. When DPPH encounters a proton-donating substance, such as an antioxidant, the radical is scavenged and the absorbance is reduced (Shimada et al., 1992). Quadratic model with an R^2 of 0.83 and MSE of 9.09 was used to describe the variations in DPPH radical scavenging activity ($p < 0.05$). Linear regression model of X_1 and X_2 were found to be significant ($p < 0.05$) and suitable for describing variations of this response in the given range of analysis. Regression coefficient of the models and response graph indicated a strong influence with linear relation for X_1 (19.74) in predicting the variations in DPPH (Table 4.2, Fig. 4.12a). However X_2 indicated an inverse marginal relation with the response. Coincidently, a stagnation or decreasing trend was observed in DPPH values with increase in enzyme-substrate ratio towards higher E/S ratios in the response surface plots, as also indicated by negative regression coefficients (X_1^2). Similarly, DPPH increased linearly with DH ($R^2 = 0.74$) (Fig. 4.12b), from an average value of 68.7 % at DH 12.43 % to 81.14 % at DH 31.18 % (Table 4.1) thereafter showing a stagnation in the increase. For the same DH, higher E/S assisted in deriving peptides exhibiting more DPPH activity in comparison to prolonged hydrolysis time and the variations in DPPH ranged from 2.5 - 5 %, on an average. Ambigaipalan and Shahidi (2017) stated the radical scavenging activities of peptides to be influenced by several factors including hydrophobicity/hydrophilicity, amino acid sequences, the degree of hydrolysis, and molecular weights of peptides. According to Chi et al. (2014), lower average molecular weight peptides comprise shorter, more active peptides that serve as electron donors and react with free radicals, rendering them more stable substances that stop chain reactions.

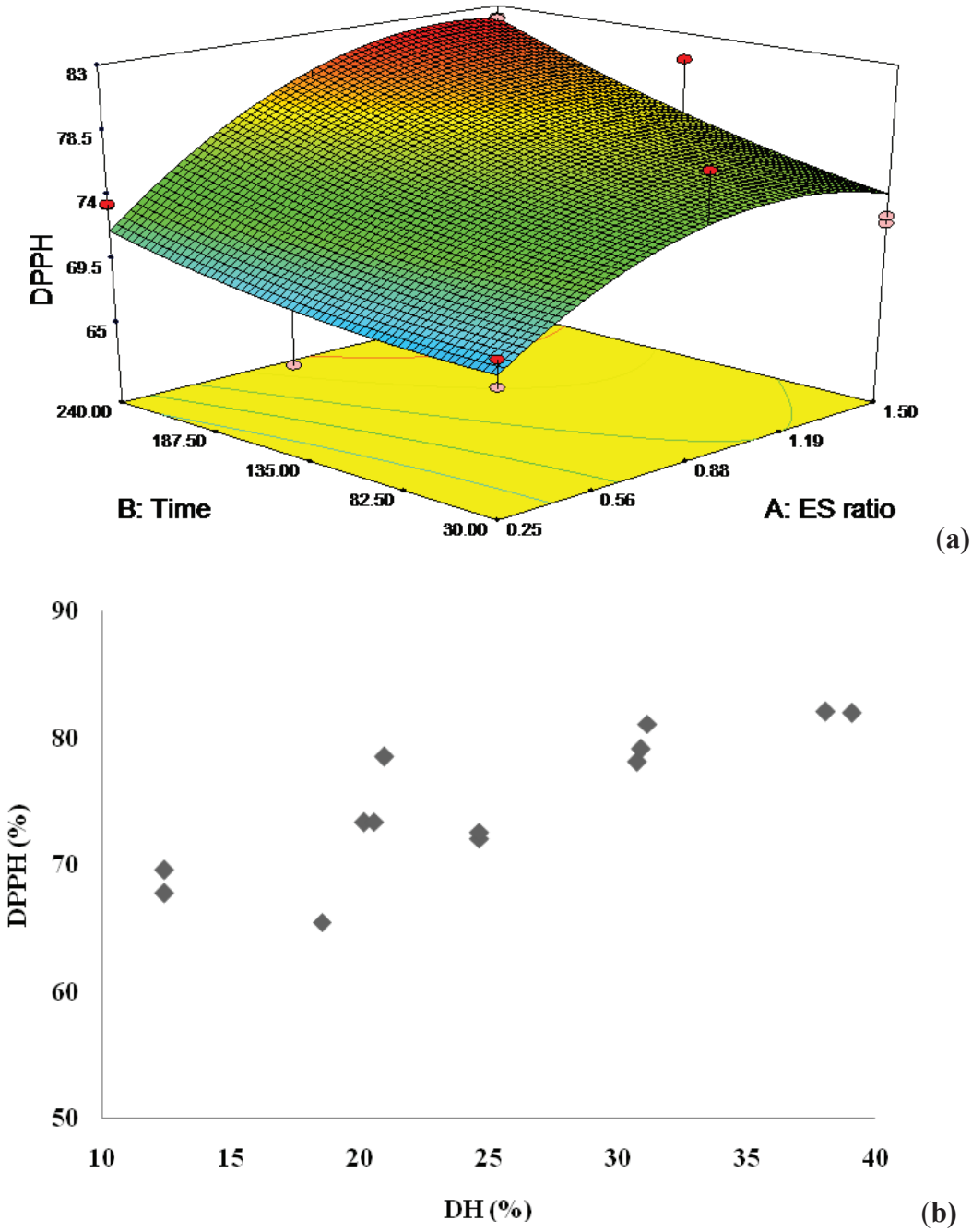


Fig. 4.12 Variations in DPPH radical scavenging activity (%) **a.** in response to enzyme-substrate ratio and hydrolysis time; **b.** in relation to DH

4.3.7.2 Ferric reducing antioxidant power

Quadratic regression model ($p < 0.05$) with an R^2value of 0.98 was best fitted to describe the variations in FRAP of hydrolysate. The predicted R^2 value (0.90) was found reasonable with adjusted R^2 value (0.96) and an adequate precision of 21.35 revealed the reliability of the fitted model. The linear terms and second order terms of X_1 and linear terms of X_2 were found to be significant at the given confidence level ($p < 0.05$). Similar to DPPH, regression coefficient of the models and response graph indicated the prominent role of X_1 in predicting the variations in FRAP while X_2 had an inverse effect even though was marginal (Table 4.2, Fig. 4.13a). Analysis of correlation between DH and FRAP indicated a distinctly linear effect with an R^2 of 0.901 (Fig. 4.13b). Similar to DPPH values, the variations in FRAP values observed for the samples having same DH values might be on account of the difference in the peptide fragments formed. Previously, Gimenez et al. (2009) reported nearly 2-fold higher ferric iron reducing ability for the hydrolysates of sole and squid gelatin compared to that of parent protein.

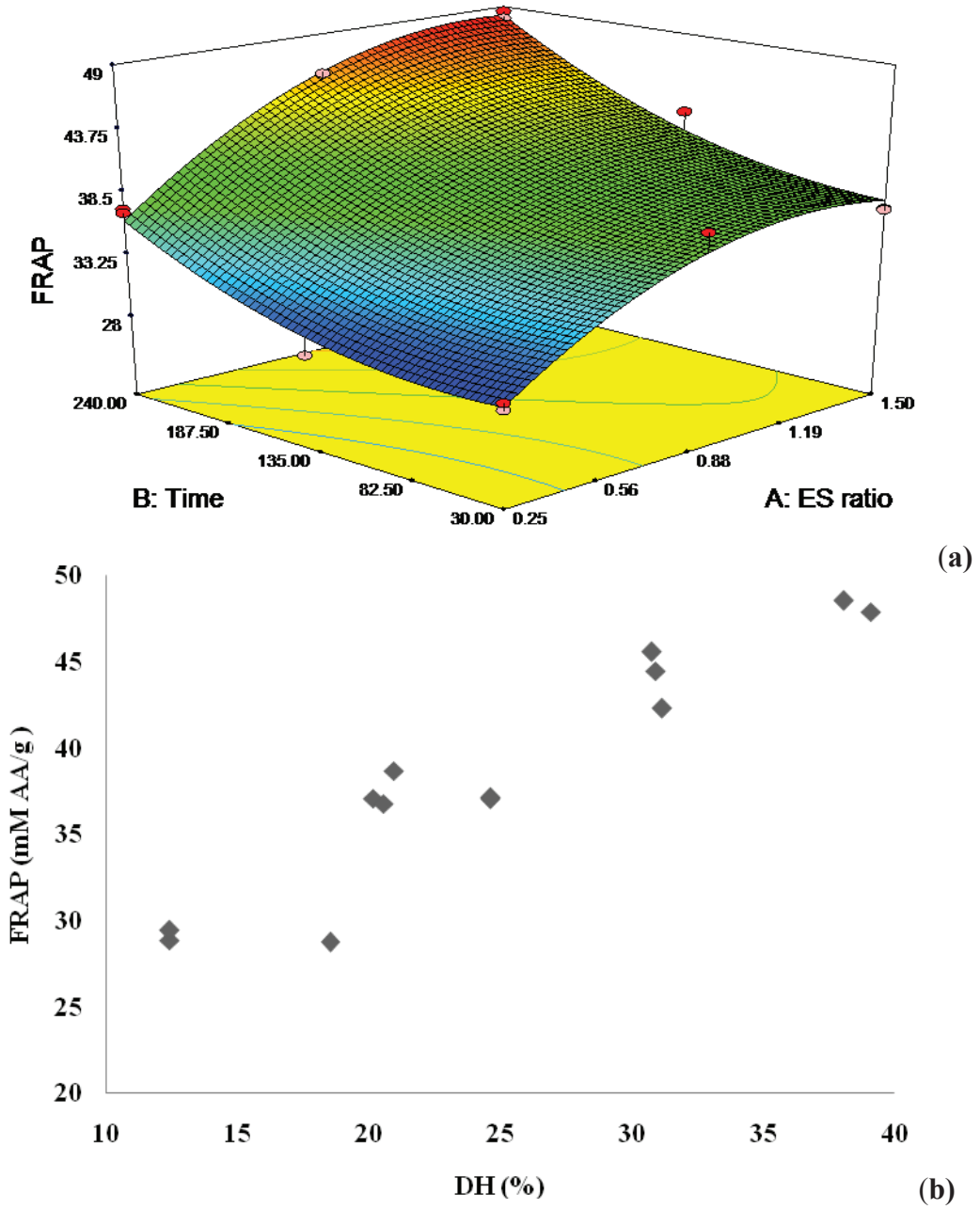


Fig. 4.13 Variations in FRAP (mM AA/g)
a. in response to enzyme-substrate ratio and hydrolysis time; **b.** in relation to DH

4.3.7.3 Metal chelating activity

Chelation of transition metal ions in foods by antioxidants help to retard the oxidation reaction by affecting both the rate of autoxidation and breakdown of hydroperoxide to volatile compounds (Gordon, 2001). Thiansilakul et al. (2007a) reported that peptides in hydrolysates could chelate the pro-oxidants, retarding the progress of lipid oxidation. Quadratic model with an R^2 value of 0.98 and MSE of 14.54 was used to best fit the variations in metal chelating activity of hydrolysate. An adjusted R^2 value of 0.96 confirmed the suitability of the model and a high precision of 23.13 was observed. All the model terms were significant (linear, second order and interaction) in explaining the variations of the response ($p < 0.05$). However X_1 with a regression coefficient of 78.85 was suggested to have a prominent role and it was inversely related to the response during the hydrolysis process (Table 4.2; Fig. 4.14a). In the present study, the metal chelating ability was comparatively higher for medium size peptides compared to larger and smaller size peptides (Fig. 4.14b). Previously in defatted salmon backbone hydrolysate, Slizyte et al. (2016) reported superior iron chelating ability for larger peptides compared to smaller peptides. Thiansilakul et al. (2007b) also observed slightly reduced metal chelating ability with increased degree of hydrolysis in round scad muscle hydrolysed with flavourzyme. On an average, the highest activity of 80 mg EDTA/g protein was observed at DH value of 20 % with X_1= 0.25; X_2 = 240 min. In contrary, the hydrolysate having similar DH value in the range of 20 % with X_1= 0.88; X_2 = 30 min showed a much lower activity of about 20 %. Similar variations were observed for several other combinations as well, having identical DH values ranging between an average of 18 - 60 mg EDTA/g protein. From the results, it is ideal to infer that metal chelating activity is highly dependent on the nature of peptides generated, rather than the degree of hydrolysis achieved during the process. Similar observations were reported by Sarmadi and Ismail (2010) proposing that

antioxidative properties of hydrolysate are affected by peptide structure and its amino acid sequence.

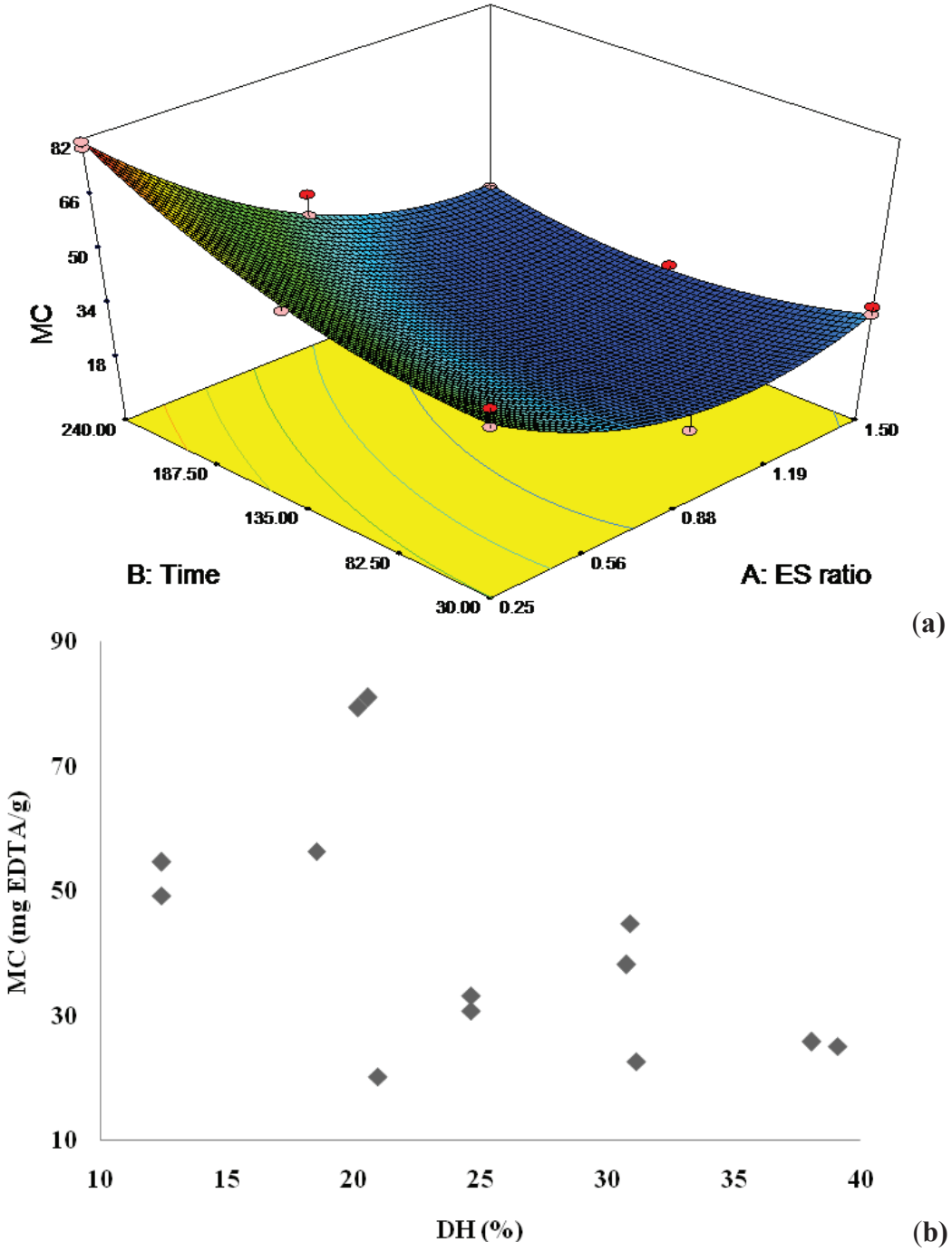
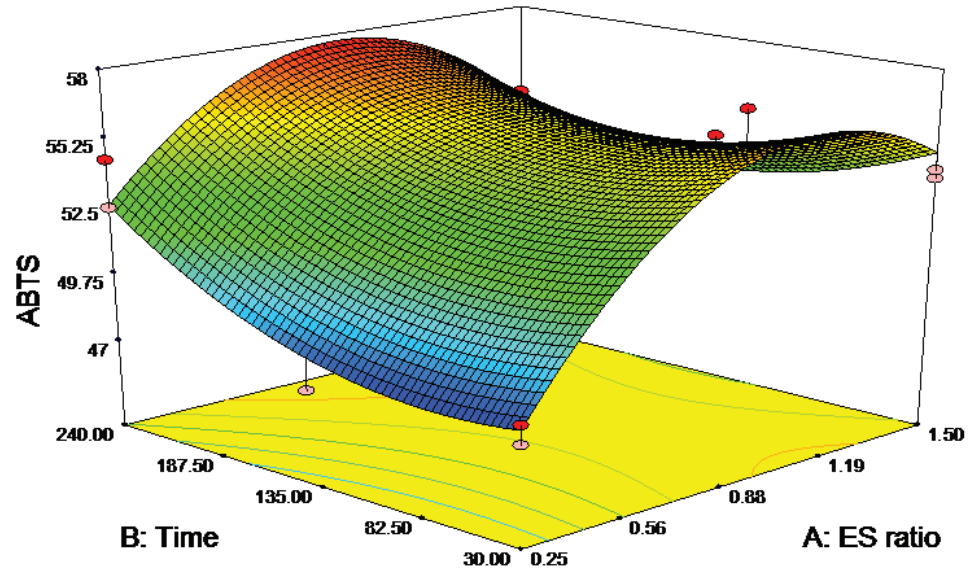


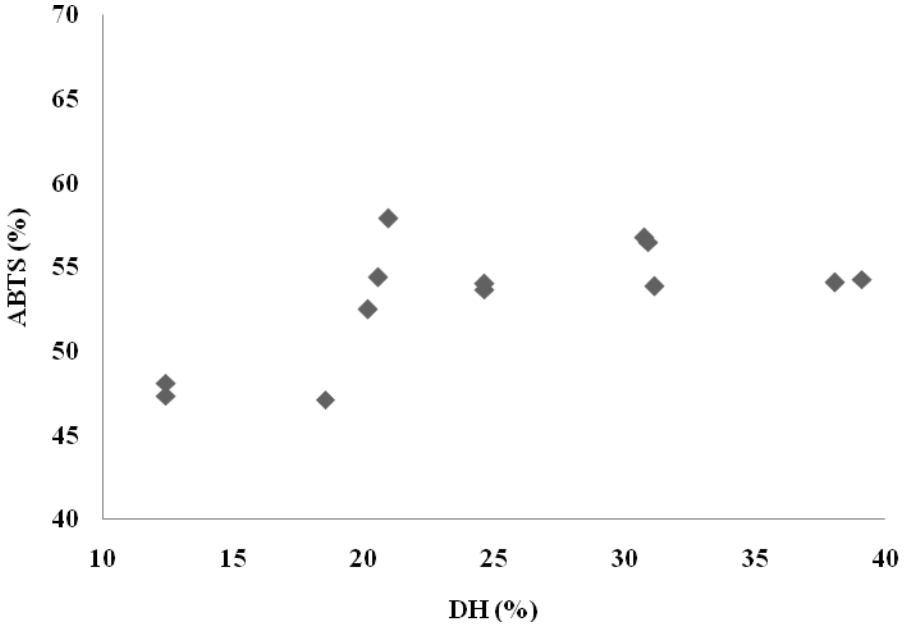
Fig. 4.14 Variations in metal chelating ability (mg EDTA/g)**a.** in response to enzyme-substrate ratio and hydrolysis time; **b.** in relation to DH

4.3.7.4 ABTS radical scavenging activity

ABTS assay is an excellent tool for determining the antioxidant activity of hydrogen-donating compounds (aqueous phase radical scavenger) and chain-breaking antioxidants (lipid peroxyl radical scavenger) (Binsan et al., 2008). Variations in ABTS radical scavenging activity was best fitted using quadratic model ($p < 0.05$) with an R^2 value of 0.89 (Table 4.2) and MSE of 2.26. A higher regression coefficient of 25.87 for X_1 ($p < 0.05$) indicated the strong and direct influence of enzyme concentration in explaining the variations in ABTS (Fig. 4.15a). Quadratic regression of X_1 as well as interaction of X_1 X_2 were the other significant factors ($p < 0.05$) influencing this response variable. However, hydrolysis time indicated very minor and insignificant effect on the ABTS values of the hydrolysates. Variations in ABTS during hydrolytic study ranged from about 47 - 58 % (Table 4.1) with maximum value at DH of 20.98 % (Fig. 4.15b), thereafter showed almost a stagnating trend with slightly lower values. However, similar to other properties described, variations in ABTS values were observed for samples having similar DH values ranging from 2.8-4.5 %. Similar to the observations in the present study, You et al. (2009) also reported an initial increase in ABTS radical scavenging activity followed by a decrease, with increasing DH values in loach protein hydrolysate. This essentially means that limited hydrolysis resulted in better antioxidant ability than extensive hydrolysis. Similar trend was observed in the present study also with comparatively higher ABTS activity for smaller peptides with limited hydrolysis (Table 4.1) as substantiated with a negative regression coefficient for second order terms of X_1.



(a)



(b)

Fig. 4.15 Variations in ABTS radical scavenging activity (%) **a.** in response to enzyme-substrate ratio and hydrolysis time; **b.** in relation to DH

From above observations, it may be inferred that optimum conditions for extracting surface-active peptides were; E/S of 0.34 % for a duration of 30 min at 60^0C and pH 6.5 (Table 4.3). The maximum desirability score obtained for this condition was 0.49, when protein recovery was included as response variable, and 0.70 without protein recovery. On the other hand, for optimized antioxidant activity, a desirability of 0.71 was observed for the hydrolytic condition viz., 0.98 % E/S for 240 min at 60^oC and 6.5 pH, with protein recovery and without including protein recovery the desirability was 0.74. This further implies that, one may opt for a serial hydrolysis process for selectively extracting larger surface-active peptides in the first step, followed by extensive hydrolysis to extract shorter peptides having higher antioxidant capacity in the subsequent steps. Further, all the response variables of the final products were validated. The experimental and predicted values were within the range, thus confirming the reliability of the optimised condition.

Table 4.3 Optimized hydrolytic conditions with the corresponding response variables

Hydrolytic conditions		**Responses**						
E/S	**Time**	**PR**	**FC**	**FS**	**EAI**	**ESI**	**OAC**	**Bitterness**
0.34	30	34.58	176.11	124.25	161	34.99	1.23	4.16
		39.64	173.33	122.0	146.2	34.59	1.24	4.0
		PR	**DPPH**	**FRAP**	**MC**	**ABTS**		
0.98	240	44.48	81.17	46.71	34.90	57.68		
		45.24	81.88	47.39	36.01	58.26		

E/S: Enzyme-Substrate ratio (%), PR: Protein recovery, FC: Foaming Capacity (%), FS: Foam Stability (%), EAI: Emulsifying Activity Index (m^2/g), ESI: Emulsion Stability Index (min), OAC: Oil Absorption Capacity (g/g), DPPH radical scavenging activity (%), FRAP (mM Ascorbic Acid/g protein), MC: Metal Chelating activity (mg EDTA/g protein), ABTS radical scavenging activity (%)

4.4 Conclusion

The objective of the study was to identify the optimised process conditions for deriving tuna protein hydrolysate having desirable range of functional and antioxidative properties separately by suitable statistical models. Moreover, emphasis was given to assess the dependency of these properties on degree of hydrolysis by comparing the values of hydrolysates obtained through different hydrolytic conditions, but having similar DH values. It was clear from the results that, for the parameters analysed, enzyme-substrate ratio (X_1) was more influential in explaining the response variations than hydrolysis time. Correlation studies between the degree of hydrolysis and responses recommended that though a general trend can be suggested, peptide properties can't be entirely explained based on the degree of hydrolysis but rather on the nature of polypeptide fragment formed under different hydrolytic conditions.

Chapter 5

Functional and antioxidant protein hydrolysates from yellowfin tuna raw red meat: Optimization by RSM

5.1 Introduction

Marine resources are considered as the cheapest and one of the richest sources of nutrients, especially protein with balanced amino acid pattern. However a major share of the marine biomass is being discarded as byproduct with low market realization. Awareness among consumers regarding the potential recovery of nutrients from fish waste has created increased interest in exploiting these sources. Among the seafood, tuna is considered as the richest source of protein. This nutritional significance together with its widespread economic impact on account of their contribution to international trade has made tuna waste of particular interest to upgrade. Tuna flesh consists of both white and red meat of which red meat is usually discarded as waste in seafood industry with negligible market value (Herpandi et al., 2011). Hence upgradation of the same in the form of protein hydrolysate, having immense properties can add more value as well as diversify its application potential.

As mentioned in Chapter 4, no comprehensive studies have been reported on the optimization of process conditions for the selective extraction of both functional and antioxidative peptides, separately from the same source. Hence, the present study focused on optimizing the key processing variables viz., enzyme-substrate ratio and hydrolysis time at pre-optimized temperature and pH, through RSM with a central composite design, to obtain process parameters for separately deriving the antioxidant and functional hydrolysates from yellow fin tuna raw red meat. Further, the variations in properties with respect to the extent of hydrolysis were statistically correlated. This study intended to have a comparative evaluation with respect to the extent of variations in properties exhibited by the derived hydrolysates with respect to the change in raw material used viz., raw tuna red meat instead of cooked tuna red meat which was used in the initial optimization study described in chapter 4.

5.2 Materials and methods

5.2.1 Raw material, enzyme and chemicals

Raw tuna red meat was used as the raw material and loins were collected as by-product from Moon Fishery Pvt. Ltd., Kochi, India and brought to the laboratory in iced condition. It was kept at -20^{o}C till further use. The tuna raw red meat was minced, treated with boiled water (1:4 (w/v)) for five min, cooled, treated with 0.2 % (w/v) sodium bicarbonate (1:4 (w/v)) for two min and pressed to original moisture content. This treated meat was used as the starting material for further optimization studies. Hydrolysis was carried out using papain enzyme (Hi Media) from papaya latex. All other chemicals used for the experiment were of analytical grade.

5.2.2 Process optimization for protein hydrolysis

Treated red meat mince was mixed well with twice the amount of water (w/v) to slurry form and was subjected to hydrolysis under optimized temperature

and pH. Optimization studies carried out (previously described in chapter 4, section 4.2.2) indicated a temperature of 60°C to be suitable (described in chapter 4, section 4.3.2). A physiological pH of 6.5 was adopted which was the initial pH of the substrate (treated meat and water slurry) without any added salt. RSM based hydrolysis using papain was initiated as per central composite design with the input factors viz., enzyme : substrate (E/S) concentration (X_1) and hydrolysis time (X_2) in the range of 0.25-1.5 % and 30-240 mins, respectively (Table 5.1). Hydrolysis was performed in a shaking water bath (Shaking bath, Neolab Instruments, Mumbai, India) with constant agitation. After each sampling, hydrolysis was terminated by heating the solution to 85-90°C for 15 - 20 min to assure enzyme inactivation. The resultant solution was cooled, coarse filtered and centrifuged at 8000 g at 10°C for 20 min (K-24A, Remi Instruments, Mumbai) to obtain supernatant containing protein hydrolysate solution which was spray dried (Lab 2 Advanced Laboratory type, Hemraj, Mumbai) and further used for analysis.

5.2.3 Determination of proximate composition

Proximate composition of raw tuna red meat and treated red meat was estimated as per AOAC (2012). Protein content of tuna red meat and hydrolysates were estimated by kjeldahl method (detailed in chapter 3; section 3.2.3 and chapter 4; section 4.2.4).

5.2.4 Determination of degree of hydrolysis and protein recovery

DH was evaluated as percentage of α-amino nitrogen in the hydrolysates with respect to the total nitrogen content in raw material. Total nitrogen content was determined by Kjeldahl method (AOAC, 2012) (detailed in chapter 3; section 3.2.3) and α-amino nitrogen was determined by formol titration method (Taylor, 1957) (detailed in chapter 3; section 3.2.5). PR in hydrolysate was defined as the percentage of protein obtained during the extraction process in hydrolysate to the total amount of protein in raw material.

Table 5.1 Experimental design and responses of the dependent variables to the hydrolysis conditions

Design point[a]	X_1	X_2	DH	PR	FC	FS	EAI	ESI	OAC	Bitterness	DPPH	FRAP	RP	ABTS
1	0.25	30	14.0	43.71	160	16	37.71	30.70	2.11	3	74.34	35.72	0.254	54.94
2	0.88	240	39.3	63.25	100	10	27.88	23.79	2.03	9	86.52	46.59	0.318	57.21
3	1.5	240	46.9	65.15	17	0	18.38	23.37	2.02	10	87.24	52.76	0.33	56.43
4	1.5	30	26.5	62.21	153	10	35.21	26.49	1.97	7	81.36	38.19	0.302	55.78
5	1.5	135	32.7	64.5	40	0	27.55	24.08	2	8	86.88	45.84	0.326	55.82
6	0.25	135	17.5	46.18	100	10	35.23	26.09	2.29	5	77.97	37.86	0.281	54.24
7	0.25	30	14.5	44.55	150	10	38.57	27.98	2.2	4	74.58	33.87	0.25	54.53
8	1.5	30	26.8	63.52	152	16	33.88	25.73	1.95	7	82.17	37.2	0.294	54.18
9	1.5	240	45.8	66.78	20	0	21.37	22.96	2.2	10	90.18	52.18	0.332	56.75
10	0.88	30	22.3	56.65	120	10	32.51	28.82	1.78	6	85.14	35.78	0.304	56.41
11	0.25	240	27.4	52.49	157	10	33.08	28.58	2.16	6.5	81.15	46.22	0.318	55.37
12	0.88	240	39.4	62.49	80	0	28.13	23.31	2.01	9	85.02	47.33	0.316	57.15
13	0.25	240	27.9	53.15	164	20	33.57	25.21	2.06	7	80.79	47.15	0.311	54.21

[a]Experiments were run at random, X_1: Enzyme-substrate ratio (%), X_2: Hydrolysis time, DH: Degree of hydrolysis (%), PR: Protein recovery, FC: Foaming Capacity (%), FS: Foam Stability (%), EAI: Emulsifying Activity Index (m^2/g), ESI: Emulsion Stability Index (min), OAC: Oil Absorption Capacity (g/g), DPPH (%), FRAP (mM Ascorbic Acid/g protein), RP: Reducing Power, ABTS (%)

5.2.5 Determination of functional and antioxidative properties

Functional properties of the hydrolysates viz., foaming properties (Sathe and Salunkhe, 1981); emulsifying properties(Pearce and Kinsella, 1978); oil absorption capacity (Shahidi et al.,1995) of the hydrolysates were determined. Ten trained panelists were assigned for the sensory analysis for bitterness adopting the methodology of Nilsang et al. (2005) with modifications. Antioxidative properties determined included DPPH radical-scavenging activity (Shimada et al., 1992); ferric reducing antioxidant power (FRAP)(Benzie and Strain, 1996); reducing power(Oyaiza, 1986) and ABTS radical (2,20 -azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) scavenging activity **(**Re et al., 1999) (detailed in chapter 3; section 3.2.8 and 3.2.9).

5.2.6 Statistical model development

RSM with central composite design consisted of 13 experimental points conducted in random order (Table 5.1). Second order regression models were fitted to the response variables as a function of input variables. The data from the CCD were explained by multiple regressions to fit the following second-order polynomial equation:

Second order regression: $Y = \beta_0 + \beta_i X_i + \beta_{ij} X_i X_j + \beta_{ii} X_i^2$, $i \neq j = 1,2$

where Y is the response; β_0 is the offset term; β_i, β_{ij} and β_{ii} are regression coefficients; and Xi and Xj are levels of the independent variables. Experimental data were fitted to generate tri-dimensional and contour plots from the regression and these response surfaces and contour plots were used to visualize the relationship between the response and experimental levels of each factor, and determine the optimum conditions. The models were fitted using software Design expert 7.0.

5.3 Results and discussion

5.3.1 Proximate composition

Nutritional evaluation of a food commodity is essential for appropriate food application as well as its subsequent processing and preservation strategies. Evaluation of the proximate composition of raw tuna red meat before and after treatment indicated an increase in moisture content by about 1.19 % after treatment whereas a decrease in protein content by 1.3 % post-treatment was observed (Table 5.2). The fat content also exhibited a significant decrease ($p < 0.05$) from 1.67 ± 0.04 % to 1.2 ± 0.04 %. Similar trend was seen in ash also reporting a decrease by about 0.85 %. A rise in moisture content must probably be on account of the hydration of myofibrillar proteins whereas the loss of water soluble proteins, fat and mineral during the leaching process might have resulted in decrease of these components. In general, a prior washing process is desirable, especially for fatty as well as pigmented raw materials for enhanced stability of hydrolysates as commented by Kristinsson and Rasco (2000). They suggested that hydrolysates derived from low fat raw material increase the oxidative stability of the final material.

Table 5.2 Proximate composition of yellowfin tuna red meat before and after treatment

Composition	Raw red meat	Treated red meat
Moisture	72.92^{a} ± 1.20	74.11^{a} ± 0.04
Protein	25.33^{a} ± 0.84	24.03^{a} ± 0.69
Fat	1.67^{a} ± 0.04	1.20^{b} ± 0.04
Ash	1.23^{a} ± 0.02	0.38^{b} ± 0.02

Values are expressed as Mean ± SD; n = 3

5.3.2 Optimization of process conditions

Evaluation of the effect of enzyme to substrate level (X_1) and hydrolysis time (X_2) under optimum conditions of temperature and pH by papain on tuna red meat protein was done using RSM with central composite design. A multiple regression analysis technique was performed to determine all the coefficients of linear (X_1, X_2), quadratic (X_1^2, X_2^2) and interaction (X_1X_2) terms to fit a full response surface model for the responses. The observed values for DH, protein recovery and associated properties are presented in Table 5.1. Variations in DH predicted from the independent variables viz., X_1 and X_2 showed a direct relation with a correlation coefficient of 0.85 and 0.92, respectively (Fig. 5.1a,b). Several authors have reported a direct and linear relationship between DH and hydrolysis time as well as E/S (Nilsang et al., 2005; Ovissipour et al., 2012). In the present study, DH increased with E/S, but the rate of increase reduced with higher E/S concentration. This fall in rate of increase in proportion to E/S is accounted to a decrease in the substrate concentration, enzyme inhibition or enzyme deactivation (Guerard et al., 2002).

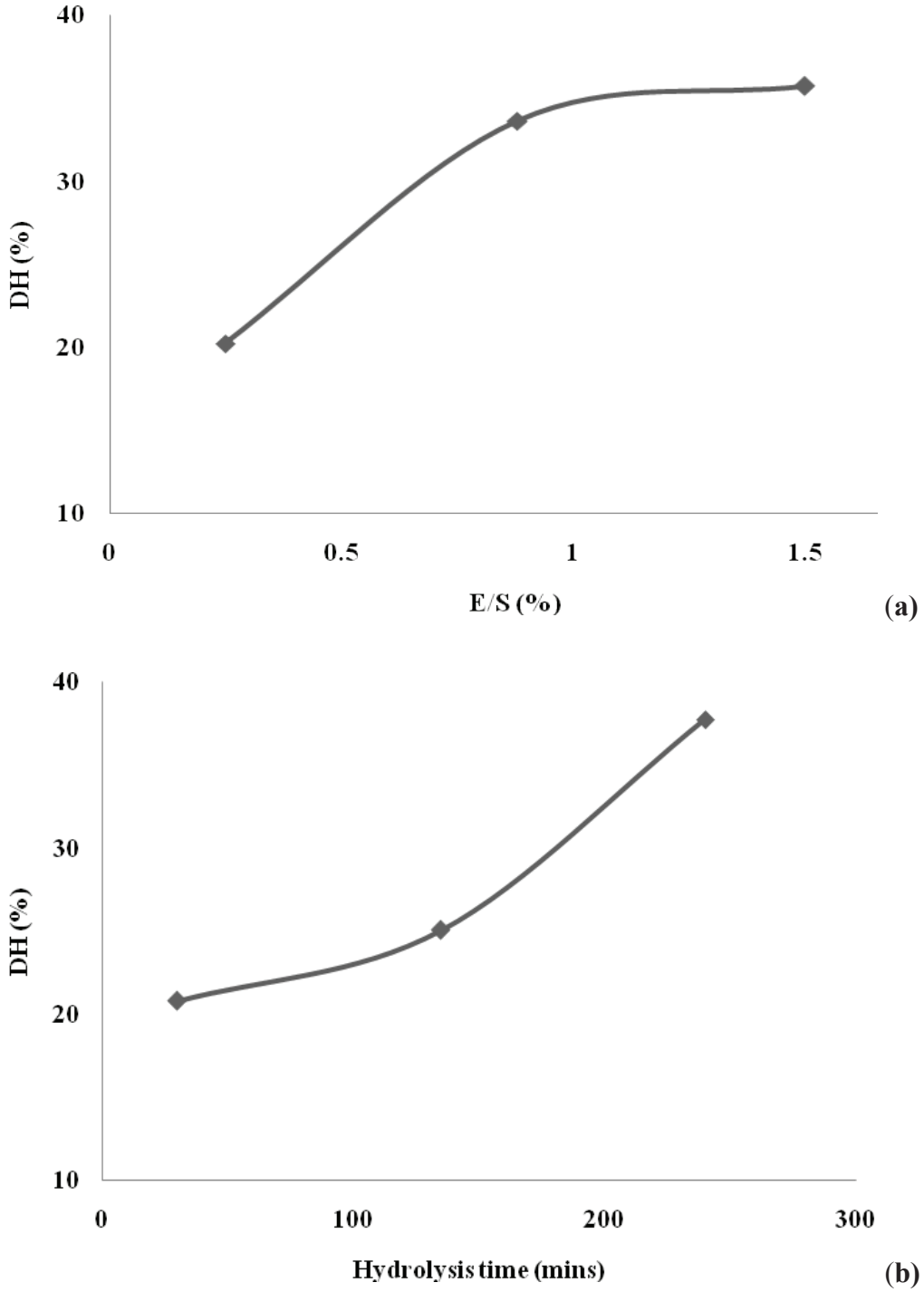


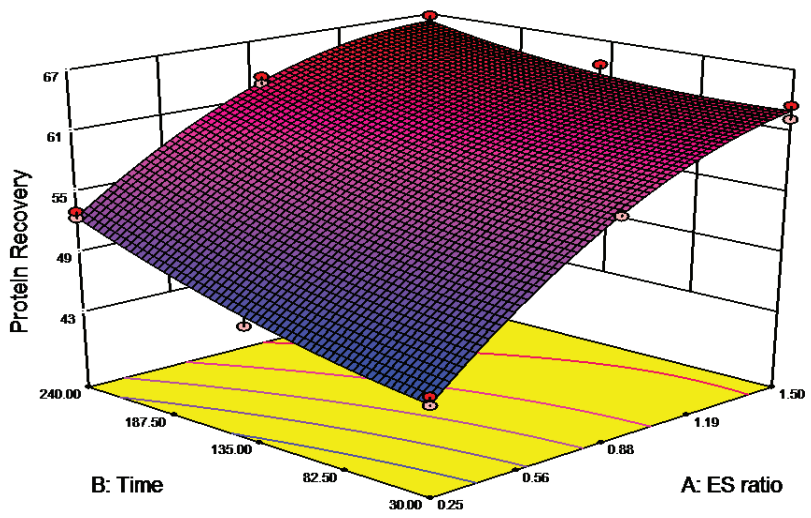
Fig. 5.1 Variations in degree of hydrolysis with **a.** E/S ratio and **b.** hydrolysis time

5.3.3 Protein recovery

Recovery of protein from raw material to the final product indicates the effectiveness of the hydrolysis process and generally a higher recovery is desirable. Variations in PR in relation to E/S and time (Fig. 5.2a) were best fitted using quadratic predictive model:

Protein Recovery = $36.154 + 30.759\ X_1^* + 0.020\ X_2^* - 0.021\ X_1X_2^* - 8.430\ X_1^{2*} + 1.002$ E-004 X_2^2

Statistical analysis reported a high coefficient of determination of 0.99 combined with a high adjusted determination coefficient of 0.99 confirming the significance and reliability of the fitted model. Linear terms of X_1 (Enzyme-substrate ratio) and X_2 (Hydrolysis time), interaction of X_1, X_2 and quadratic terms of X_1 (X_1^2) were the significant terms in the model. However X_1 was more influential than X_2 in the process for recovery of protein in hydrolysate, as indicated by the highest value of estimated regression coefficient in the model. Usually, protein recovery varies with the extent of hydrolysis, showing a direct relationship and the present study also observed a direct linear relation for this response with DH with a correlation coefficient of 0.74 (Fig.5.2b). Substantiating the higher influence of E/S from the regression equation, for similar DH, hydrolytic condition with higher E/S indicated a variation with about 10 % more protein recovery from substrate. For DH of 22 % at E/S of 0.88 % and time of 30 min had a higher recovery of 4 % in comparison to lower E/S and higher time (0.25 % and 240 min) indicating a DH of about 28 %.



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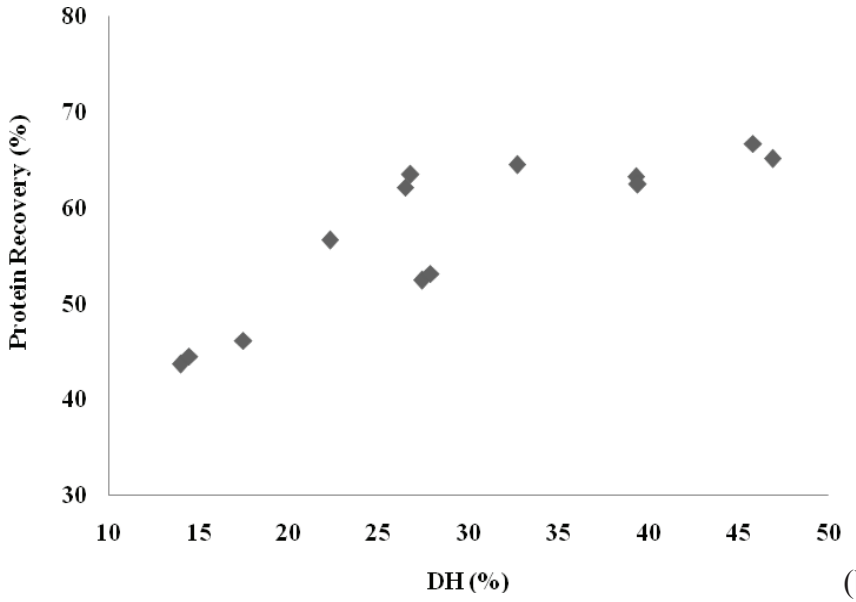


(b)

Fig. 5.2 Variations in protein recovery (%) **a.** in response to enzyme-substrate ratio and hydrolysis time; **b.** in relation to DH.

5.3.4 Functional properties

5.3.4.1 Foaming properties

Foaming properties account to be key attributes influencing the applicability of protein hydrolysates in food systems especially in beverages and aerated bakery products.The property of proteins to form stable foams is a main functional property facilitating uniform distribution of fine air cells in food structure. Foaming properties are usually expressed in terms of foaming capacity (FC) and foam stability (FS). Quadratic model with an R^2 of 0.97 and 0.75 was best fitted to explain the changes in FC (Fig. 5.3a) and FS after 3 min (Fig. 5.4a), respectively using the equation:

$$FC = 188.712 - 37.909\ X_1^* - 1.064\ X_2^* - 0.531\ X_1X_2^* + 30.709\ X_1^2 + 0.005\ X_2^{2*}$$

$$FS = 18.518 - 10.551\ X_1^* - 0.104\ X_2 - 0.057\ X_1X_2^* + 6.779\ X_1^2 + 4.762\ E\text{-}004\ X_2^2$$

The linear terms of both X_1 and X_2; interaction between X_1,X_2 and quadratic term of X_2 (X_2^2) were the significant terms ($p < 0.05$) for variations in FC whereas X_1 as well as X_1X_2 were the significant terms ($p < 0.05$) in explaining the variations in FS. The foaming properties of hydrolysate had an inverse relation with the factors and the response was more influenced by X_1 than X_2. With DH, though there was a general trend of decrease in foaming properties ($R^2 = 0.57$), hydrolysates with similar DH showed variations indicating that the properties are influenced to a higher extend by the nature of peptides formed (Fig. 5.3b). FC ranged from 17-160 % with a drastic decrease from 160 % to < 100 % from DH 28 % and higher. FC values decreased initially with E/S and then increased to reach a threshold value and further decreased. FS indicated a scattered pattern with DH with a constant stability at low DH (14 %) up to 32 % DH and thereafter showed a drastic reduction in this response from 18 % to no stability at higher DH (Fig. 5.4b). For similar range

of DH, variations of 10 % were noted. Previous reports by Vander Ven et al. (2002) suggested high molecular weight peptides to be generally positively related to FS of protein hydrolysates.

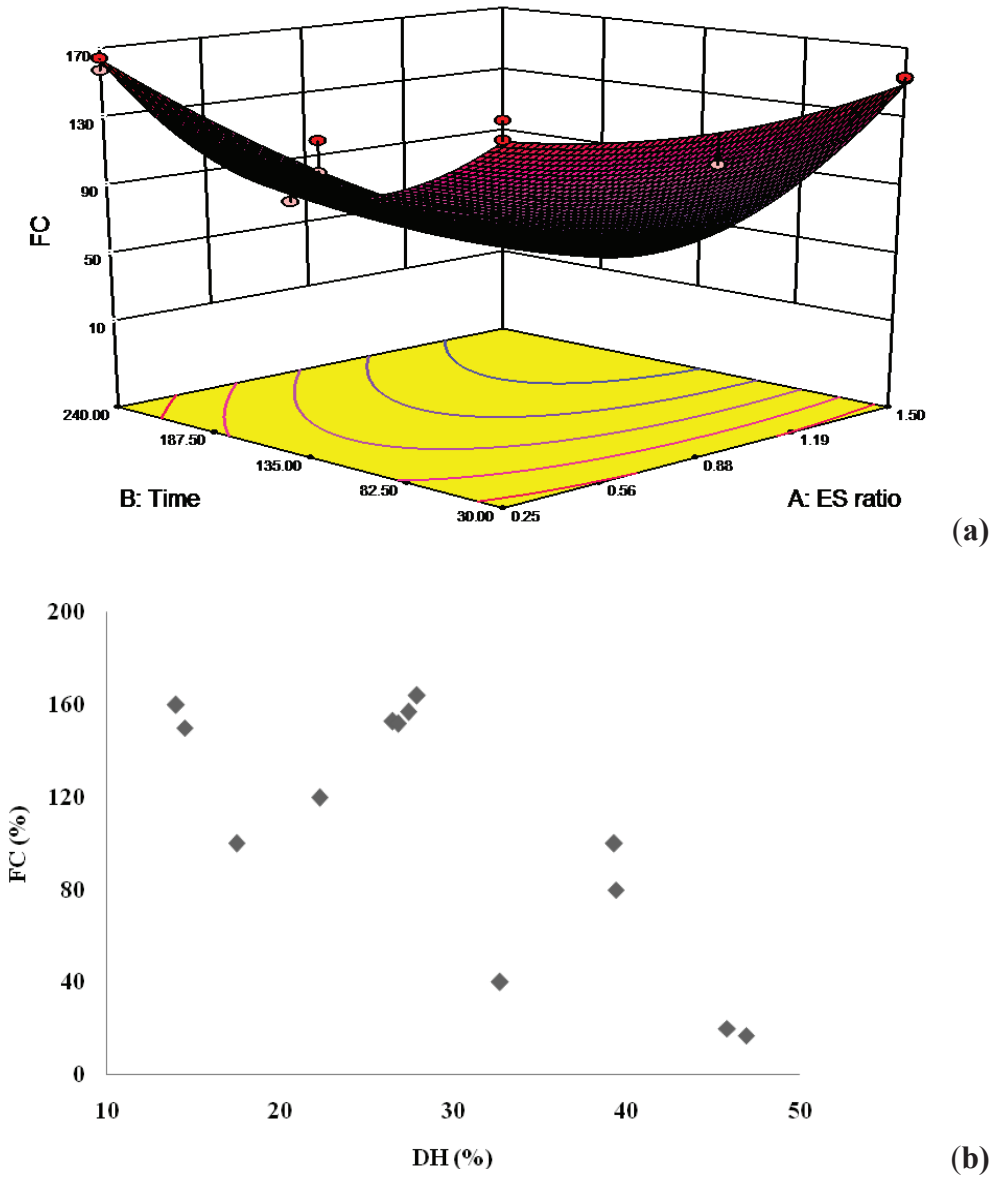
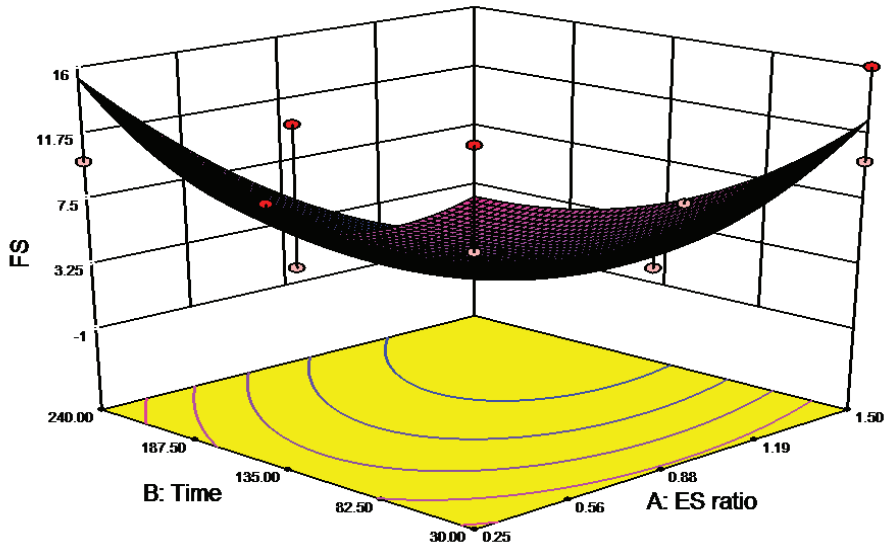


Fig. 5.3 Variations in foaming capacity (%) **a.** in response to enzyme-substrate ratio and hydrolysis time; **b.** in relation to DH.



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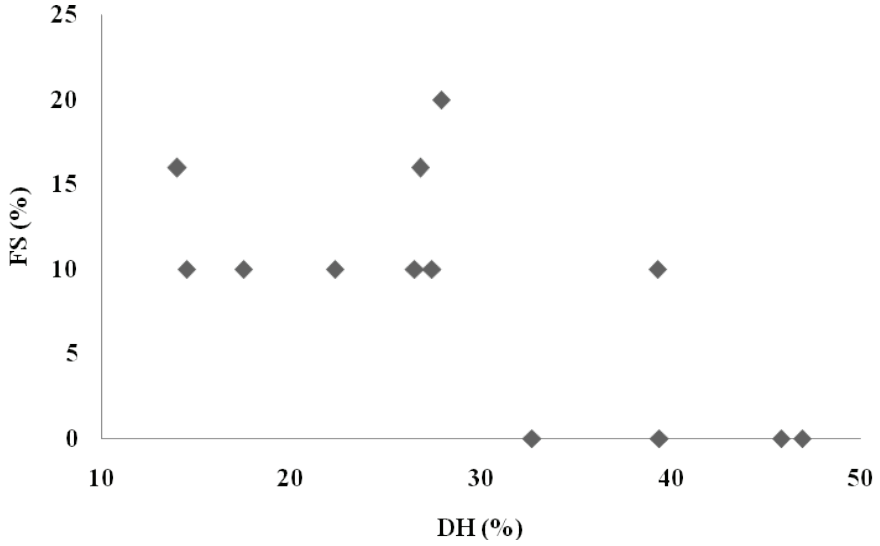


(b)

Fig. 5.4 Variations in foam stability (%) **a.** in response to enzyme-substrate ratio and hydrolysis time; **b.** in relation to DH

5.3.4.2 Emulsifying properties

Emulsifying ability determines the maximum oil quantity with a fixed amount of the protein and its stability can be determined by measuring the velocity of phase separation into water and oil during storage (Chen et al., 2014). Quadratic model with an F value of 29.37 ($p < 0.05$) was best fitted for explaining the changes in Emulsifying Activity Index (EAI). Linear and interaction of $X_1$and X_2 were the significant ($p < 0.05$) factors influencing this response (Fig. 5.5a). The model was explained using the following quadratic equation:

$$EAI = 38.862–3.959X_1^*–9.321\ E\text{-}003\ X_2^*–0.038X_1X_2^*+1.333\ X_1^2+7.370\ E\text{-}006\ X_2^2$$

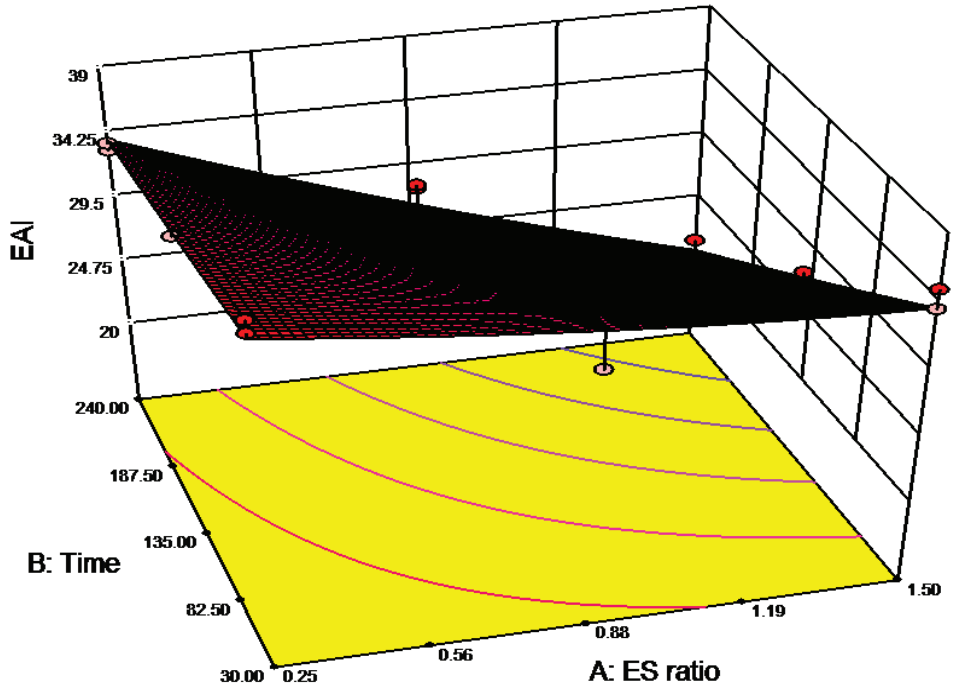
The high coefficient of determination value ($R^2 = 0.96$) as well as adjusted R^2 (0.92) indicated the reliability of this fitted model. The negative regression coefficient for X_1 and X_2 indicated that with progress of hydrolysis, the response decreased and it was more influenced by X_1 than X_2.

Emulsion stability index (ESI) was explained using quadratic regression model with an R^2 of 0.81. The equation explaining the variations in ESI in terms of coded factors was:

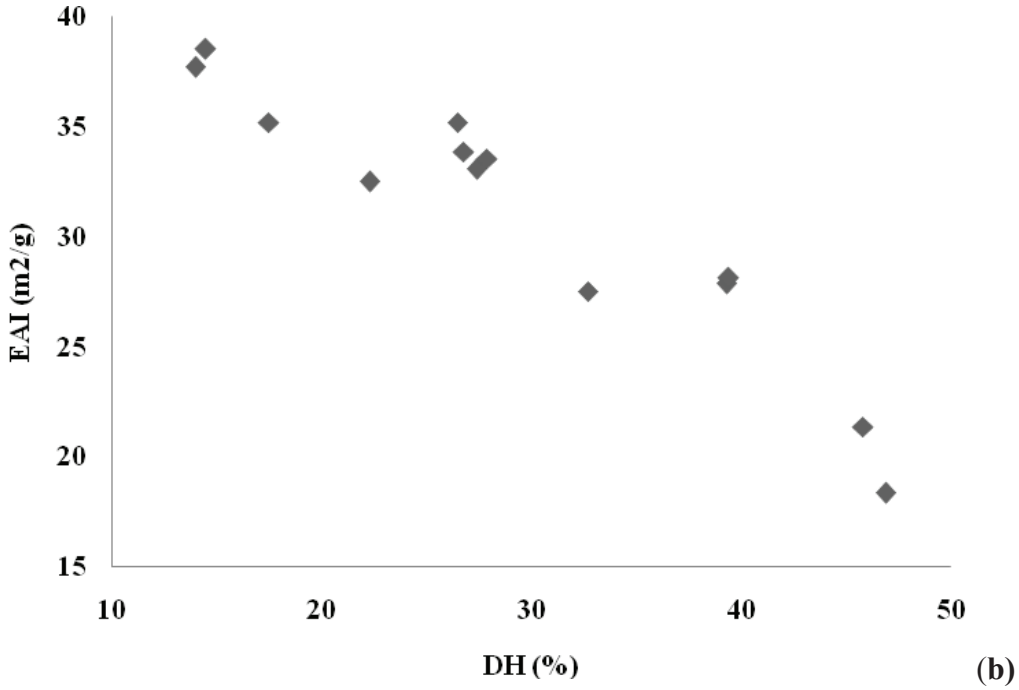
$$ESI = 31.850–4.533X_1^*–0.046X_2^*–1.944E\text{-}003X_1X_2+1.284X_1^2+1.172E\text{-}004X_2^2$$

Linear terms of X_1 and X_2 were significant ($p < 0.05$) for variations of ESI during hydrolysis (Fig. 5.6a). The study indicated an inverse relation for the factors with emulsifying properties and enzyme-substrate ratio had a better role than hydrolysis time in controlling this response. EAI and ESI had a correlation coefficient of 0.89 (Fig. 5.5b) and 0.73 (Fig. 5.6b), respectively with DH, exhibiting higher activity at lower extent of hydrolysis. On an average it decreased with time as well as enzyme-substrate concentration from 38.57 to 18.38 m^2/g and 30.7 to 22.96 min, respectively (Table 5.1). For similar DH, the resultant hydrolysates exhibited only slight variations in the properties. Rate of decrease in the emulsifying properties was higher initially followed by a lower rate of decrease as substantiated from the regression equation with negative regression coefficients for the linear terms of X_1 and X_2 followed by a positive value for their respective quadratic terms. Several authors have reported careful control on the extent of hydrolysis, as excessive hydrolysis can decrease the emulsifying capacity of protein hydrolysates. Higher the extent of hydrolysis, more the amount of smaller peptides and amino acids formed, which are less efficient in reducing the interfacial

tension due to the lack of unfolding and reorientation at the interface (Gbogouri et al., 2004; Klompong et al., 2007).



(a)



(b)

Fig. 5.5 Variations in emulsifying activity index (m^2/g) **a.** in response to enzyme-substrate ratio and hydrolysis time; **b.** in relation to DH

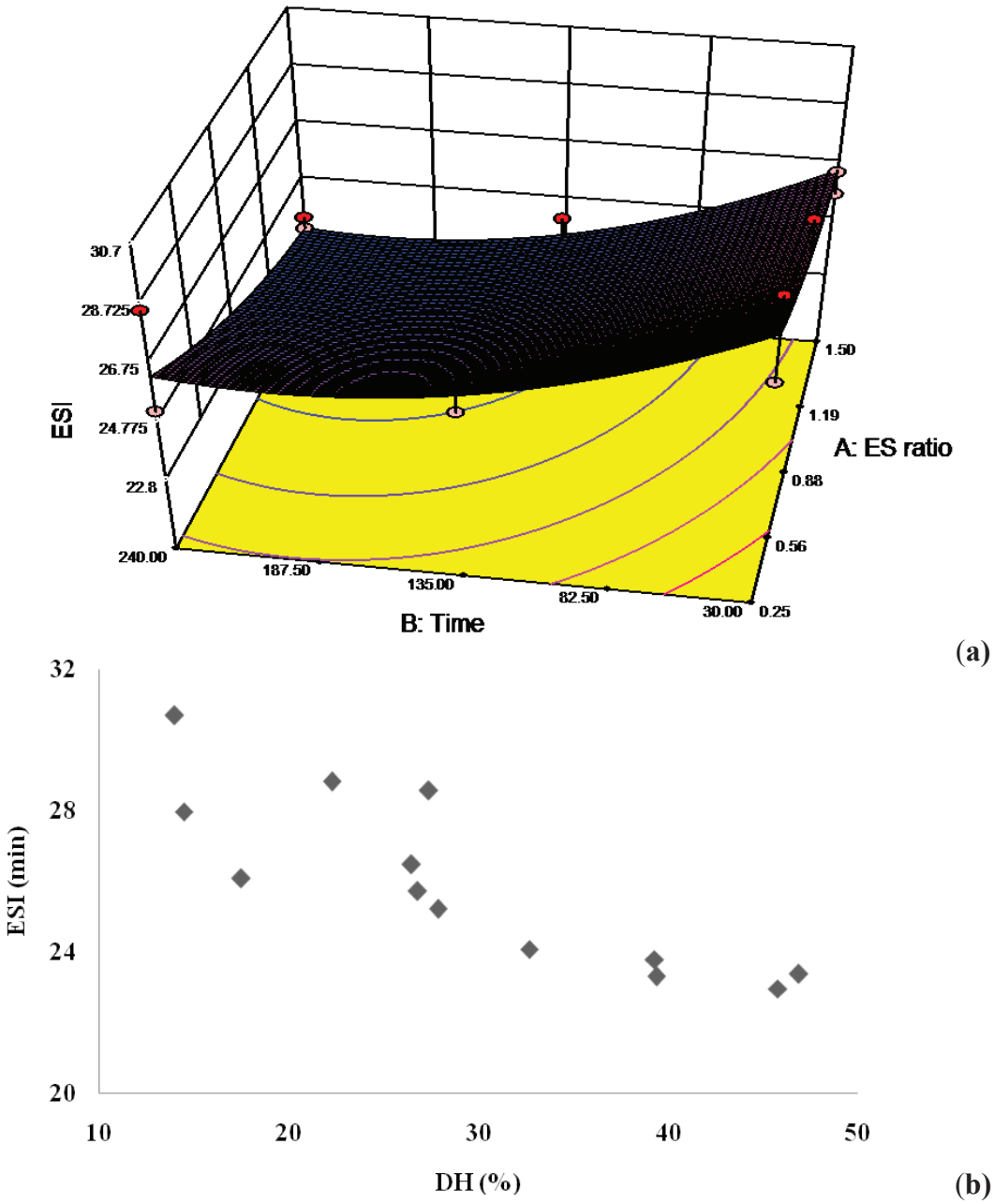


Fig. 5.6 Variations in emulsion stability index (min) **a.** in response to enzyme-substrate ratio and hydrolysis time; **b.** in relation to DH

5.3.4.3 Oil absorption capacity

OAC is correlated with surface hydrophobicity which is facilitated by exposure of more internal hydrophobic groups during hydrolysis of protein. Second order regression model with a coefficient of determination of 0.73 (Fig. 5.7a) was best fitted for explaining the variations in OAC of hydrolysates as a factor of E/S and hydrolysis time using the equation:

$OAC = 2.317 – 0.926X_1 + 1.320E\text{-}003X_2 + 7.457E\text{-}004X_1X_2 + 0.409X_1^{2*} – 5.556E\text{-}006\ X_2^2$

Second order of X_1 was the significant ($p < 0.05$) factor influencing this response with an estimated regression coefficients of 0.41 whereas the effect of linear terms of X_1 and X_2 were insignificant with respect to OAC variations. During hydrolysis, OAC ranged from 1.78 – 2.29 g/g without any specific trend of increase or decrease with the changes in factors viz., X_1 or X_2. Hydrolysates derived under the same range of DH showed high variation in the response (Fig. 5.7b) of up to 0.15 g/g which confirms that this response was dependent on type of polypeptide fragments formed. Similar findings were reported by Amiza et al. (2012) in cobia frame hydrolysates with varying range of OAC between 2.4 – 2.8 ml/g without being DH dependent.

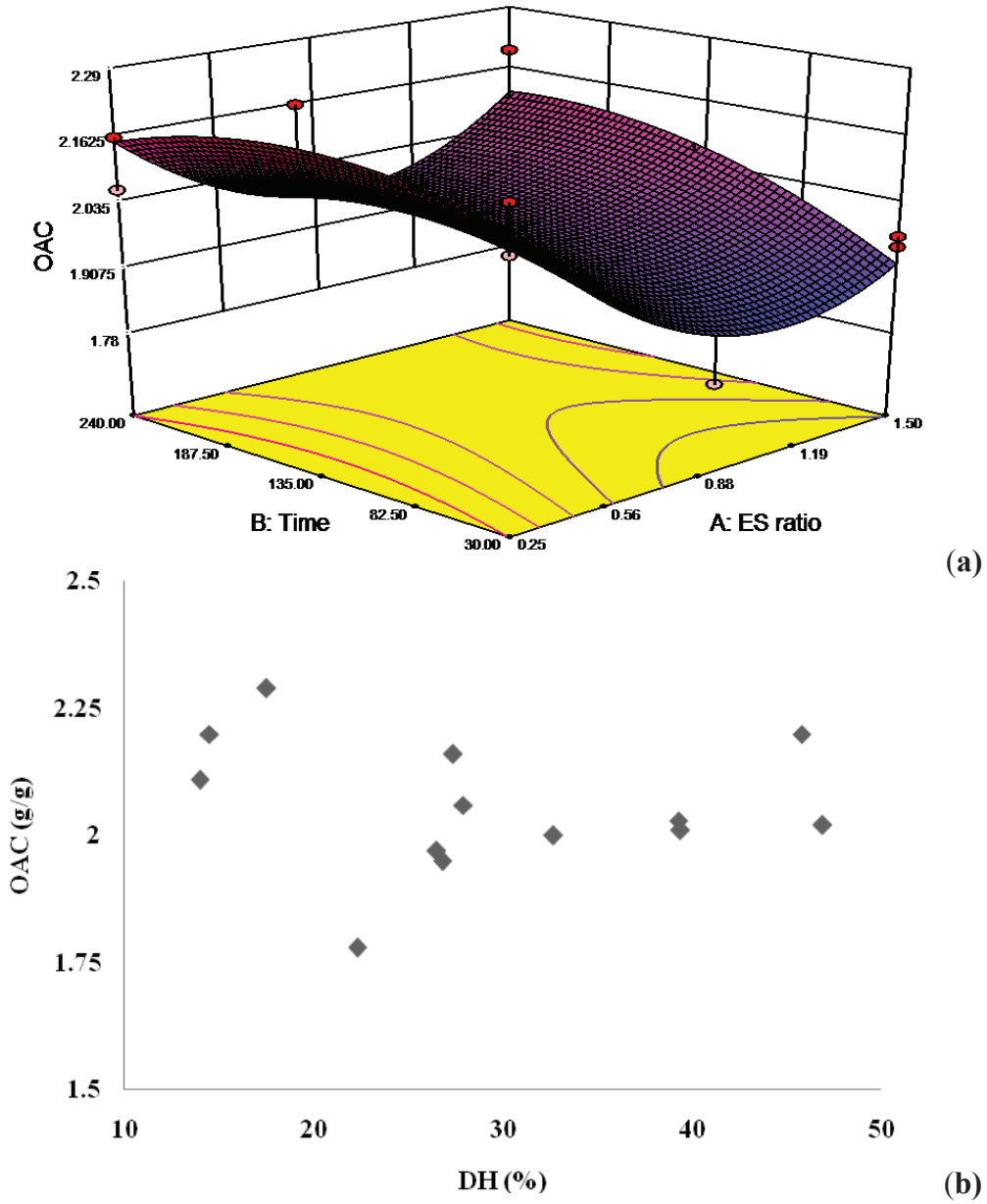


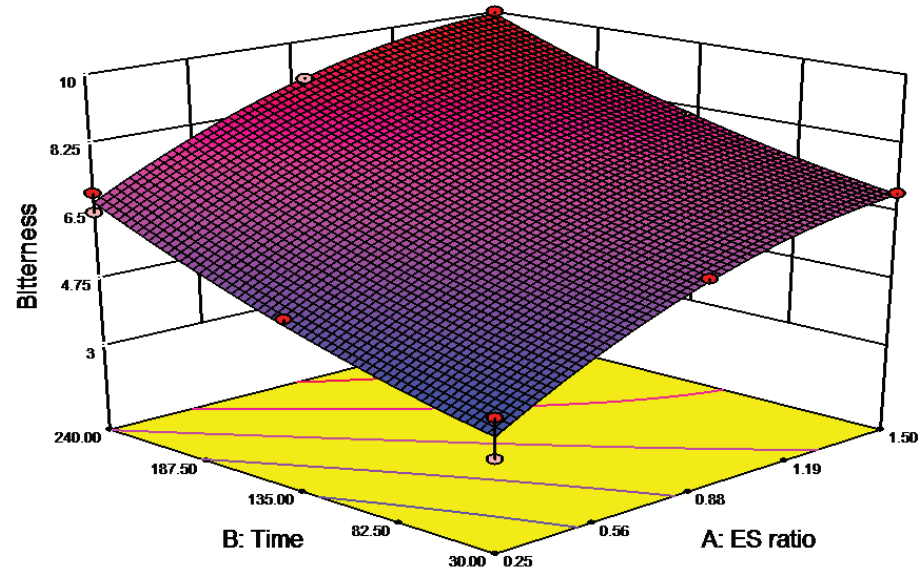
Fig. 5.7 Variations in oil absorption capacity (g/g) **a.** in response to enzyme-substrate ratio and hydrolysis time; **b.** in relation to DH

5.3.4.4 Sensory properties

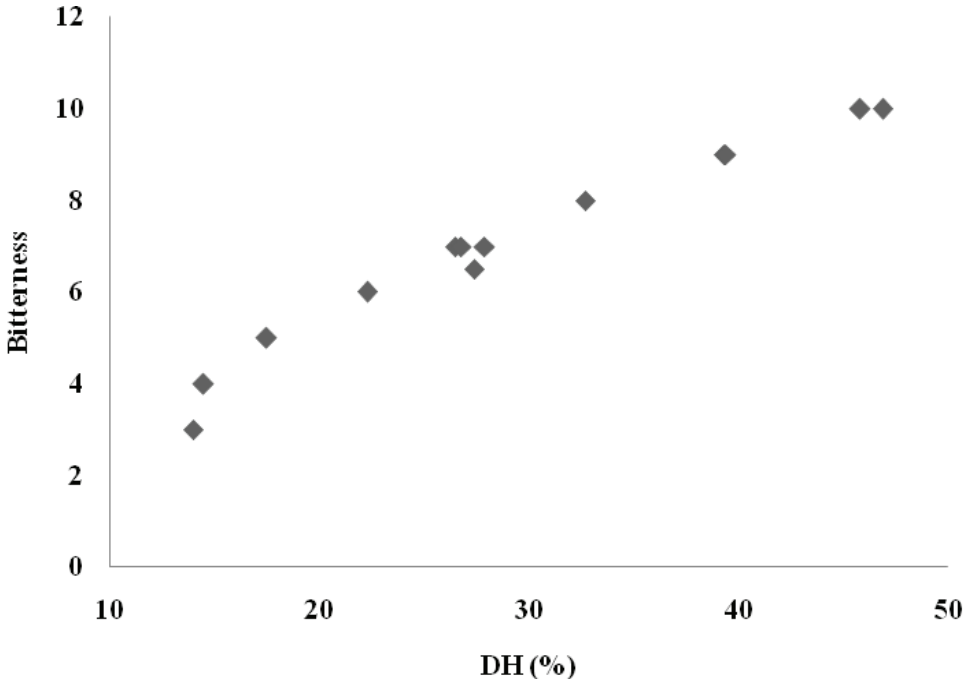
Effective utilization and commercialization of protein hydrolysate can be possible only by overcoming the major hindrance of bitterness formation which is a major characteristic associated with hydrophobic amino acids of peptides liberated during hydrolysis (Dauksas et al., 2004; Kim and Wijesekara, 2010). Many techniques have been suggested to reduce or mask bitterness in hydrolysates (Adler-Nissen, 1986; Saha and Hayashi, 2010) of which strict control of any hydrolysis experiment and termination at low degree of hydrolysis is a common and desirable method to prevent the development of a bitter taste and the retention of functional properties. Quadratic regression model with a high determination coefficient of 0.99 supported by the adjusted R^2 value of 0.98 was fitted to explain the variation of this response:

$$\text{Bitterness} = 1.972 + 5.720X_1^* + 7.915\text{E-}003X_2^* - 9.543\text{E-}004X_1X_2 - 1.687X_1^{2*} + 2.835\text{E-}005X_2^2$$

Linear term of $X_1$and X_2 as well as second order term of X_1 were the significant terms ($p < 0.05$) influencing the bitterness variations of hydrolysate. A positive regression coefficient for linear terms of X_1 and X_2 inferred that both the factors had a direct relation on the degree of bitterness. Response surface graphs generated by the predictive model to predict the critical points and the effectiveness of each factor indicated an increase in the bitterness with X_1 and X_2 (Fig. 5.8a) and a positive correlation graph between DH and bitterness ($R^2 = 0.96$) substantiated the findings (Fig. 5.8b). Response in general ranged from a score 3.0 - 9.0 and the variations between the samples having same DH under different combinations of X_1 and X_2 were minimum implying that sensory attributes of hydrolysate are more DH dependent.



(a)



(b)

Fig. 5.8 Variations in bitterness **a.** in response to enzyme-substrate ratio and hydrolysis time; **b.** in relation to DH

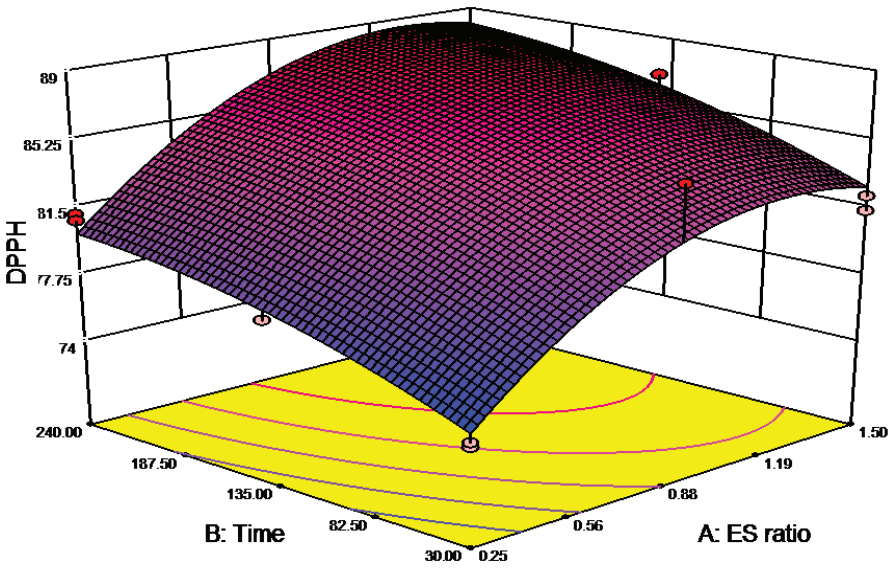
5.3.5 Antioxidative properties

5.3.5.1 DPPH radical scavenging activity

DPPH radical scavenging assay is a widely used technique to assess the efficacy of antioxidative property of a substance. This assay determines the hydrogen-donating ability of protein hydrolysates which assists in breaking of the radical chain reaction (Yarnpakdee et al., 2015). Second order regression model with an F value of 14.47 ($p < 0.05$) and R^2 of 0.91 (Fig. 5.9a)was best fitted to describe the variations in DPPH radical scavenging activity using the equation:

$DPPH = 69.179 + 20.299X_1^* + 0.047X_2^* + 1.564\ E\text{-}003X_1X_2 - 8.155X_1^{2*} - 8.605\ E\text{-}005X_2^2$

Linear regressions terms viz., X_1 and X_2 as well as second order term of X_1 were found to be significant ($p < 0.05$) and suitable for describing variations in DPPH. Regression coefficient of the models indicated direct and a better influencial role for X_1in predicting the variations in DPPH while X_2had a very marginal role. However the negative regression values for second order terms of X_1 and X_2 indicated that the rate of DPPH increased to reach a threshold beyond which there was a reduced rate of increase. DH had a direct and linear relation with DPPH indicating a correlation coefficient of 0.80 (Fig. 5.9b) and on an average it increased from 74.34 to 90.18 % (Table 5.1). The rate of increase in response was higher (up to 22 % DH) indicating upto 10 % increase and reached a stagnation with higher E/S and time with a further of only 4 % increase (40 % DH) as confirmed by the negative regression values of quadratic terms of X_1 and X_2. However the variations in this property for the hydrolysates derived under different hydrolytic conditions for the same DH was less prominent. Similar to the findings in the present study, in Nile tilapia hydrolysate, Yarnpakdee et al. (2015) observed an increase in DPPH activity with the extend of hydrolysis up to 30 % beyond which it decreased.



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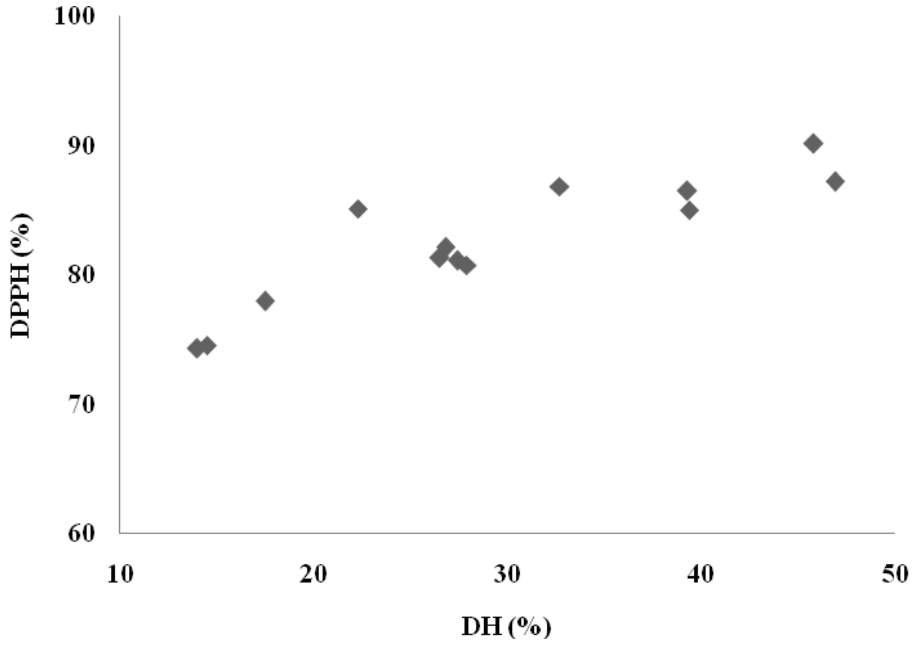


(b)

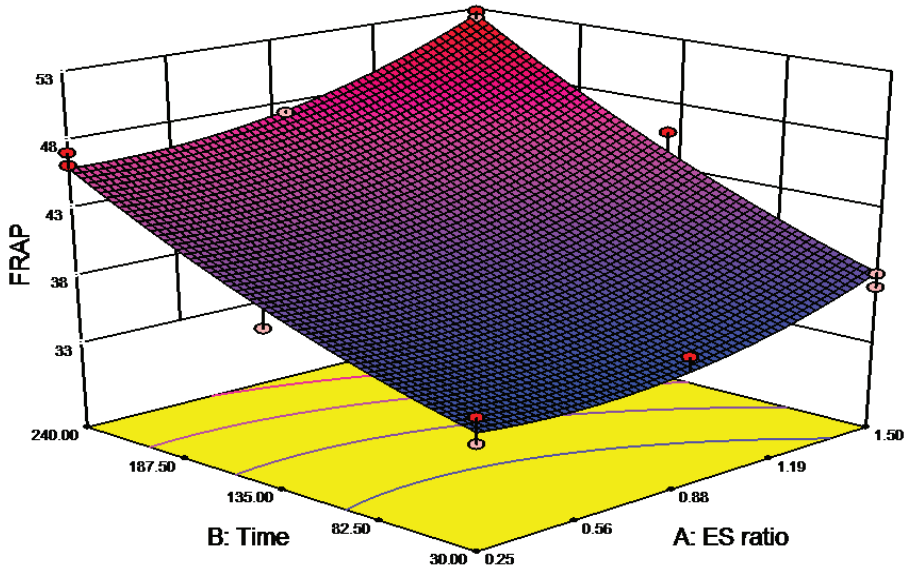
Fig. 5.9 Variations in DPPH radical scavenging activity (%) **a.** in response to enzyme-substrate ratio and hydrolysis time; **b.** in relation to DH

5.3.5.2 Ferric reducing antioxidant power

Quadratic model with a significant F value of 66.33 ($p < 0.05$) and high coefficient of determination value of 0.98 explained the variations in FRAP of hydrolysate (Fig. 5.10a) using the equation:

$$FRAP = 34.885 - 5.631\ X_1^* + 0.025\ X_2^* + 0.011\ X_1X_2 + 4.690\ X_1^2 + 9.626\ E\text{-}005\ X_2^2$$

The linear terms of E/S and hydrolysis time were the significant ($p < 0.05$) factors. Further the regression model indicated that linear term of X_1 was inversely related to FRAP while second order term (X_1^2) directly influenced this response. This inferred that though an increase in FRAP with E/S was not observed initially, with progress there was an increase in this property with E/S where as hydrolysis time had a marginal influence on this response. From the results, the potentiality of protein hydrolysate to act as a natural antioxidant was evident with a linear increase (R^2= 0.82) in FRAP with DH (Fig. 5.10b) from 33.87 to 52.76 mM AA/g protein. The rate of increase in response was not prominent initially 38 mM AA/g (up to 27 % DH) but followed a prominent increase thereafter. For similar DH, the hydrolysates exhibited a marked difference of about 9 mM AA/g protein higher for higher E/S than hydrolysis time confirming the influential role of E/S on the property. Observations made in yellow stripe trevally protein hydrolysate by Klompong et al. (2007) also suggested that the reducing power of hydrolysate was dependent on the DH and enzyme used.



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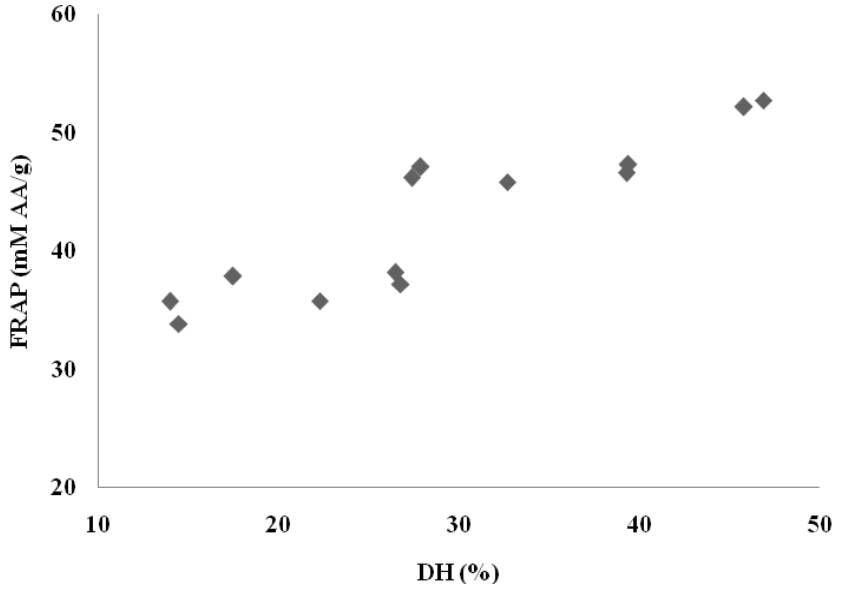


(b)

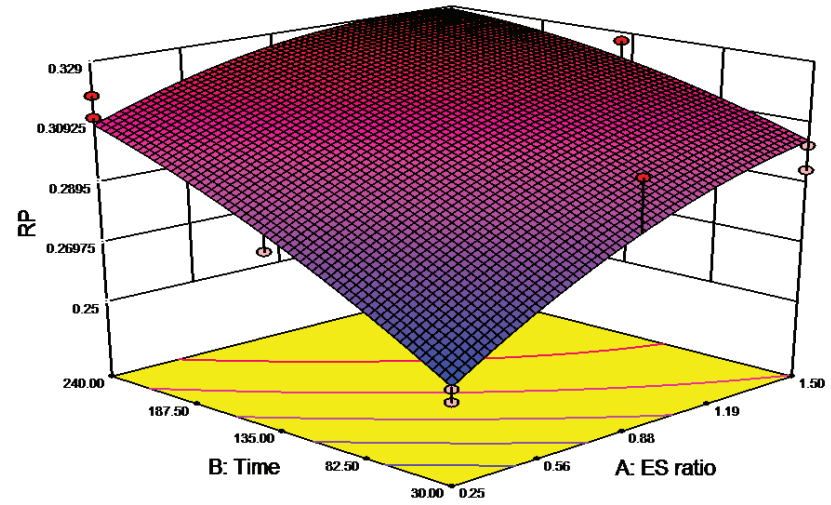
Fig. 5.10 Variations in FRAP (mM AA/g) **a.** in response to enzyme-substrate ratio and hydrolysis time; **b.** in relation to DH

5.3.5.3 Reducing power

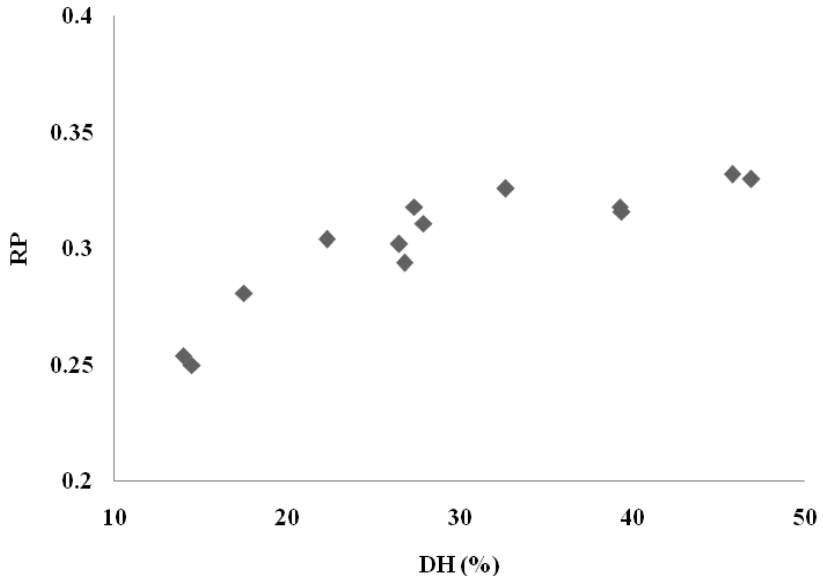
Protein hydrolysates donate electrons to scavenge the free radicals that promote oxidation. Quadratic model with a significant F value ($p < 0.05$) of 14.71 and $R^2$of 0.91 was best fitted (Fig. 5.11a) to explain the variations in reducing power using the equation:

$$RP = 0.227+0.075X_1^*+3.982\ E\text{-}004\ X_2^*-1.129\ E\text{-}004\ X_1X_2-0.018\ X_1^2-4.195\ E\text{-}007\ X_2^2$$

Linear order of X_1 and X_2 had direct, marginal but significant ($p < 0.05$) role in the explaining the variations of RP. In the present study, the reducing power was comparatively lower for large molecular weight peptides and it ranged from 0.250 – 0.332, showing a linear and direct relation ($R^2 = 0.76$) with the degree of hydrolysis (Fig. 5.11b). The rate of increase was rapid initially from 14-27 % DH reaching 0.315 followed by a stagnation. Similar to FRAP, in RP also for hydrolysates with similar DH under different hydrolytic conditions, a variation of upto 0.017 units were observed with higher property when E/S was higher in comparison to hydrolysis time. Hence the results indicate that reducing properties of hydrolysates are influenced by the nature of peptide fragments formed during hydrolysis and that it varied with the hydrolysis conditions, for similar DH. Sarmadi and Ismail (2010) proposed that antioxidative properties of hydrolysate are affected by peptide structure and its amino acid sequence.



(a)



(b)

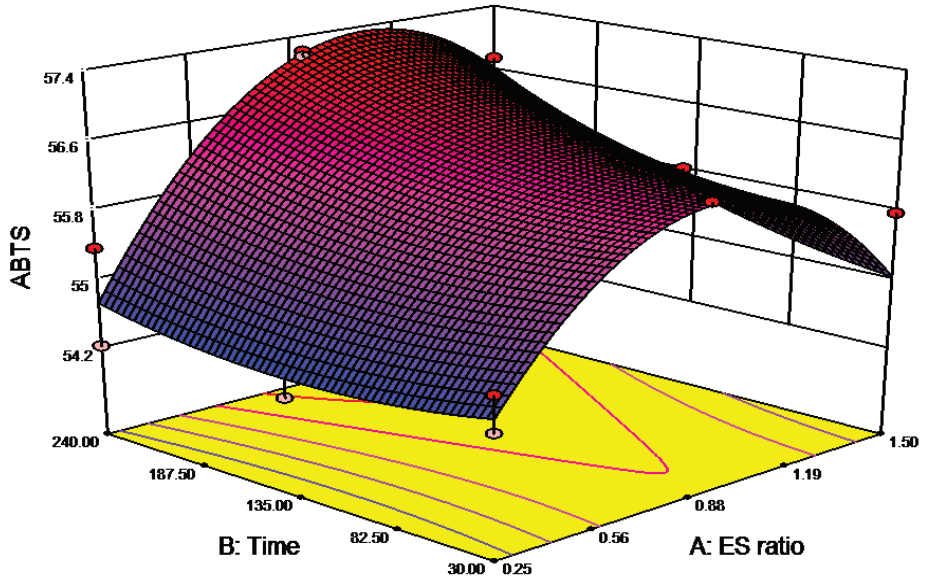
Fig. 5.11 Variations in reducing power **a.** in response to enzyme-substrate ratio and hydrolysis time; **b.** in relation to DH

5.3.5.4 ABTS radical scavenging activity

ABTS radical is relatively stable and gets readily reduced by antioxidants. Second order regression model with an R^2 value of 0.85 (Fig. 5.12a) explained the variations of this response using the equation:

$$ABTS = 53.367+6.865X_1^{*}-7.269\ E\text{-}003X_2^{*}+5.923\ E\text{-}003X_1X_2-3.861X_1^{2*}+2.211\ E\text{-}005\ X_2^2$$

Linear and second order regression of and linear terms of X_2 were significant ($p < 0.05$) for the variations in ABTS of hydrolysates with X_1being more influencial in explaining the variations in ABTS and was directly related. However the rate of increase in ABTS with $X_1$was found to be affected with hydrolysis as indicated by negative regression coefficient for X_1^2. This indicates that higher ABTS radical scavenging activity is obtained up to a limited extent of hydrolysis. Similar reports in loach protein hydrolysis substantiate these findings (You et al., 2009). Though a general trend of increase in ABTS activity was observed with hydrolysis, a wide variation in this response for similar DH was observed as in the case of other properties (Fig. 5.12b) ranging on an average, from 54 – 57 %. Up to 0.88 % E/S, a rapid increase was seen followed by a slight decline. This indicates that the individual hydrolytic factors viz., E/S, time etc. as well as their combination effect determine the type of polypeptides formed during hydrolysis which in turn influence the properties of the resultant hydrolysate.



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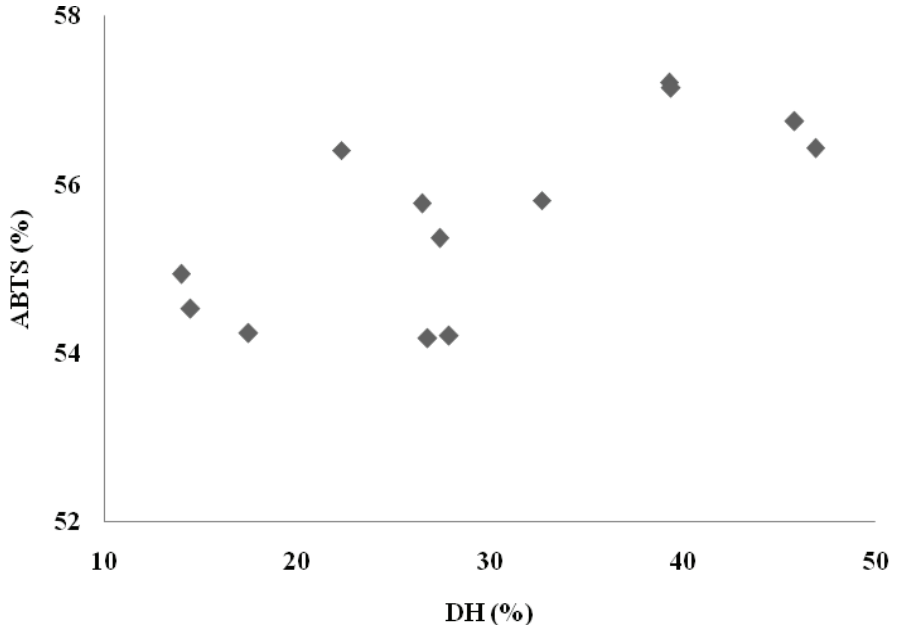


(b)

Fig. 5.12 Variations in ABTS radical scavenging activity (%) **a.** in response to enzyme-substrate ratio and hydrolysis time; **b.** in relation to DH.

The hydrolytic condition was optimised to derive protein hydrolysates with superior functional and antioxidative property, separately giving emphasis to the protein recovery (Table 5.1). Optimized hydrolytic conditions for functional properties was obtained with an enzyme-substrate ratio (E/S) of 0.41% and 30 min hydrolysis time at 60^{o}C and pH of 6.5 with a desirability of 0.611. Similarly, the optimum conditions to exhibit the highest antioxidative properties with a desirability of 0.932 were: 1.28 % E/S and 240 minutes hydrolysis time. This combination was used to derive the desired tuna red meat protein hydrolysates. Further all the response variables of the final product were validated. The experimental and predicted values were within the range and did not differ statistically ($p < 0.05$), thus confirming the reliability of the optimised condition. As suggested by Binsi et al. (2016), sequential hydrolysis can be explored as a feasible option to derive functional and antioxidative peptides from a single hydrolysis process.

Table 5.3 RSM optimized hydrolytic conditions and corresponding responses

Hydrolytic conditions		**Responses**						
E/S	**Time**	**PR**	**FC**	**FS**	**EAI**	**ESI**	**OAC**	**Bitterness**
0.41	30	47.81	144.05	11.93	36.72	28.91	2.05	4.29
		47.05	146.67	10.67	35.81	27.75	2.09	4.17
		PR	**DPPH**	**FRAP**	**RP**	**ABTS**		
1.28	240	65.76	88.52	50.36	0.329	57.17		
		64.89	89.01	49.87	0.334	56.65		

E/S: Enzyme-Substrate ratio (%), PR: Protein recovery, FC: Foaming Capacity (%), FS: Foam Stability (%), EAI: Emulsifying Activity Index (m^2/g), ESI: Emulsion Stability Index (min), OAC: Oil Absorption Capacity (g/g), DPPH (%), FRAP (mM Ascorbic Acid/g protein), RP: Reducing Power, ABTS (%)

5.4 Conclusion

Valorization of tuna red meat, a protein rich by product from tuna processing industry, to the form of protein hydrolysate by optimization of the process conditions viz., E/S and time, based on protein recovery, functional and antioxidative activities were attempted. Hydrolytic conditions for optimized functional and antioxidant properties were separately derived based on RSM for further food and nutraceutical applications. Determinants of coefficients of the fitted models explaining the variations in the responses ranged from 0.73-0.99, indicating the fitness of the models. Based on the observations in the present study, it may be concluded that enzyme-substrate ratio (X_1) is more influential in explaining the differences in the properties of hydrolysates than hydrolysis time during the hydrolysis of yellowfin tuna red meat using the enzyme papain. Comparative studies done on cooked and raw tuna red meat indicated protein recovery during hydrolysis to be higher from raw tuna red meat in comparison to cooked meat. Hydrolysate from cooked tuna red meat exhibited superior functional properties except OAC, whereas except ABTS radical scavenging activity, hydrolysates from raw tuna red meat exhibited superiority with regard to antioxidative activities. The present study widens the possibility for further exploration of tuna red meat hydrolysates for commercialization purpose.

Chapter 6

Characterization and storage stability of the optimized functional and antioxidant tuna protein hydrolysates

6.1 Introduction

Tuna represents an important element of the global fish and seafood economy with an annual value of 42.2 billion US dollar (Galland et al., 2016). The demand for thermally processed commodities has boosted the importance of tuna species in seafood industry. However as mentioned earlier, the enormous quantities of throw-outs generated during its processing has urged in the effective recovery and its intelligent utilization and in this respect deriving bioactive hydrolysates is a promisive option.

In the present study, attempts were made to characterize the optimized functional hydrolysate and antioxidant hydrolysate from yellowfin tuna cannery waste (chapter 4) with respect to the nutritional, structural, morphological, thermal as well as physico-chemical aspects. Although the specific health benefits from different hydrolysates are mostly supportable scientifically, the consistency of these benefits is debatable on account of the quality changes during storage. Hence considering this aspect, the optimized hydrolysates were subjected to storage stability studies under chilled as well as ambient conditions. Further, based on the

laboratory-scale evaluations, attempts were made to model large scale production of the optimized tuna protein hydrolysates and economic feasibility analysis was worked out.

6.2 Materials and methods

6.2.1 Raw materials and chemicals

Spray dried hydrolysates derived from tuna red meat using papain enzyme according to an RSM based protocol (previously discussed in chapter 4) for optimum functional and antioxidative properties were used for the study. The optimized hydrolytic conditions for deriving functional hydrolysate (FTPH) were viz., enzyme: substrate (E/S) ratio of 0.34 %, hydrolysis time of 30 min, temperature of 60^{o}C and pH of 6.5. For antioxidant hydrolysate (ATPH), the hydrolytic conditions were enzyme: substrate (E/S) ratio of 0.98 %, hydrolysis time of 240 min, temperature of 60^{o}C and pH of 6.5. All the chemicals used for the study were of analytical grade.

6.2.2 Characterization studies

6.2.2.1 Degree of hydrolysis and proteolytic activity

Degree of hydrolysis was estimated as per the methodology described by Hoyle and Merritt (1994) (described in chapter 4, section 4.2.5). Proteolytic activity of the sample was projected from the tyrosine content of the protein hydrolysate which measured the extent of hydrolysis under given conditions (Gajanan, 2014) (described in chapter 3, section 3.2.5).

6.2.2.2 Protein recovery and yield

Protein recovery and yield in hydrolysate was evaluated (described in chapter 3, section 3.2.3 and 3.2.4).

6.2.2.3 Determination of molecular weight

Molecular weight cutoff devices (Amicon®Ultracel®, Merck Millipore Ltd, Ireland; Fig. 6.1) viz., 100kDa, 50kDa, 30kDa, 10kDa and 3kDa, with the capability for high concentration factors and easy concentrate recovery from dilute and complex sample matrices were used. Processing time varied from 10 to 40 min depending on the cut-offs. A known concentration of the protein solution was taken in centrifuge tubes with cut-off filters and subjected to pre-set centrifuging conditions. The device was spun in a fixed-angle rotor centrifuge (K-24A, Remi Instruments, Mumbai). The concentrate was collected from the filter device sample reservoir using a pipettor, while the ultrafiltrate was collected from the provided centrifuge tube. Further the volume and protein concentration of the filtrate collected was analysed for determining the molecular weight distribution pattern of peptides.

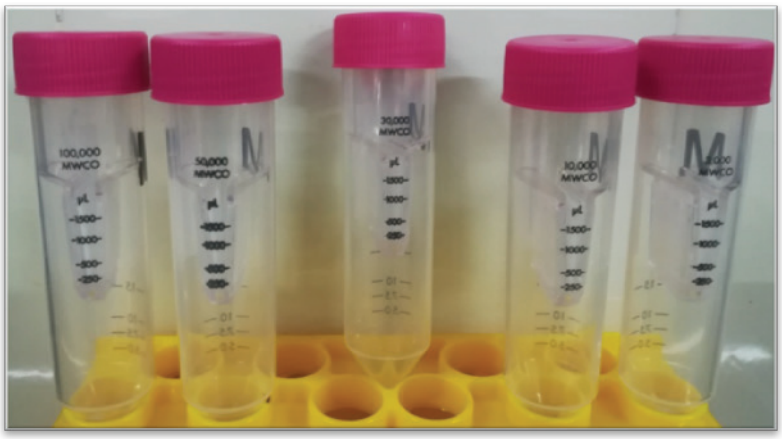


Fig. 6.1 Molecular weight cut-off devices

6.2.2.4 Nutritional profiling

6.2.2.4.1 Proximate composition

Proximate composition of tuna protein hydrolysates were estimated as per AOAC (2012). Protein content of tuna red meat and hydrolysates were estimated by kjelhdahl method.

6.2.2.4.2 Amino acid profile

HPLC (high-performance liquid chromatography) (Shimadzu Prominence, Japan) was employed for amino acid profiling of the hydrolysate (Ishida et al., 1981). A precolumn derivatization method was developed for the high-performance liquid chromatographic (HPLC) determination of amino acids using o-phthalaldehyde (OPA) and 9-fluorenylmethyl-chloroformate (FMOC). Poroshell HPH-C_{18} column with the following specifications: 4.6 mm dia, 100 mm length and 2.7 µ particle size; gradient mobile phase using phosphate and borate buffer at a pH of 8.2 was employed. The instrument was equipped with UV detector (Shimadzu SPD-20A, Japan) and a wavelength of 338 nm was employed for detecting amino acids except for proline which was detected at 262 nm. The oven temperature was maintained at 40°C and the run time was 30 min.

Fig. 6.2 Amino acid analyser

6.2.2.4.3 Mineral profile

Inductivity Coupled Plasma–Optical Emission Spectrometer (iCAP 6300 Duo, Thermo fisher Scientific, Cambridge, England) with dual configuration (axial and radial) and iTEVA (version 2.8.0.97) operational software was used for

elemental analysis. For mineral profiling, samples were digested under specific conditions (Table 6.1) for 40 min in a microwave assisted extraction system, Milestone START D (Milistone Srl., Italy), set with easy CONTROL software and HPR 1000/10S high pressure segmented rotor. The digested samples were diluted with ultra-pure water in known volume prior to elemental profiling.

The experimental conditions used in the determination of the above elements are shown in Table 6.2. ICP multi-element standard solution (CertiPUR, Merck, Mumbai, India) was used for the preparation of calibration solutions. Yttrium was used as internal standard.

Table 6.1 Microwave digestion conditions in Milestone START D[a]

Sample	1.0 g
Nitric acid (HNO_3) (TraceMetal™ Grade, Fisher Scientific)	8.0 ml
Hydrogen peroxide (H_2O_2) (30-32%, Optima, Fisher Scientific)	2.0 ml
Pressure	400 psi (max.)
Power	1200 W
Temperature [b]	Step I: Ramp to 150°C over 30 minutes Step II: Hold at 150°C for 10 minutes Step III: Allow to cool to room temperature over 1 hour

[a] microwave condition and digestion procedures were adapted from Milestone Cookbook Digestion Rev. 03_04

[b] Temperature and pressure sensors were used to monitor digestion conditions and to prevent over-pressurization of vessels

Table 6.2 Experimental conditions for elemental analysis using ICP-OES

Optics temperature	38°C
Camera temperature	-44°C
Nebulizer	MiraMist, Cyclonic Chamber
Main Argon flow rate	15 L min^{-1}
Auxiliary Argon flow rate	0.5 L min^{-1}
Nebulizer gas flow	0.5 L min^{-1}
Maximum integration time	30 sec

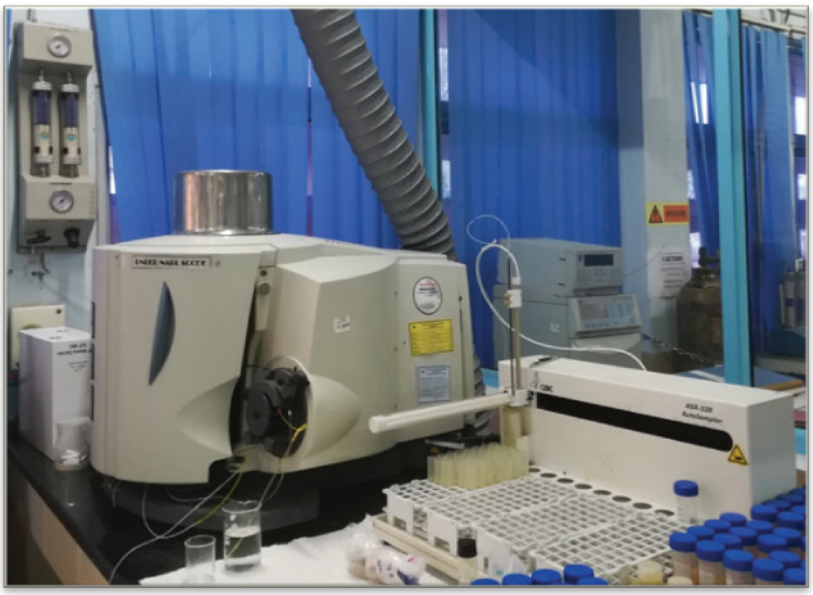


Fig. 6.3 Inductivity Coupled Plasma–Optical Emission Spectrometer

6.2.2.5 Morphological and thermal characteristics

6.2.2.5.1 Scanning electron microscopy

Surface morphology of hydrolysate sample was determined in SEM (Philips XL 30, The Netherlands). Samples fixed onto double-sided adhesive carbon tabs mounted on SEM stubs were further coated with gold in vacuum using sputter coater, and examined at 10 kV under a magnification of 650 x.

6.2.2.5.2 Differential scanning colorimetry

Thermal characteristics of the sample were determined by employing differential scanning calorimeter. Samples (about 3-5 mg, dry basis) were weighed and placed on DSC aluminum pans and equilibrated over saturated salt solutions in desiccators at 25°C. The samples were then hermetically sealed with lids for analysis and weighed. The DSC analysis was performed in a TA-MDSC-2920(Ta Instruments, New Castle, De, USA) in an inert atmosphere (45 ml/min of N_2). Instrument calibration was carried out with Indium (T melting = 156.6°C). Thermo-analytical curves were obtained by heating/cooling the samples at a constant rate of 10°C/min and a temperature ramp of 20°C to 170°C under nitrogen atmospheric condition. From the DSC thermogram, the temperature at onset (To), peak (Tp), the end (Tf), and the enthalpy (DH) were determined.

6.2.2.5.3 Fourier-transform infrared spectroscopic analysis

FTIR spectrometer (Shimadzu IR-Prestige-21) by KBr method using diffuse reflectance assembly was adopted to determine the spectra of the sample in the wavelength range of 4500 to 400 cm^{-1}. A known quantity of sample mixed with potassium bromide (1:100 (w/w)) was analysed for the reflectance spectra with recordings of upto 64 scans under a resolution of 4 cm^{-1}. By using Kubelka-Munk algorithm, the reflectance spectra were further transformed to transmission spectra.

6.2.2.6 Physico-chemical characteristics

6.2.2.6.1 Hygroscopicity

Sample hygroscopicity was adopted from the methodology of Cai and Corke (2000) with slight modifications. A known quantity of the sample was kept in desiccators containing saturated solution of NaCl (relative humidity of 75.3 %)

at 25 °C for a period of one week. Sample was re-weighed after the observation period and hygroscopicity was measured as percentage of the amount of moisture absorbed to that of the initial dry sample.

6.2.2.6.2 Bulk density and tapped density

Flow properties exhibited by the samples were determined by measuring the bulk density (ρB) and tapped densities (ρT) (Chinta et al., 2009). For this, a known quantity of sample powder was loosely filled into a graduated cylinder using a funnel by slight tapping so as to assemble the powder sticking to the wall of the cylinder. Bulk density of the sample was measured from weight of the sample to the volume it occupied. Similarly, protein hydrolysate powder was filled into the cylinder with tapping until it reached a constant volume to measure the tapped density.

Hausner ratio and Carr Index, suggestive of the flow properties were determined from the bulk and tapped densities as:

$$\text{Hausner ratio} = \rho T / \rho B$$

$$\text{Carr index} = 100\ (1 - \rho B / \rho T)$$

6.2.2.6.3 Colour and browning intensity

Colour of the samples were measured using Hunter- Lab scan XE – Spectrocolorimeter (Color Flex, Hunter Associates Laboratory, Reston, USA.) at D-65 illuminant and 10° observer. Pre-determined quantity of samples were filled in a 64 mm glass sample cup and results were expressed in terms of L*(lightness), a*(+redness/-greenness), b*(+yellowness/-blueness). The instrument was calibrated using white and black standard ceramic tiles and the readings were recorded in the inbuilt software.

Absorbance of known concentration of filtered samples (80 mg ml^{-1}) were measured at 420 nm in spectrophotometer (Lambda 25 UV/Vis, Perkin Elmer Life and Analytical Sciences, Singapore) and expressed as browning intensity.

6.2.2.7 Functional and bioactive characteristics

6.2.2.7.1 Foaming properties

Foaming capacity and stability of fish protein hydrolysate were determined according to the modified method of Sathe and Salunkhe (1981). Protein solution (1.0 %) was prepared and the pH was adjusted to 2, 4, 6, 8 and 10. This protein solution was whipped for 2 min at a speed of 16,000 rpm using a homogenizer (230 VAC T-25 digital Ultra-turrax, IKA, India) and poured into a 100 ml graduated cylinder. The total sample volume was taken immediately for foam capacity. The foaming capacity was calculated according to the following equation:

$$FC\ \% = \frac{V_2 - V_1}{V_1} \times 100$$

where V_2 is the volume after whipping (ml) and V_1 is the volume before whipping (ml). The whipped sample was allowed to stand at room temperature for 3 min and the volume of whipped sample was then recorded. Foam stability was calculated as follows:

$$FS\ \% = \frac{V_2 - V_1}{V_1} \times 100$$

where V_2 is the volume after standing (ml) and V_1 is the volume before whipping (ml). Similarly the foaming capacity and foam stability of protein solution at different concentration viz., 0.1, 0.5, 1.0, 2.0 and 3.0 %, under neutral pH was determined.

6.2.2.7.2 Emulsifying properties

Emulsifying properties were determined according to the method of Pearce

and Kinsella (1978). Vegetable oil (10 ml) and protein solution (30 ml, 1%) adjusted to a pH of 2, 4, 6, 8 and 10 were mixed and homogenized using a homogenizer (230 VAC T-25 digital Ultra-turrax, IKA, India) at a speed of 20,000 rpm for 1 min. An aliquot of the emulsion (50 µl) was pipetted from the bottom of the container at 0 and 10 min after homogenization and mixed with 5 ml of 0.1% sodium dodecyl sulphate (SDS) solution. The absorbance of the diluted solution was measured at 500 nm using a spectrophotometer (Lambda 25 UV/Vis, Perkin Elmer Life and Analytical Sciences, Singapore). The absorbance was measured immediately (A_0) and 10 min (A_{10}) after emulsion formation was used to calculate the emulsifying activity index (EAI) and the emulsion stability index (ESI) as follows:

$$EAI\ (m/g^2) = \frac{2 \times 2.303 \times A_0}{0.25 \times Wt\ of\ protein}$$

$$ESI\ (min) = \frac{A_{10} \times \Delta t}{\Delta A}$$

Δt =Time, $\Delta A = A_0 - A_{10}$

Similarly, the emulsifying properties of protein solution at different concentrations viz., 0.1, 0.5, 1.0, 2.0 and 3.0 % at neutral pH were determined.

6.2.2.7.3 Antioxidative properties: pH and thermal stability studies

The methodology described by Yarnpakdee et al. (2015) was adopted for pH and thermal stability studies. About 25 mg of sample was dispersed in distilled water (8 ml), previously adjusted to different pHs (2, 4, 6, 8, 10) using 1 M HCl or NaOH. The final volume was made up to 10 ml with the water having the corresponding pH and allowed to stand at room temperature for 30 min. Further the pH of the mixtures was adjusted to neutral (7.0) and made up to 25 ml with distilled water. The residual antioxidant activities were determined viz., DPPH radical scavenging activity and ABTS radical scavenging activity. To determine thermal stability, 10 ml of protein hydrolysate solution (1 mg /ml) was placed in a temperature controlled water

bath at different temperatures (30, 40, 50, 60, 70, 80 90 and 100 °C) for 30 min. Further the solutions were rapidly cooled by placing in cold water and the residual antioxidant activities were determined viz., DPPH radical scavenging activity and ABTS radical scavenging activity.

6.2.3 Storage stability studies

Changes in physical, chemical, or microbiological properties of a product can be considered as loss of stability. Optimized hydrolysate samples viz., FTPH and ATPH were kept in plastic (polypropylene) bottles and closed air tightly. Further they were stored at chilled conditions (4^{o}C) and ambient conditions (25^{o}C) for a period of six months and samples were drawn periodically viz., every month for quality analysis viz., moisture, pH, colour, solubility, TBARS, TMA, sensory and microbiological indices.

6.2.3.1 Moisture

Moisture content was determined as per AOAC (2012) using oven drying method wherein the loss in weight of food sample on account of evaporation of water by drying was done. A known quantity of sample was subjected to 105°C in a thermostatically controlled hot air oven until constant weight. From the difference in weight, moisture content was estimated.

6.2.3.2 pH

A known quantity of the sample was finely blended with distilled water in 1:9 ratio (w/v) and pH was measured using pH meter (ECPH S1042S, Eutech Instruments, Singapore).

6.2.3.3 Colour

Hydrolysate powder was analysed for its colour characteristics viz., L* (the degree of lightness), a* (redness(+)/greenness (-)) and b* (yellowness (+) or blueness (-)) using Hunter Lab colorimeter (Colorflex EZ 45/0, Hunter Associates Lab inc., Reston, Virginia, USA).

6.2.3.4 Solubility

Protein solubility of the samples were calculated as per the methodology of Morr et al. (1985) (described in chapter 3; section 3.2.8.1)

6.2.3.5 Thio-barbituric Acid Reactive Substances

TBARS was estimated by distillation method described by Tarladgis et al. (1960). This is one of the most widely used tests to evaluate the extent of lipid oxidation in meats, based on the reaction between important oxidation product malonaldehyde with TBA reagent to produce a coloured complex.

Sample of 10 g was taken and homogenised ((230 VAC T-25 digital Ultra-turrax, IKA, India) with 50 ml distilled water. Then mixed with 2.5 ml of 4 N HCl and 47.5 ml distilled water in a 250 ml round bottom flask which was connected to a TBA distillation unit. About 50 ml distillate was collected within 10 min duration. Five ml of distillate was then mixed with 5 ml TBA reagent and kept in boiling water for 30 min. After cooling the solution, the optical density was measured in 1 cm quartz cuvette against a reagent blank at 538 nm by a UV Vis Spectrophotometer (Lambda 25 UV/Vis, Perkin Elmer Life and Analytical Sciences, Singapore). The results were expressed as mg malonaldehyde (MDA) per kg of sample.

$$\text{TBARS} = \frac{\text{Absorbance} \times 7.8 \times 5}{\text{Volume taken}}$$

6.2.3.6 Tri-methylamine nitrogen

TMA-N contents were determined using the Conway micro – diffusion assay (Conway, 1950). TCA extract of the sample was prepared. About 1 ml of $N/_{100}$ sulphuric acid was taken in the inner chamber of the conway's unit. Simultaneously, 1 ml of TCA extract together with equal quantity of saturated potassium carbonate and formaldehyde was taken in the outer chamber. TMA-N gets liberated while

all other amines and ammonia are held back on addition of formaldehyde. TMA absorbed in standard acid is estimated by titration

$$\text{TMA-N (mg \%)} = \frac{(\text{Blank} - \text{Titre volume}) \times 0.01 \times 14 \times \text{Volume made up} \times 100}{\text{Volume of aliquot taken} \times \text{Weight of sample}}$$

6.2.3.7 Sensory analysis

Sensory analysis of samples during storage was performed by a group of 10 trained panellists using a 9-point hedonic scale for attributes viz., appearance, colour, texture, aroma and taste using score sheets as prescribed by Meilgaard et al. (2006) (Annexure 3). The overall acceptability was evaluated on the basis of these attributes. Final judgments were made based on the average scores given by all panellists.

6.2.3.8 Microbiological analysis

Aerobic plate count was enumerated following the methodology prescribed by USFDA (2001) adopting serial dilution of blended sample using pour plate technique. For analysis, phosphate buffer was used in ratio (1: 9:: sample : buffer (w/v)) and the homogenized sample was serially diluted using 9 ml sterile phosphate buffer. Three consecutive dilutions were pipetted into clean dry petri dishes. About 12-15 ml plate count agar (cooled to 45 ± 1°C) was added to each plate and mixed thoroughly and uniformly. Plates were then incubated at 35°C ± 2°C for 48 ± 2 h for evaluating aerobic plate count and results expressed as log cfu/g.

6.2.4 Economic feasibility analysis

The parameters of the enzymatic process viz., enzyme-substrate concentration, hydrolysis time, temperature and pH, previously identified as optimum to produce TPH with superior functional properties viz., FTPH (0.34 % E/S, 30 min, 60°C, pH 6.5) and superior antioxidant properties viz., ATPH (0.98

% E/S, 240 min, 60°C) in laboratory-scale evaluations, were used to model large scale production utilizing the pilot facilities available at ICAR-CIFT. The economic feasibility of producing the optimized tuna fish protein hydrolysate (TPH) on an industrial scale, in order to identify the total investment cost, operation cost, return on investment and the most importantly, the investment payback time was carried out.

6.2.5 Statistical analysis

The analytical data obtained in triplicate were subjected to analysis of variance (ANOVA). The differences between means were evaluated by duncan's multiple range test and were considered significant at 5 % levels. SPSS statistic programme (SPSS 16.0 for Windows, SPSS Inc., Chicago, IL) was used for interpretation of the results obtained.

6.3 Results and discussion

6.3.1 Characteristics of optimized tuna protein hydrolysates

6.3.1.1 Degree of hydrolysis and proteolytic activity

Degree of hydrolysis is an indicator of the efficiency of the hydrolysis. It measures the extent of breakage the protein molecules undergo to liberate a mixture of high and low molecular weight peptides and free amino acids, responsible for the bioactivity of the derived peptides. DH of the optimized functional hydrolysate was observed to be 14.35 ± 0.15 % and the corresponding proteolytic activity, in terms of amount of tyrosine liberated was 0.329 ± 0.003 (Table 6.3). DH of the optimized antioxidant hydrolysate and its corresponding amount of tyrosine liberated was observed to be 31.59 ± 1.06 % and 0.515 ± 0.006, respectively. The physicochemical condition of the reaction determines the degree of hydrolysis as well as the molecular weight of the peptides which are contributors to the bioactive property exhibited by the peptides (Ren et al., 2008a). Opheim et al. (2015) reported short-chain peptides produced on account of higher degree of hydrolysis to be associated with higher bioactivity such as antioxidativity. Similarly authors like Nalinanon et al. (2011) reported that functional properties like emulsion and foaming properties are governed by their DH and hydrolysate concentrations. In concurrence with these reports, the present study suggested limited degree of hydrolysis to result in larger peptides (> 10 kDa) of higher proportion (60 %) in functionally optimized hydrolysate. Similarly the higher degree of hydrolysis adopted for optimized antioxidant hydrolysate resulted in higher proportion of smaller peptides (< 3 kDa), accountable for superior antioxidant properties (Table 6.3).

6.3.1.2 Protein recovery and yield

Recovery of protein during hydrolysis is a major determinant factor that indicates its efficacy. Generally a high protein recovery ensures efficiency of the processing conditions adopted. Protein recovery reported for the optimized

hydrolysate sample was 39.64 % (FTPH) and 45.24 % (ATPH), respectively (Table 6.3). Correspondingly a yield of 5.9 % and 8.6 % was observed for FTPH and ATPH from their respective solutions while it was about 6.9 % and 14.4 % from their raw material. These variations in protein recovery and associated yield can be linked to the extent of hydrolysis undergone wherein the proteins from the parent substrate was extracted into the resultant hydrolysate solution upon hydrolysis, with higher peptide cleavage proportional to the degree of hydrolysis. The optimized hydrolytic conditions followed by subsequent filtration and centrifugation promoted the concentration of desirable protein in the derived sample with removal of other undesirable components like fat, minerals etc. Similar reports by Haslaniza et al. (2010) indicated an increase in DH caused increased cleavage of peptide bonds thus increasing the peptide solubility in the resultant hydrolysate solution. Longer hydrolytic conditions along with higher enzyme concentration resulted in papain to act more extensively on the substrate resulting in more recovery of protein into the hydrolysate solution. Simultaneously the yield was also influenced by the amount of protein recovered into the solution which was further spray dried to powder. Generally reports suggested a yield ranging from 3 - 15 % (Gajanan et al., 2016; Parvathy et al., 2016; 2018a) based on hydrolytic conditions followed by subsequent drying adopted. Generally these lower yields are due to the reality that only the soluble fractions are dried and further associated to the solid losses occurring during spray drying operation.

6.3.1.3 Molecular weight

Molecular weight of the protein hydrolysate is regarded as one of the main factors determining the functional and bioactive properties of hydrolysates which inturn is crucial for its effective utilization (Klompong et al., 2007; Li et al., 2013 and Taheri et al., 2014). Analysis of the molecular weight pattern of

the derived hydrolysate using molecular weight cut-offs revealed a distribution of 60 % peptides above 10 kDa in functional hydrolysate (Table 6.3). However, in antioxidant hydrolysate, in contrary to FTPH, major share of peptides (45 %) were of lower chain length (< 3 kDa), 30 % in the range of 3-10 kDa and about 25 % peptides between 10-30 kDa. Chi et al. (2014) in their studies reported a positive correlation between the peptide molecular weight and functional properties viz., emulsion stability index, emulsifying activity index, foam stability, and foam capacity. Studies on the foam stability of casein hydrolysates was correlated to their molecular weight distribution where a high proportion of peptides of molecular weight >7 kDa, (intact protein as well as high molecular weight peptides) to be positively related to foam stability (Van der Ven et al., 2002). Similarly, the findings from the present study strengthened the reason for higher antioxidant activity exhibited by the hydrolysate (ATPH) as supported by previous studies which mentioned the antioxidant peptides to be highly influenced by their molecular weight distribution (Li et al., 2008; Zhao et al., 2011). Studies by Picot et al. (2010) reported that short peptides of molecular mass less than 3 - 4 kDa usually contain bioactive properties. Lower molecular weight fish protein peptides ranging between 0.5 and 1.5 kDa proved to exhibit higher antioxidant activity (Nalinanon et al., 2011; Li et al., 2013; Centenaro et al., 2014). Chi et al. (2014) also reported hydrolysates with lower molecular weight to be composed of shorter and more active peptides, which could serve as electron donors and react with free radicals to transform them into more stable substances and end the chain reactions.

Table 6.3 Characteristics of functional tuna protein hydrolysate and antioxidant tuna protein hydrolysate

Parameters	FTPH	ATPH
DH (%)	14.35 ± 0.15	31.59 ± 1.06
Proteolytic Activity (µmoles tyrosine/mg protein)	0.329 ± 0.003	0.515 ± 0.006
Protein Recovery (%)	39.64	45.24
Yield (%)	5.9	8.6
Molecular weight	10% (>30 kDa) 50 % (10-30 kDa) 35 % (3-10 kDa) 5 % (< 3 kDa)	25 % (10-30 kDa) 30 % (3-10 kDa) 45 % (< 3 kDa)

6.3.1.4 Nutritional profile

6.3.1.4.1 Proximate composition

Proximate composition of optimized tuna protein hydrolysates viz., FTPH and ATPH is given in Table 6.4. Numerous studies have been carried out on the proximate analysis of protein hydrolysate indicating similar range of nutrients (Khantaphant et al., 2011; Parvathy et al., 2018b, c). Enzymatic hydrolysis of parent protein facilitates their selective extraction by proper solubilisation yielding higher protein content in the derived hydrolysate. As the other unwanted components are removed during hydrolysis and subsequent centrifugation process, generally protein hydrolysates will be a concentrated form of protein ranging between 60 – 90 % based on the process conditions adopted (Dong et al., 2005; Choi et al., 2009; Khantaphant et al., 2011). A protein content of 88.57±0.66 % and 89.09±0.74 % was observed in FTPH and ATPH, respectively. High protein content of fish protein hydrolysates demonstrates its potential use as protein supplements for human nutrition. A lower fat content of about 0.5 % was observed in the derived hydrolysates (Table 6.4). A low fat content is desirable in hydrolysate as it may influence its keeping quality. The

low fat content of the resultant fish protein hydrolysates is on account of removal of lipids with insoluble protein fractions by centrifugation as well as the removal of fat in the parent source viz., tuna red meat by treatment (Chapter 4; Section 4.3.1). On account of the final drying of the optimized hydrolysate solution, a lower moisture content of 7.59 ± 0.18 % (FTPH) and 8.23 ± 0.14 % (ATPH) was observed. Most of the studies demonstrated that protein hydrolysates from various fish proteins contain moisture below 10 % (Chalamaiah et al., 2010; Foh et al., 2011; Parvathy et al., 2016). Low moisture content facilitates better handling as well as storage stability to the hydrolysates. The ash content of fish protein hydrolysates reported in the present study was 2.42 ± 0.08 % (FTPH) and 2.36±0.14 % (ATPH), respectively. Ash content in a wide range of 0.45 % to 27 % of total composition was previously reported which is on account of variations with respect to the application of acid or base for pH adjustment of medium (Choi et al., 2009; Ovissipour et al., 2009b; Yin et al., 2010; Mazorra-Manzano et al., 2012).

Table 6.4 Proximate composition of functional tuna protein hydrolysate and antioxidant tuna protein hydrolysate

Composition	FTPH	ATPH
Moisture	7.59±0.18	8.23±0.14
Protein	88.57±0.66	89.09±0.74
Fat	0.49±0.09	0.51±0.08
Ash	2.42±0.08	2.36±0.14

6.3.1.4.2 Amino acid profile

Hydrolysates, composed of a mixture of long and short chain peptides as well as free amino acids exhibits many advantages as nutraceuticals or functional foods on account of their amino acid profile (Wiriyaphan et al., 2015). Several authors have described the amino acid composition of protein hydrolysates produced from different fish species (Yarnpakdee et al., 2015; Johnrose et al.,

2016) However variations were observed in their amino acid compositions which is mainly accountable to factors viz., source of raw material, enzymes used as well as hydrolysis conditions (Klompong et al., 2009a). Among all the amino acids, aspartic acid and glutamic acid were found to be higher in most of the reported fish protein hydrolysates (Yin et al., 2010; Ghassem et al., 2014). Similar to fish muscle hydrolysates, other body parts like head, skin and visceral hydrolysates were reported to contain all the essential and non-essential amino acids (Sathivel et al., 2005a; Ovissipour et al., 2009a; Yin et al., 2010). Present study revealed the richness in amino acids like glutamic acid, aspartic acid, lysine and leucine while amino acids viz., phenyl alanine, tyrosine, methionine and cysteine were found to be in lower amounts in the hydrolytes (Table 6.5). Previous studies conducted by us on the amino acid profile of the source of hydrolysate viz., tuna red meat also revealed similar profile with its abundance in amino acids like glutamic acid, aspartic acid, lysine and leucine while tyrosine, methionine and cysteine were found in low amounts. Similar to the present study, Sathivel et al. (2003) reported higher levels of glutamic acid, aspartic acid, lysine and leucine in hydrolysates prepared from the whole herring as well as herring body. Reports by Gamarro et al. (2013) suggested that tuna red meat contained all the essential amino acids contributing to about 49–52 % of the total amino acids. The EAA values viz., 49.96 % in functional hydrolysate and 50.33 % in the antioxidant hydrolysate, exceeded the reference value of 40 % recommended by WHO/FAO/UNU (2007). The present study also indicated a high essential amino acid/non-essential amino acid ratio of 1.01 (FTPH) and 1.03 (ATPH) strengthening its suitability as a dietary protein supplement. Further, the hydrolysates had an extremely high content of flavour enhancers viz., glutamic acid, aspartic acid, glycine and alanine (38.31 % and 37.33 % of the total

amino acids) in FTPH and ATPH, respectively. Moderately high levels of lysine viz., 9.42 % and 9.43 % were present in the functional and antioxidant hydrolysate, respectively. This is an amino acid required for appropriate growth and a precursor for carnitine production, a nutrient necessary for converting fatty acids into energy and regulating cholesterol levels. Protein hydrolysate also exhibited fairly higher levels of hydrophobic amino acids viz., leucine, alanine, valine, proline, glycine contributing to its antioxidant activity. Chi et al. (2015) confirmed that the higher contents of hydrophobic and aromatic amino acids facilitated the radical scavenging activities of protein hydrolysate facilitated by greater interaction between the peptide and fatty acids as well as their ability to donate protons to electron-deficient radicals and maintain their stabilities via resonance structures and enhance radical scavenging activities.

Table 6.5 Amino acid profile of functional tuna protein hydrolysate and antioxidant tuna protein hydrolysate

Amino acid composition	Percentage of total amino acids	
	FTPH	**ATPH**
Essential Amino acids (EAA)		
Arginine	6.36	6.09
Histidine	4.88	4.91
Isoleucine	3.77	3.25
Leucine	8.23	8.22
Phenyl alanine	2.76	2.84
Threonine	4.40	4.99
Valine	5.73	5.87
Methionine	2.34	2.37
Lysine	9.42	9.43
Tyrosine	2.07	2.36
Total	**49.96**	**50.33**
Non Essential Amino acids (NEAA)		
Alanine	6.86	6.42
Aspartic acid	9.70	9.41
Glycine	4.90	4.42
Glutamic acid	16.85	17.08
Proline	6.38	6.66
Serine	3.67	3.97
Cysteine	0.90	0.78
Total	**49.26**	**48.74**

6.3.1.4.3 Mineral profile

Minerals are important components in human diet so as to maintain a healthy life status. They are known to play a major role in regulation of bodily functions and are also significant for metabolic processes. Mineral profiling of optimized tuna protein hydrolysate viz., FTPH and ATPH indicated higher levels of Na, K, P, Ca and Mg (Table 6.6). Parvathy et al. (2016) observed similar results of higher levels of elements like Na, K, Ca, Mg and P in bromelain treated protein hydrolysate from the waste of yellowfin tuna. Thiansilakul et al. (2007a) also reported higher levels of Na, K, Ca and Mg in freeze-dried round scad protein hydrolysate. Similar results were also reported by Sathivel et al. (2003) in herring and herring byproduct hydrolysates with an abundance in minerals like K, Mg, P, Na, S and Ca. Foh et al. (2011) observed higher levels of Na and K in Tilapia protein hydrolysate. Iron content in tuna protein hydrolysate was in the range of 20 ppm which must be on account of the raw material used for the hydrolysate viz., tuna red meat which was myoglobin rich which contains heme proteins rich in iron. Similarly Foh et al. (2011) also reported iron content in similar range (21 -26 ppm) in tilapia protein hydrolysate. TPH was also found to be a good source of zinc. Similar range of zinc (16.48 – 17.88 ppm) was observed in tilapia fish protein hydrolysates from fresh mince as well as hot water dipped raw material (Foh et al., 2011). The lead content in ATPH was slightly higher (1.13 ppm). This must be because the tuna protein hydrolysate derived is a dry powder having low moisture content in the range of 7.5 – 8.0 %. However it didn't cross the acceptability level of 0.5 ppm, on wet weight basis as per current FSSAI regulations.

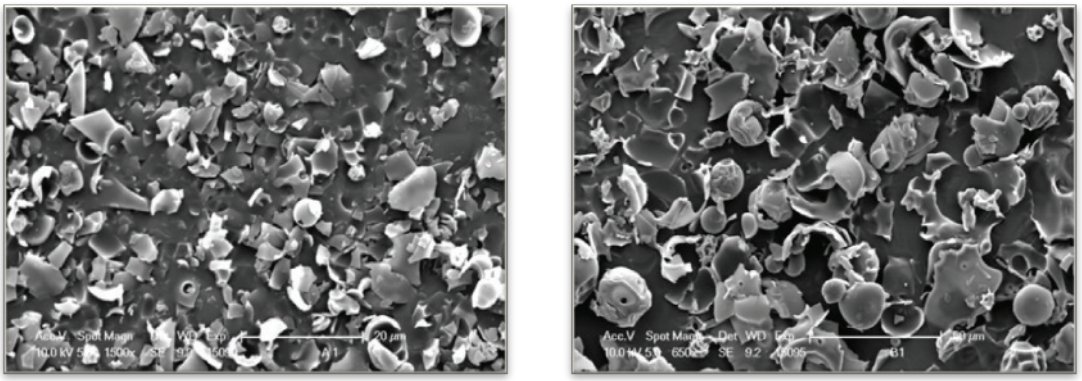
Table 6.6 Mineral profile of functional tuna protein hydrolysate and antioxidant tuna protein hydrolysate

Element (ppm)	Functional hydrolysate	Antioxidant hydrolysate
Barium	3.03±0.093	2.22±0.16
Calcium	514.4±8.993	536.7±9.652
Cadmium	0.207±0.090	0.227±0.058
Cobalt	0.011±0.167	BDL
Copper	4.483±1.007	4.332±0.854
Iron	20.86±1.186	21.14±1.118
Potassium	2261±23.43	1528±20.13
Magnesium	364.2±3.009	241.1±3.072
Manganese	0.739±0.173	0.429±0.068
Sodium	2800.85±89.08	2725.62±20.55
Nickel	1.082±0.100	1.266±0.076
Phosphorous	1587±9.379	876.2±2.599
Selenium	7.694±1.907	6.460±0.674
Strontium	0.690±0.333	1.312±0.386
Zinc	19.83±0.241	21.04±0.098
Lead	0.12±0.04	1.13±0.08
Aluminium	BDL	BDL
Arsenic	BDL	BDL
Boron	BDL	BDL
Chromium	BDL	BDL
Titanium	BDL	BDL
Zirconium	BDL	BDL
Silver	BDL	BDL
Cadmium	BDL	BDL
Mercury	BDL	BDL

6.3.1.5 Morphological and thermal characteristics

6.3.1.5.1 Scanning electron microscopy

Particle size of protein powders is a key physical property that affects its flowability (Kuakpetoon et al., 2001). The microstructure analysis of hydrolysate revealed predominantly ruptured flake like structures having a size range of 5 -12 µm (Fig. 6.4). It is ideal to infer that, the higher extent of hydrolysis resulted in breakage of complex protein molecules to low molecular weight peptides which failed to retain the uniform spherical structure from the sprayed droplets on rapid drying. Particle size of powders are found to be dependent on the spray drying conditions viz., feed temperature, air inlet temperature and outlet temperatures (Sathivel et al., 2009). Similar observations correlating the degree of hydrolysis and the morphology of the spray dried particles were reported by Arias-Moscoso et al. (2015). The shorter the peptide, lesser the ability to form a continuous network at the droplet interphase. They reported an average hydrolysate particle size at pH 7.0 to be 4.0 to 1.5 µm, and the results to be consistent with the DH and electrophoretic patterns.

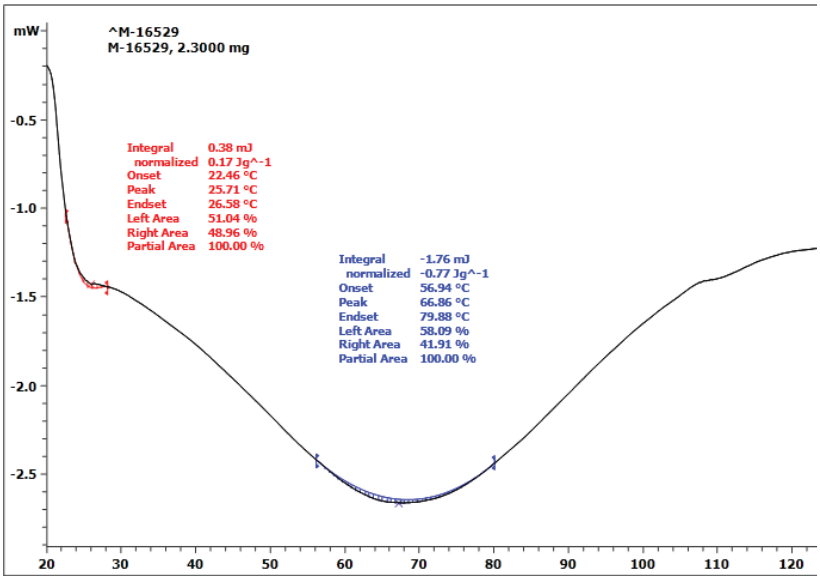


FTPH **ATPH**

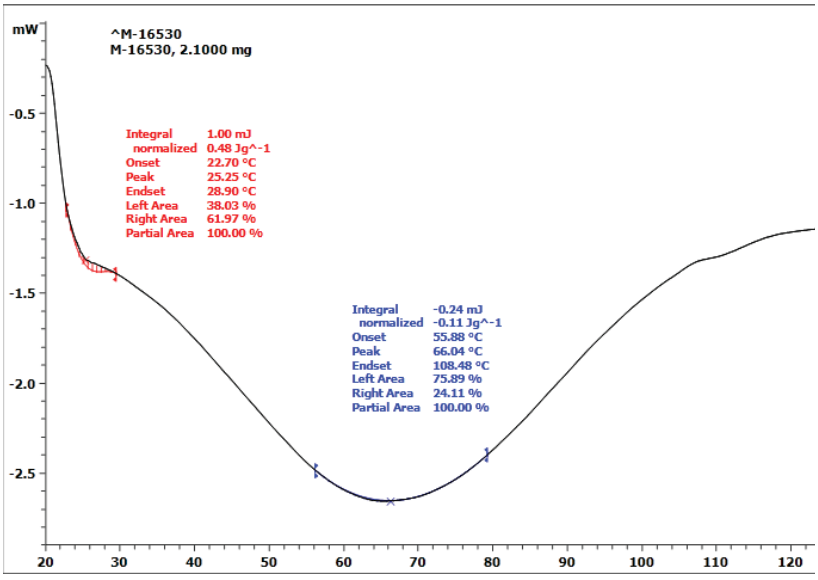
Fig. 6.4 SEM image of tuna protein hydrolysates

6.3.1.5.2 Differential scanning colorimetry

The thermally induced phase transitions and associated energetics as well as the conformational changes of a sample as a function of temperature can be well understood by employing differential scanning calorimetry (Chiu and Prenner, 2011). The DSC thermogram of tuna protein hydrolysates were characterized by two endothermic peaks; the first one corresponding to glass transition point, considered to be the temperature at which the protein polymer undergoes transition from glassy to rubbery state. The onset and peak temperature for the hydrolysates were similar with a value indicating 22.46 ^{o}C and 25.71 ^{o}C for FTPH and 22.70^{o}C and 25.25^{o}C for ATPH (Fig. 6.5). This was accounted to be due to the commencement of long-range coordinated molecular motion of disordered (amorphous) structure (Sperling, 2006). Powder stickiness is identified as one of the main physical phenomenon related to occur 10- 20^{0}C above this point (Roos, 1995; Hogan and O'callaghan, 2013). The second prominent endothermic temperature transition was at 56.94^{o}C and 66.86 ^{o}C for FTPH while for ATPH it was observed at an onset temperature at 55.88^{o}C and peak at 66.04 ^{o}C. This point is related to the protein thermal resistance, where the onset temperature indicated the beginning of sample melting as a function of the heating rate. Foh et al. (2012) reported a lower denaturation temperature of 52.84^{o}C for hydrolysate prepared from Nile tilapia mince. On denaturation of protein, the water molecules compete with the central and lateral chains of the protein molecule resulting in breakage of hydrogen bonds exposing the hydrophobic groups which alters the transition temperature (Pechkova et al., 2007).



FTPH



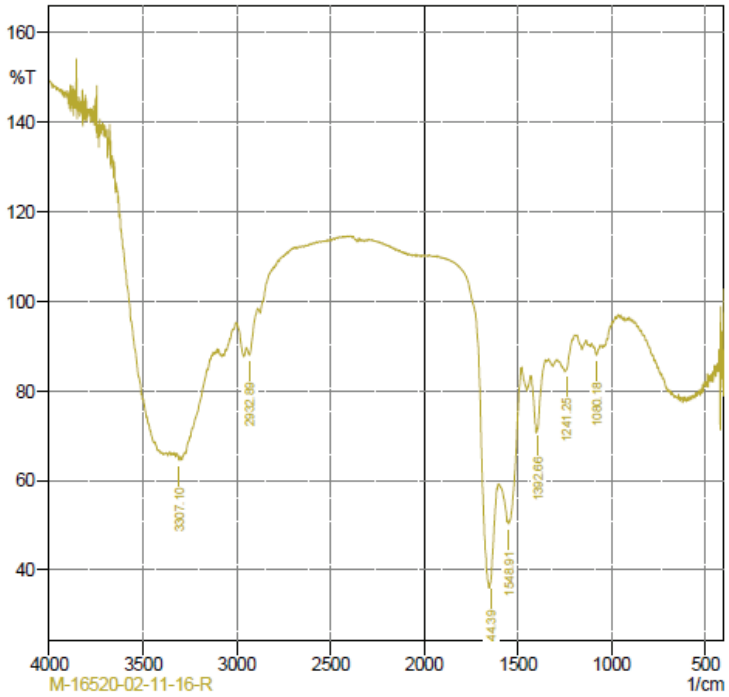
ATPH

Fig. 6.5 DSC curve of tuna protein hydrolysates

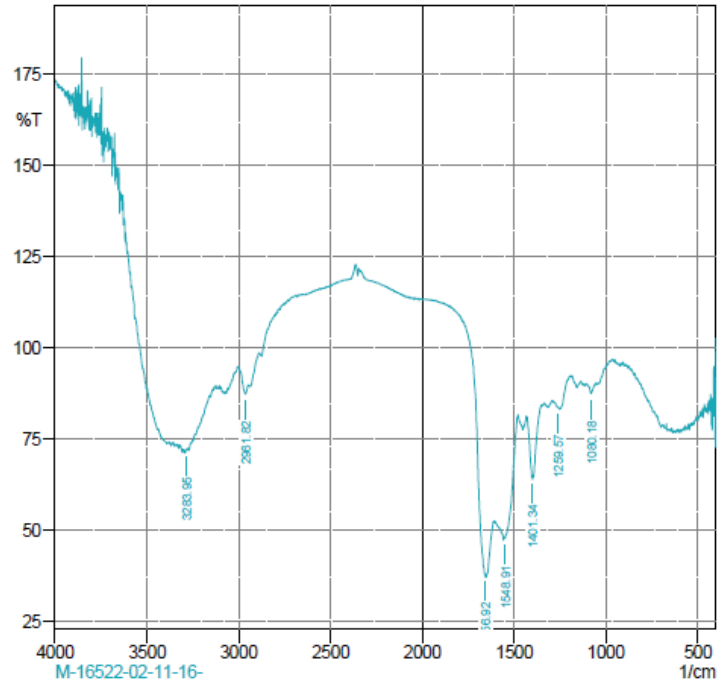
6.3.1.5.3 Fourier-transform infrared spectroscopic analysis

The alterations in the secondary structure of proteins and polypeptides can be well revealed by Fourier Transform Infrared (FTIR) spectroscopy (Nikoo et al., 2011). Heat treatment and enzymatic hydrolysis promote protein denaturation causing shifts in absorption spectrum peaks towards shorter wavelengths, referred to as blue shifts (Damodaran and Paraf, 1997). FTIR spectra of the hydrolysate molecule in the range of 4500 – 400 cm^{-1} were analysed (Fig. 6.6). The FTIR spectrum fingerprint region from 1,800 to 800 cm^{-1} is useful for analyzing proteinaceous material as this range is adsorbed by bond formation with the amide group. These absorptions are mainly due to C=O stretching, C-N stretching, N-H stretching, and O-C-N bending. Moreover, the region from 3,300 to 3,070 cm^{-1} almost exclusively indicates the N-H group with contributions from O-H stretching by the intermolecular hydrogen bonding related to free water. Among these absorption bands, the Amide-I band between 1600 and 1700 cm^{-1} and Amide-II between 1565 and 1520 cm^{-1}, are the most useful peaks for infrared analysis of the secondary structure of proteins (Muyonga et al., 2004). The functional and antioxidant hydrolysate samples exhibited Amide-I band at 1644.39 cm^{-1} and 1656.92 cm^{-1}, respectively. Amide-II band of FTPH and ATPH were observed at 1548.91 cm^{-1}. Amide-I represents C=O stretching/hydrogen bonding coupled with COO- and Amide-II arises from bending vibration of N–H groups and stretching vibrations of C–N groups. The frequency range of 1660–1650 cm^{-1} is characteristic of α-helical structures (Hashim et al., 2010) and hence the present study confirmed the sample to have helical confirmation even after hydrolysis and subsequent spray drying process. Binsi et al. (2017c) observed gelatin short peptides or collagen like peptides to have undergone self-assemblage during freeze-drying process resulting in high helical content of pure gelatin.

Amide A and B bands were observed in the wavelengths of 3283.95 and 2961.82, respectively for both the hydrolysates. A free NH stretching vibration is generally observed in the range of 3400–3440 cm^{-1}. Nevertheless, studies by Muyonga et al. (2004); Nikoo et al. (2011) reported band shifts to lower frequencies when an NH group of peptide is involved in hydrogen bonding, essentially indicating the peptide hydrogen bonding involved during tuna protein hydrolysis in the current investigation.



FTPH



ATPH

Fig. 6.6 FTIR spectra of tuna protein hydrolysates

6.3.1.6 Physico-chemical properties

6.3.1.6.1 Hygroscopicity

Dry powders are generally hygroscopic in nature which inturn affects its other physico-chemical properties like powder reconstitution as well as storage stability. Proteins, on hydrolysis results in the formation of lower average molecular weight peptides with greater number of available water binding sites (Hogan and O'callaghan, 2013). This in turn facilitates its hygroscopic tendency in comparison to the parent protein. Netto et al. (1998) reported an increase in moisture sorption behaviour due to protein hydrolysis. The smaller particle size of powders resulting in a greater surface area enhances the exposure of powders to the atmospheric moisture facilitating higher absorption. The results of the present study indicate fine flaked particles for the resultant hydrolysate which resulted in a hygroscopicity of about 10.66 ± 0.05 % and 8.83 ± 0.10 % for FTPH and ATPH, respectively (Table 6.6). The drier the sample, more is the tendancy to absorb moisture from the surrounding. As previously reported FTPH was drier with a moisture content of 7.59 ± 0.18 % in comparison to ATPH which reported a slightly higher moisture of 8.23 ± 0.14 %. Similar results were reported by Suzihaque et al. (2015) for spray dried pineapple powders where the powders produced at higher inlet temperature were more hygroscopic compare to the lower inlet temperature on account of the variations in moisture content in the resultant powder and corresponding water concentration gradient between the product and the surrounding air.

6.3.1.6.2 Bulk density and tapped density

Bulk density (apparent or packing density) indicating the behavior of a product in dry mixes, is a measure of the mass of powder which occupies a fixed volume. It is an economically, commercially and functionally important property which is dependent on particle density, particle internal porosity as well as

arrangement of particles in the container (Sharma et al., 2012). A lower bulk density (0.022 ± 0 and 0.031 ± 0.001 g/cc) with nearly parallel tapped density values (0.024 ± 0 and 0.035 ± 0.002) were observed for the functionally optimized hydrolysate as well as antioxidant hydrolysate, respectively, indicating finer particles with lesser uniformity (Table 6.6). This was evident from the SEM images which indicated a mixture of particles of both flake as well as spherical shape. A higher bulk density values were reported by Sathivel et al. (2008) for pollock skin hydrolysate samples ranging between 0.12–0.14 g/cm^3. Low bulk density, as influenced by agglomeration, favours the formulation of weaning foods and hence is a vital characteristic of instant powders (Barbosa-Canovas and Juliano, 2005). The carr index and hausner ratio, indicative of the flow properties of powders were observed to be 1.10 ± 0 and 8.77 ± 0.02, respectively for FTPH. The carr index and hausner ratio, for ATPH were 1.14 ± 0.01 and 12.09 ± 0.68, respectively. The flow property indices revealed functional tuna protein hydrolysate to have excellent/very free flow nature {1.0-1.11 (Hausner ratio); ≤10 (Carr index)}. However the flow property characteristics, as revealed by these indices revealed good/free flow {1.12-1.18 (Hausner ratio); 11-15 (Carr index)} nature for the antioxidant hydrolysate. This essentially indicates that flow properties were comparatively superior for functional hydrolysate.

6.3.1.6.3 Colour and browning intensity

Colour, as represented by indices viz., L*(lightness), a*((+) redness/ (-) greenness), b*(yellowness) value were 90.87 ± 0.02, 0.01 ± 0.006 and 15.36 ± 0.06, respectively for FTPH whereas ATPH exhibited an L*, a*, b* value of 90.62 ± 0.05, -0.61 ± 0.01 and 17.16 ± 0.11, respectively indicating the hydrolysate samples to reveal a creamish white appearance (Table 6.6; Fig. 6.7). He et al. (2013) reviewed lighter colour with creamish white appeal for hydrolysate from fish processing co-products, in general. Several factors like the raw material used, hydrolysis

conditions and subsequent drying adopted are influential in deciding colour of the resultant hydrolysate.

Fig. 6.7 Colour of tuna protein hydrolysates

The derived hydrolysate indicated a browning intensity value of 0.137 ± 0.001 and 0.145 ± 0.001 for FTPH and ATPH, respectively (Table 6.7). These variations in browning intensity must be on account of variations in hydrolytic conditions viz., higher enzyme concentration as well as longer hydrolysis period for deriving ATPH in comparison to FTPH. Parvathy et al. (2018b) reported a higher browning index of 0.200±0.002 for red meat derived hydrolysate from *Euthynnus affinis* while that of tuna white meat hydrolysate was 0.045±0.001 indicating the influence of raw material compositional variation with higher pigments in former than later. Further, studies suggest the formation of aldehydes during the hydrolysis process resulting in oxidation followed by a further oxidation taking place during subsequent drying contributing to the interaction of free amino groups and aldehydes leading to non-enzymatic browning of products (Elavarasan and Shamasundar, 2016).

Table 6.7 Physico-chemical properties of functional tuna protein hydrolysate and antioxidant tuna protein hydrolysate

Parameters	FTPH	ATPH
Hygroscopicity (%)	10.66 ± 0.05	8.83 ± 0.10
Bulk Density (g/cc)	0.022 ± 0	0.031 ± 0.001
Tapped Density (g/cc)	0.024 ± 0	0.035 ± 0.002
Hausner Ratio	1.1 ± 0	1.14 ± 0.01
Carr Index	8.77 ± 0.02	12.09 ± 0.68
L*(Lightness) **a*(Redness/Greenness)** **b*(Yellowness)** **a*/b***	90.62 ± 0.05 -0.61 ± 0.01 17.16 ± 0.11 -0.037 ± 0.006	90.87 ± 0.02 0.01 ± 0.006 15.36 ± 0.06 0
Browning Intensity	0.145 ± 0.001	0.137 ± 0.001

6.3.1.7 Functional and bioactive characteristics

6.3.1.7.1 pH stability of functional hydrolysate

Variations in foaming properties viz., foaming capacity as well as foam stability at different pH levels viz., 2, 4, 6, 8 and 10 indicated these properties to be maximum at a pH 6.0 (Fig. 6.8). However deviation from the neutral to acidic and alkaline range exhibited a lowering of these properties. Similar to the present study, Parvathy et al. (2016) reported foaming properties to be maximum at pH 6.0 in protein hydrolysate from yellowfin tuna waste and indicated the property to decrease with deviations in pH. Klompong et al. (2007) reported maximum foaming capacity for yellow stripe trevally hydrolysate at pH 6 with a slight decrease at alkaline pH using alcalase. The lowering of foaming properties of proteins can be coincided with the lowest solubilities at or near their isoelectric pH of 4.0.

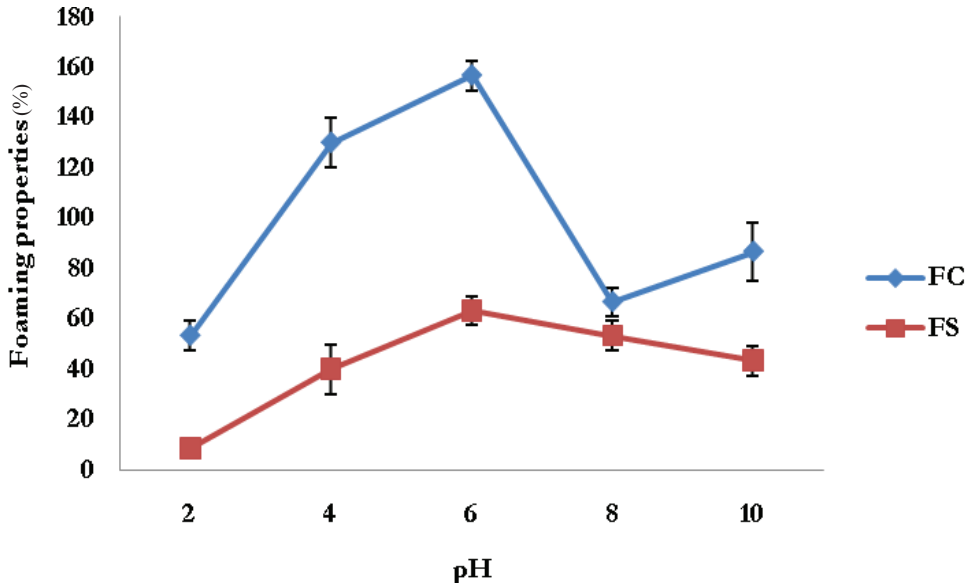


Fig. 6.8 Variations in foaming properties of functional tuna protein hydrolysate

However in contrast, variations in emulsifying properties viz., EAI increased with pH. Increase in EAI was linearly related with pH from 46.43± 1.74 m^2/g (pH 2.0) to 162.01± 2.43 m^2/g (pH 6.0) thereafter showing a reduced rate of increase reaching 192.78± 7.56 m^2/g at pH 10.0 (Fig. 6.9). ESI, in contrary was highest at a pH of 6.0 viz., 48.09 ± 2.69 min and was lower towards the extremes of pH, more decrease noted at a pH of 2.0 (20.1 ± 1.66 min). Similarly Cho et al. (2014) reported a decrease in EAI of protein hydrolysate from egg white with decrease in pH. The solubility was lowest at pH 4 affecting the movement of peptides rapidly to the interface, and their net charge was minimized.

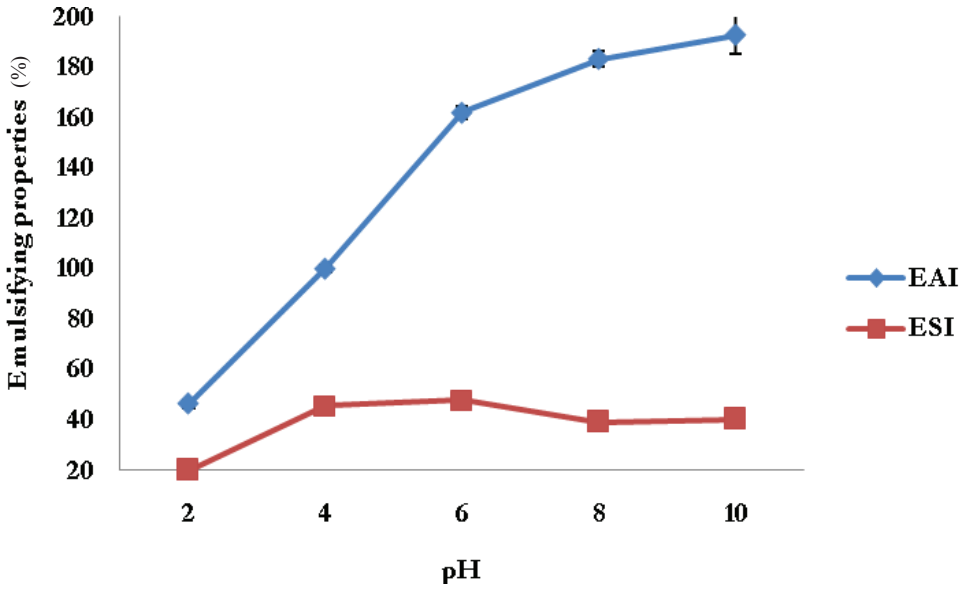


Fig. 6.9 Variations in emulsifying properties of functional tuna protein hydrolysate

6.3.1.7.2 pH stability of antioxidant hydrolysate

The influence of pH on antioxidant activity of antioxidant hydrolysate as monitored by DPPH radical scavenging activity and ABTS radical scavenging activity is depicted in Fig. 6.10 and 6.11, respectively. DPPH radical scavenging activity ranged between 20.54 -23.74 % over the pH range of 2-10 with a slight increase from acidic to basic range. However ABTS radical scavenging activity of hydrolysate indicated a decrease from 26.46 % (pH 2) to 21.77 % (pH 8) followed by an increase to 28.64 % at pH 10. This must be associated with the charge variations at N- and C-terminal of peptides as reported by Yarnpakdee et al. (2015). They reported ABTS radical scavenging activity of Nile tilapia hydrolysate to be quite stable over the pH range of 1–11.

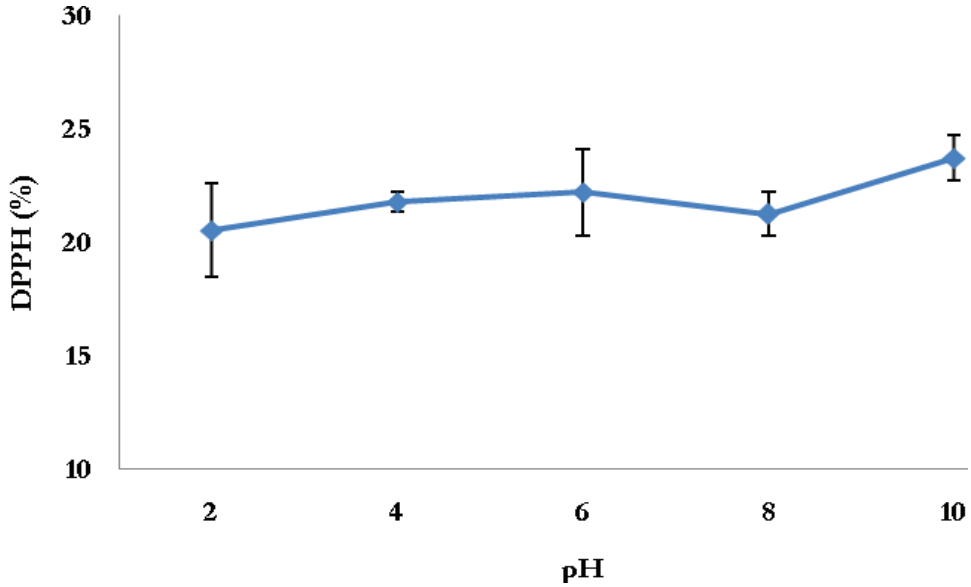


Fig. 6.10 Variations in DPPH radical scavenging activity of antioxidant tuna protein hydrolysate at different pH

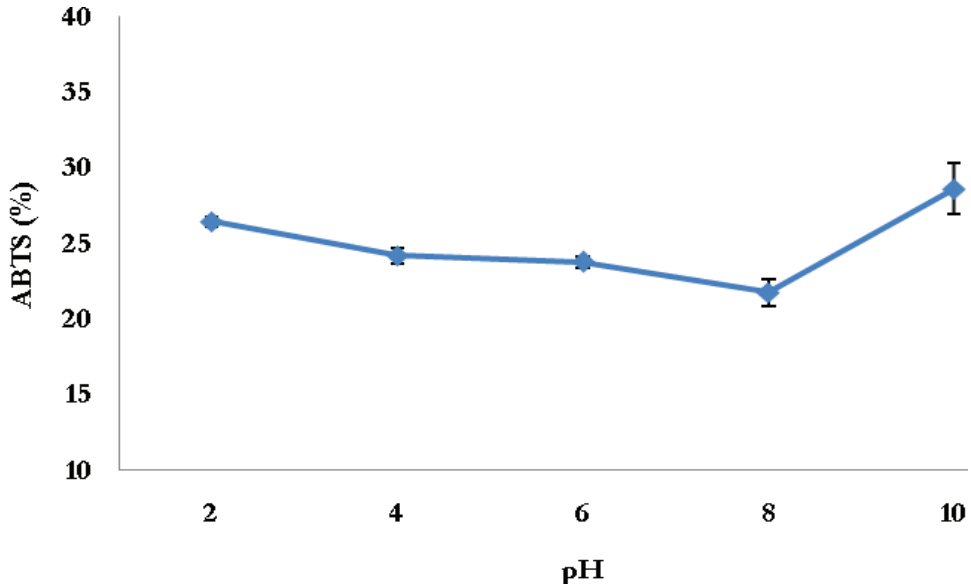


Fig. 6.11 Variations in ABTS radical scavenging activity of antioxidant tuna protein hydrolysate at different pH

6.3.1.7.3 Thermal stability of antioxidant hydrolysate

Thermal stability of the optimized hydrolysate indicated an increase in DPPH radical scavenging activity by 6.55 % from 30 to 50°C thereafter maintained a stable antioxidative potential ranging from 31.62 - 33.33 % upto 100°C (Fig. 6.12). ABTS radical scavenging activity was reported to increase from 15.10 % at 30°C to 24.10 % at 90°C thereafter abruptly decreased by 12.28 % on exposure to 100°C (Fig. 6.13). This decrease reported must be due to the degradation or aggregation of antioxidant peptide and the exposure of hydrophobic groups on account of heat treatment. Yarnpakdee et al. (2015) reported ABTS radical scavenging and metal chelating activities of Nile tilapia hydrolysate to remain constant when subjected to the heating at 30–100 °C for 30 min. Reports suggest peptides with smaller sizes to be more stable to aggregation at high temperature (Nalinanon et al., 2011). The present study indicates the suitability of using this optimized hydrolysate as antioxidant supplement in thermally processed foods as well as acidic foods.

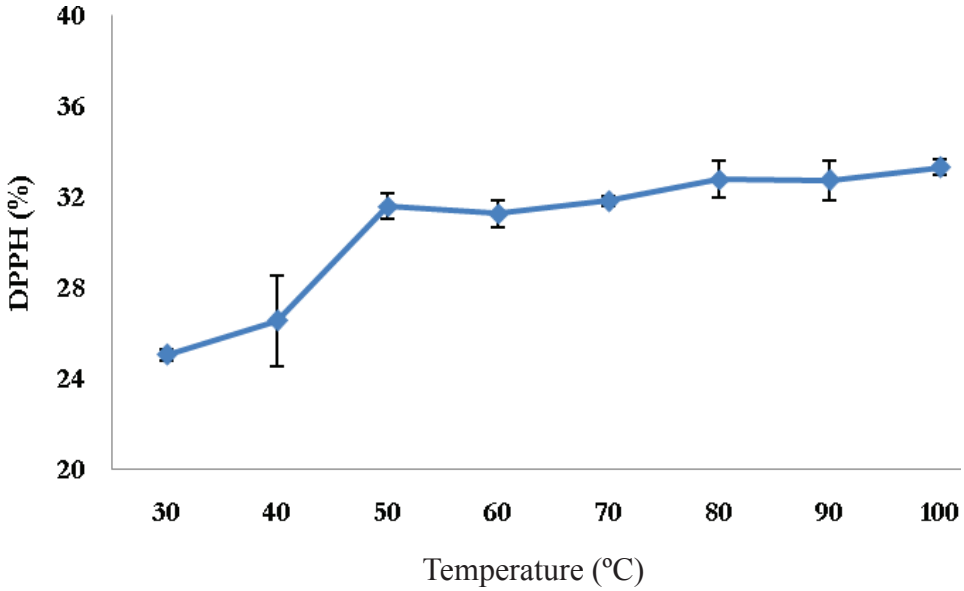


Fig. 6.12 Variations in DPPH radical scavenging activity of antioxidant tuna protein hydrolysate at different temperature

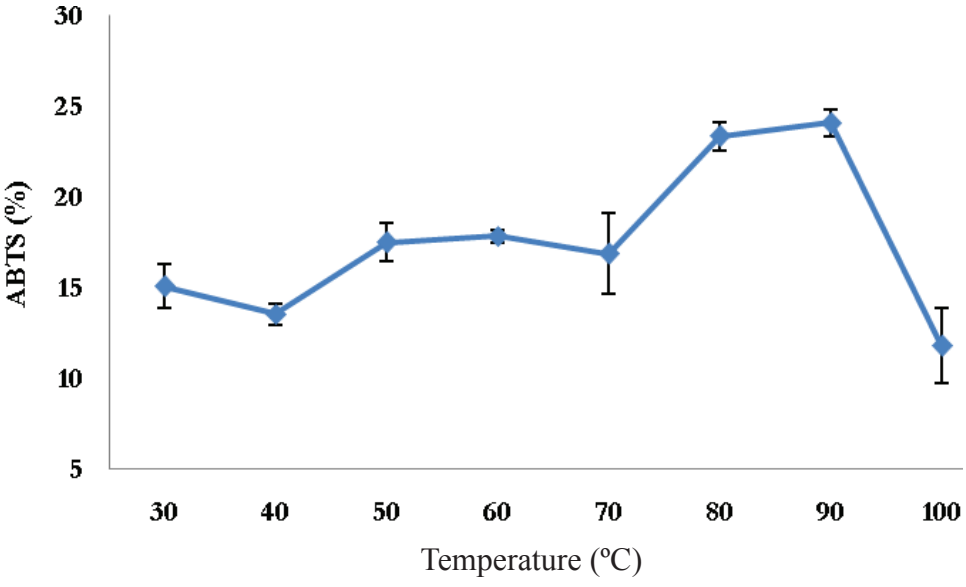


Fig. 6.13 Variations in ABTS radical scavenging activity of antioxidant tuna protein hydrolysate at different temperature

6.3.1.7.4 Effect of concentration on functional properties

The effect of concentration on the functional properties of the derived FTPH was assessed. Functional properties of optimized functional protein hydrolysate exhibited a proportional increase in foaming properties (foaming capacity and foam stability) as well as emulsifying properties viz., EAI and ESI with protein concentration. Foaming capacity of the hydrolysate varied from 90 ± 10 % (0.1 %) to 170± 10 % (3 % protein concentration) (Fig. 6.14). However, though the rate of increase in foaming capacity was higher following a linear pattern from 0.1 % concentration to 0.5 %, there was a decrease in the rate with further increasing concentration to 0.5 % and higher. Similarly a linear increase in foam stability with concentration from 0.1 to 1.0 % was observed with further stagnation in the changes up to 3 %. Foam stability varied from 16.7 ± 2.9 % for 0.1 % protein concentration to 140 ± 10 % for 3 % concentration (Fig. 6.14). Studies conducted by Salem et al. (2017) in octopus protein hydrolysate reported an increase in foaming properties with increase in protein concentration from 0.5 to 2 %. Emulsifying properties viz., EAI exhibited direct and linear relation with protein concentration (R^2 = 0.960). It increased from 104.46 ± 3.39 m^2/g for 0.1 % protein concentration to 200.76 ± 8.43 m^2/g for 3 % protein concentration. Similarly ESI ranged linearly from 28 ± 1.63 min (0.1 %) to 43.46 ± 0.35 min (3 %) with an R^2 of 0.916 (Fig. 6.15).

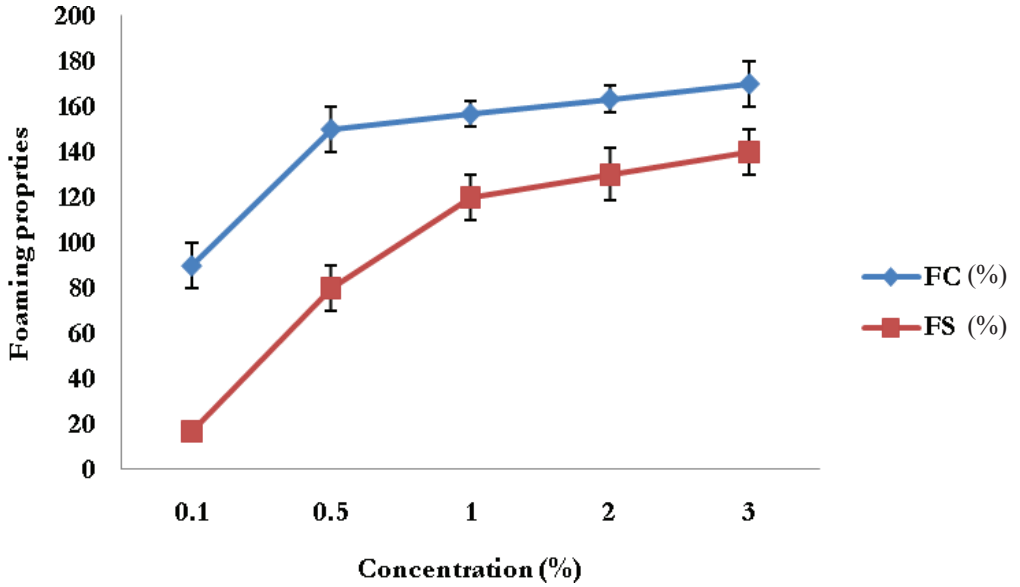


Fig. 6.14 Variations in foaming properties of functional tuna protein hydrolysate at different concentration

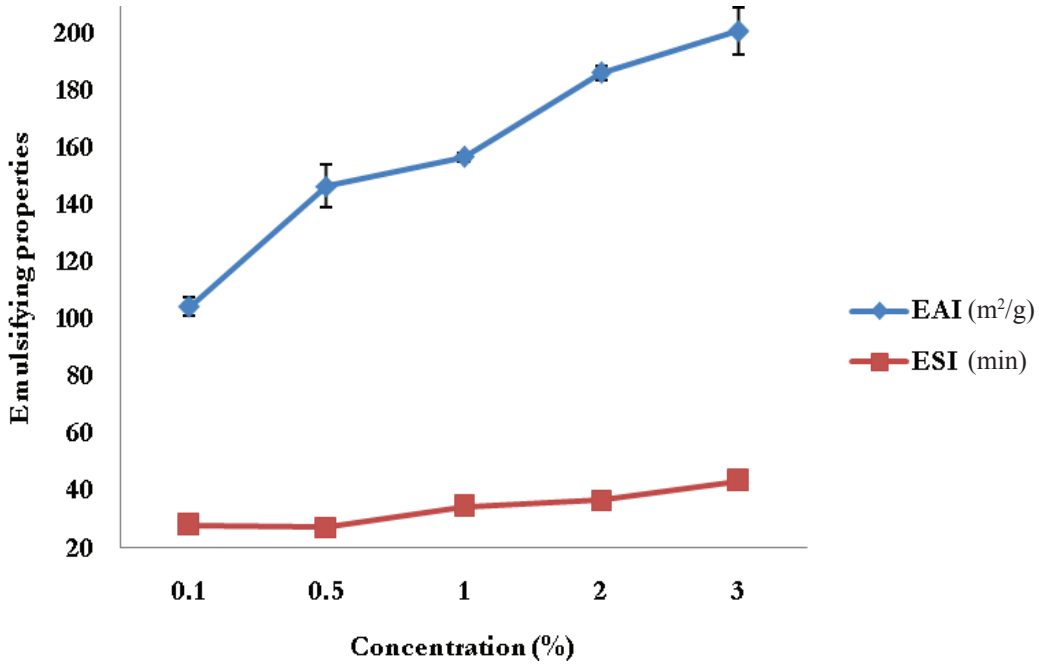


Fig. 6.15 Variations in emulsifying properties of functional tuna protein hydrolysate at different concentration

6.3.1.7.5 Effect of concentration on antioxidative properties

Variations in antioxidant properties viz., DPPH radical scavenging activity indicated an increasing trend in the property with protein concentration. The rate of increase was higher initially (0.5 to 10 mg/ml) and it got stagnated towards higher concentration viz., 10 mg/ml and above (Fig. 6.16). Similar to DPPH radical scavenging activity, ABTS radical scavenging activity was also directly related to the protein concentration. However it was linearly related to the concentration (R^2 = 0.99) with an increase from 15.98 ± 2.28 % for 0.5 mg/ml to 92.37 ± 0.36 % for 40 mg/ml (Fig. 6.17). Haldar et al. (2018) reported an increase in DPPH radical scavenging activity of mussel protein hydrolysate with increase in protein concentration viz., 11.12 ± 0.37 % for 1 mg/ml to 56.12 ± 0.02 % for 10 mg/ml on treating with alcalase for 120 mins. Similarly, pepsin digested mussel protein exhibited an increase in DPPH radical scavenging activity from 17.58 ± 0.37 (1 mg/ml) to 92.04 ± 0.28 (10 mg/ml). Reports by Naqash and Nazeer (2013) on pink perch hydrolysate indicated an increase in antioxidative properties viz., DPPH radical scavenging activity, metal chelating ability and reducing power with increase in protein hydrolysate concentration ranging from 0.5 to 3.0 mg/ml. Previous works also reported an increase in antioxidative properties viz., DPPH radical scavenging activity, reducing power etc. with increasing amount of protein hydrolysates from different fish species (Morales Medina et al., 2016; Salem et al., 2017). According to Blois (1958), samples having IC_{50} lower than 50 μg ml^{-1} are regarded as very strong antioxidants. Investigation of DPPH IC_{50} of Ascorbic acid and BHT in the current work indicated a value of 4.9 and 52.5 μg ml^{-1}, respectively while that of the derived hydrolysate was 1.59 mg/ml. Extracting the antioxidant hydrolysate fractions based on their activity can facilitate to refine their ability for incorporation in food systems. Further synthetic antioxidants have approval for use in food at low concentrations based on complex toxicity studies conducted. The recommended levels of synthetic antioxidants like BHA/BHT in food are 200 ppm whereas on account of safety, higher levels of natural antioxidants can be used in food systems. Hence in the present study, the lower antioxidant property exhibited by hydrolysate

in comparison to commercial ones can be met by higher levels of their incorporation in foods and nutraceuticals.

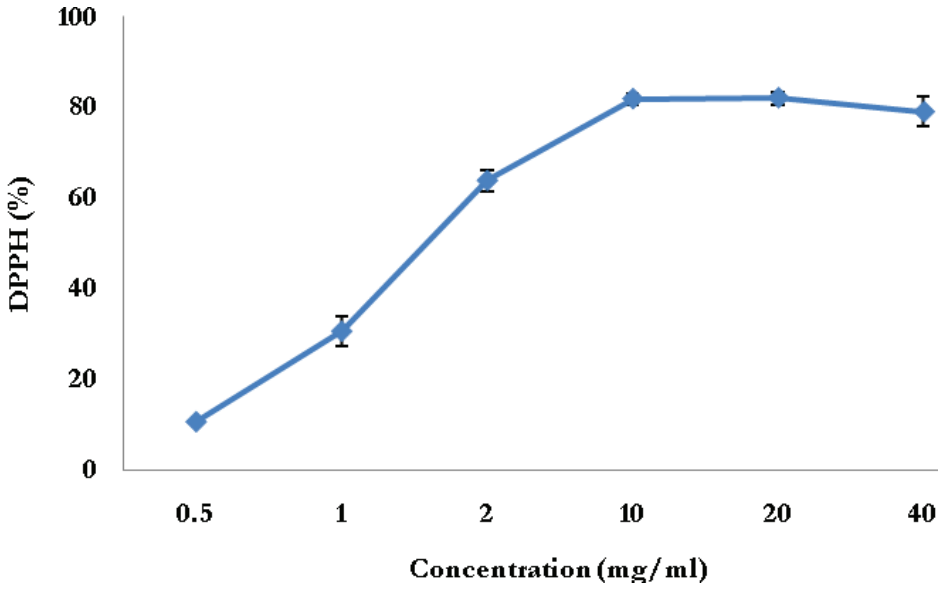


Fig. 6.16 Variations in DPPH radical scavenging activity of antioxidant tuna protein hydrolysate at different concentration

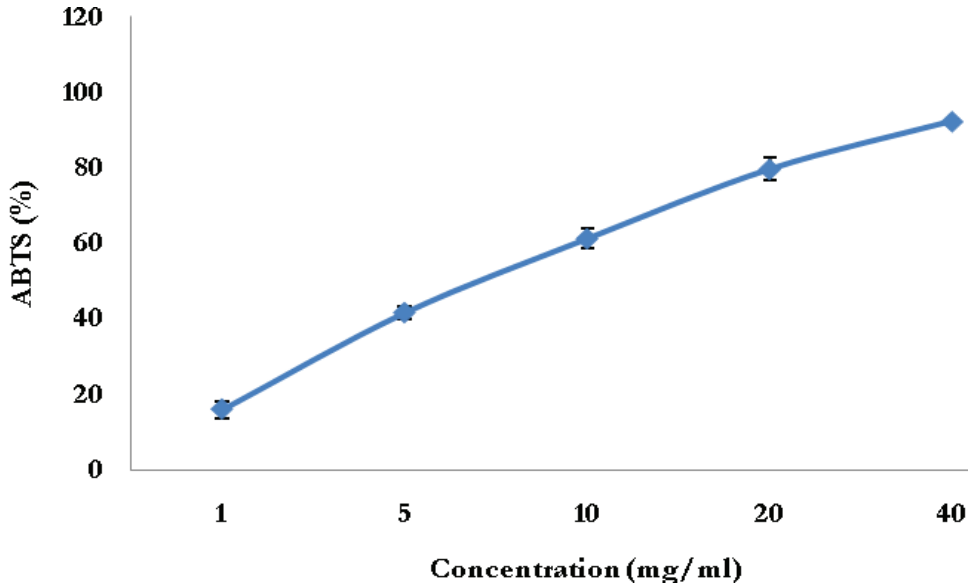


Fig. 6.17 Variations in ABTS radical scavenging activity of antioxidant tuna protein hydrolysate at different concentration

6.3.2 Storage stability studies

6.3.2.1 Moisture

Moisture content is one of several important parameters affecting stability of products as it influences other physico-chemical parameters. Moisture content is the combination of free and bound moisture present in a product. Under unfavourable storage conditions, on account of the hygroscopic nature of protein hydrolysate, moisture absorption occurs in the product which results in rapid physical and chemical changes. In the present study, an increase in moisture content was observed in both samples at both chilled and ambient conditions (Fig. 6.18; Table 6.8). In FTPH, during the initial period, the variations in moisture content were not significant but towards the fourth month there was a significant difference was observed ($p < 0.05$) (Fig. 6.18a). Between the storage period also, there was no significant difference till three months of storage. However, the increase was more prominent at ambient conditions suggesting further possibilities of related physico-chemical reactions to occur. In the case of ATPH, there was a significant increase ($p < 0.05$) in the moisture content observed throughout the storage period, though during initial chilled storage it was not prominent resulting in significant variations between the samples stored under these two varying conditions (Fig. 6.18b).

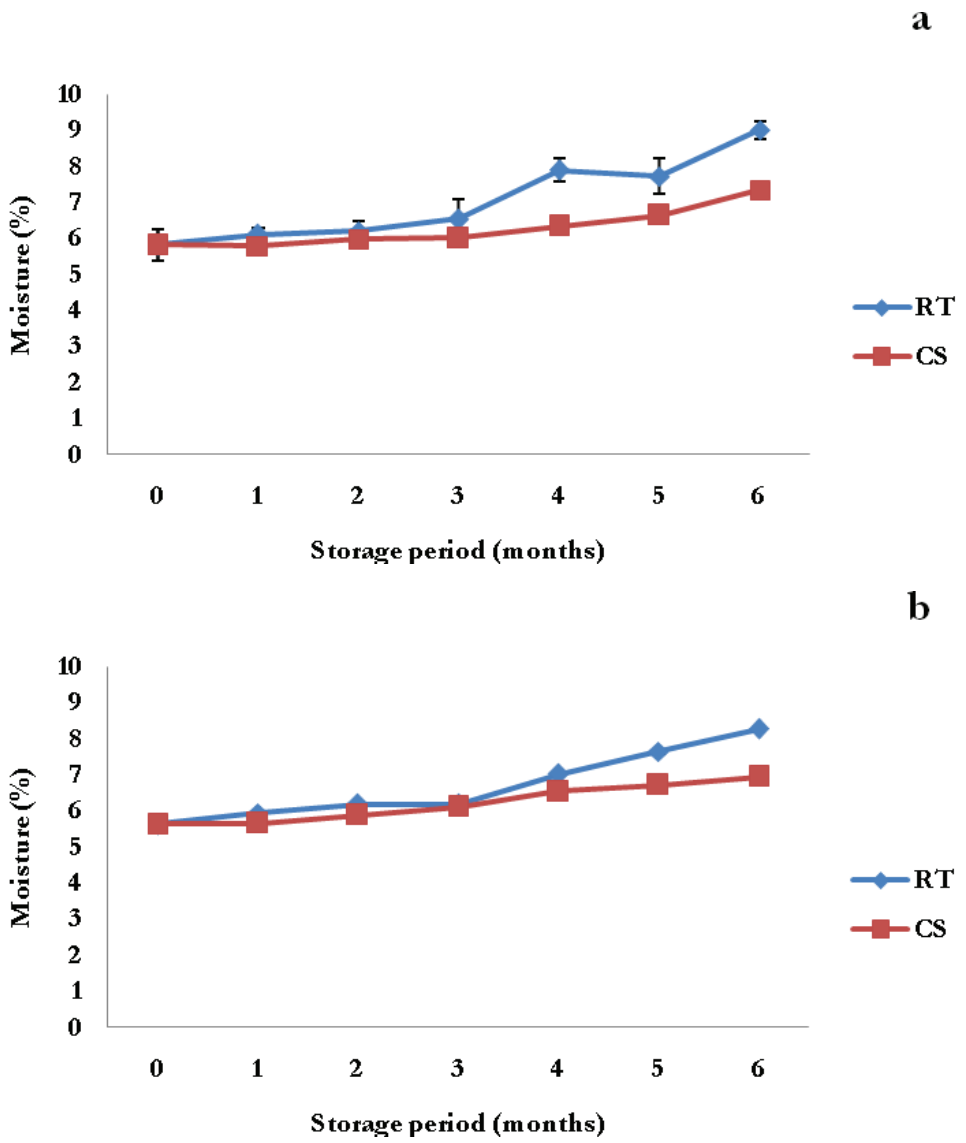


Fig. 6.18 Variations in moisture content of optimized tuna protein hydrolysates **a.** FTPH; **b.** ATPH at ambient (28°C) and chilled conditions (4°C)

6.3.2.2 pH

The variations in pH during storage could be used as a factor determining the stability of protein hydrolysate during storage (Klompong et al., 2012). pH of the hydrolysate samples showed a slight increase ranging from 5.75 – 6.01 at room temperature and 5.75 – 5.92 at chilled condition for FTPH (Fig. 6.19a). pH of ATPH

ranged from 5.71-5.92 (RT) and 5.71-5.89 (CS) (Fig. 6.19b). Studies conducted by Klompong et al. (2012) reported no marked changes in pH throughout the storage of 12 weeks at room temperature for yellow stripe trevally protein hydrolysate.

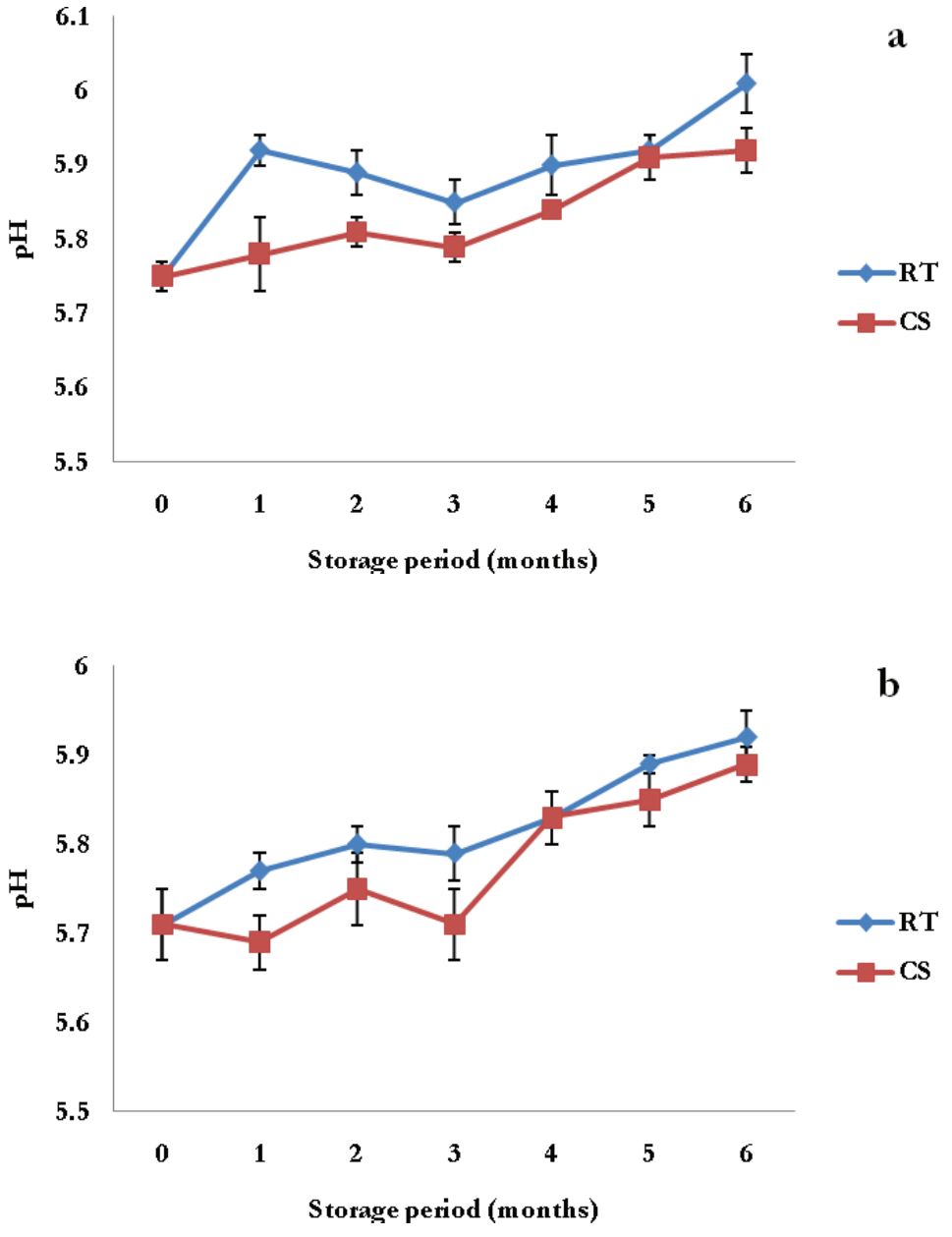


Fig. 6.19 Variations in pH of optimized tuna protein hydrolysates **a.** FTPH; **b.** ATPH at ambient (28°C) and chilled conditions (4°C)

6.3.2.3 Colour

Colour variations in samples were observed to be more prominent during ambient storage conditions (Table 6.8). Lightness values decreased (Fig. 6.20) while a proportional increase in redness (Fig. 6.21) as well as yellowness (Fig. 6.22) was observed ($p < 0.05$). This was substantiated from the observations made by Hoyle and Merritt (1994) who found that hydrolysates from herring exhibited decrease in lightness and increase in yellowness indicating sample darkening during storage. Studies conducted by Klompong et al. (2012) also indicated trevally protein hydrolysate to exhibit a slight decrease in lightness while redness and yellowness gradually increased during storage. The decrease in lightness and the increases in redness and yellowness might be associated with non-enzymatic browning. The formation of brown pigments might result from aldol condensation of carbonyls produced from lipid oxidation upon reaction with basic groups in proteins via Maillard reaction. In addition, the decrease in lightness was probably due to the oxidation of myoglobin and the melanin pigment present in the sample. Authors have reported nonenzymatic browning in different protein hydrolysates during storage at medium to high aw and these changes to be dependent on storage temperature and relative humidity (Rao and Labuza, 2012; Rao et al., 2012). Similar to the present study, Thiansilakul et al. (2007a) observed yellowness of the protein hydrolysates became more intense with storage time, being more pronounced at 25^{o}C than at 4^{o}C.

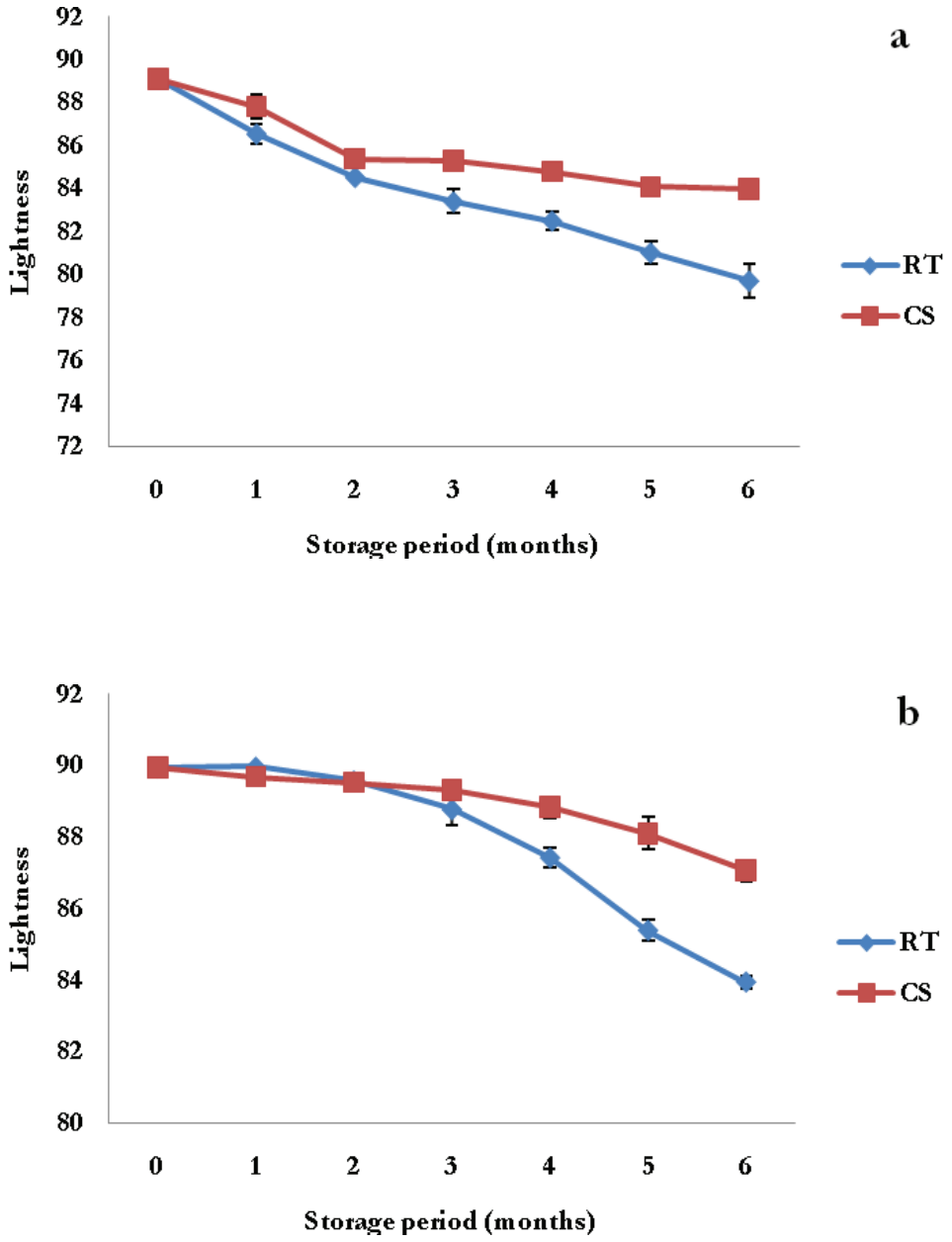


Fig. 6.20 Variations in lightness of optimized tuna protein hydrolysates **a.** FTPH; **b.** ATPH at ambient (28°C) and chilled conditions (4°C)

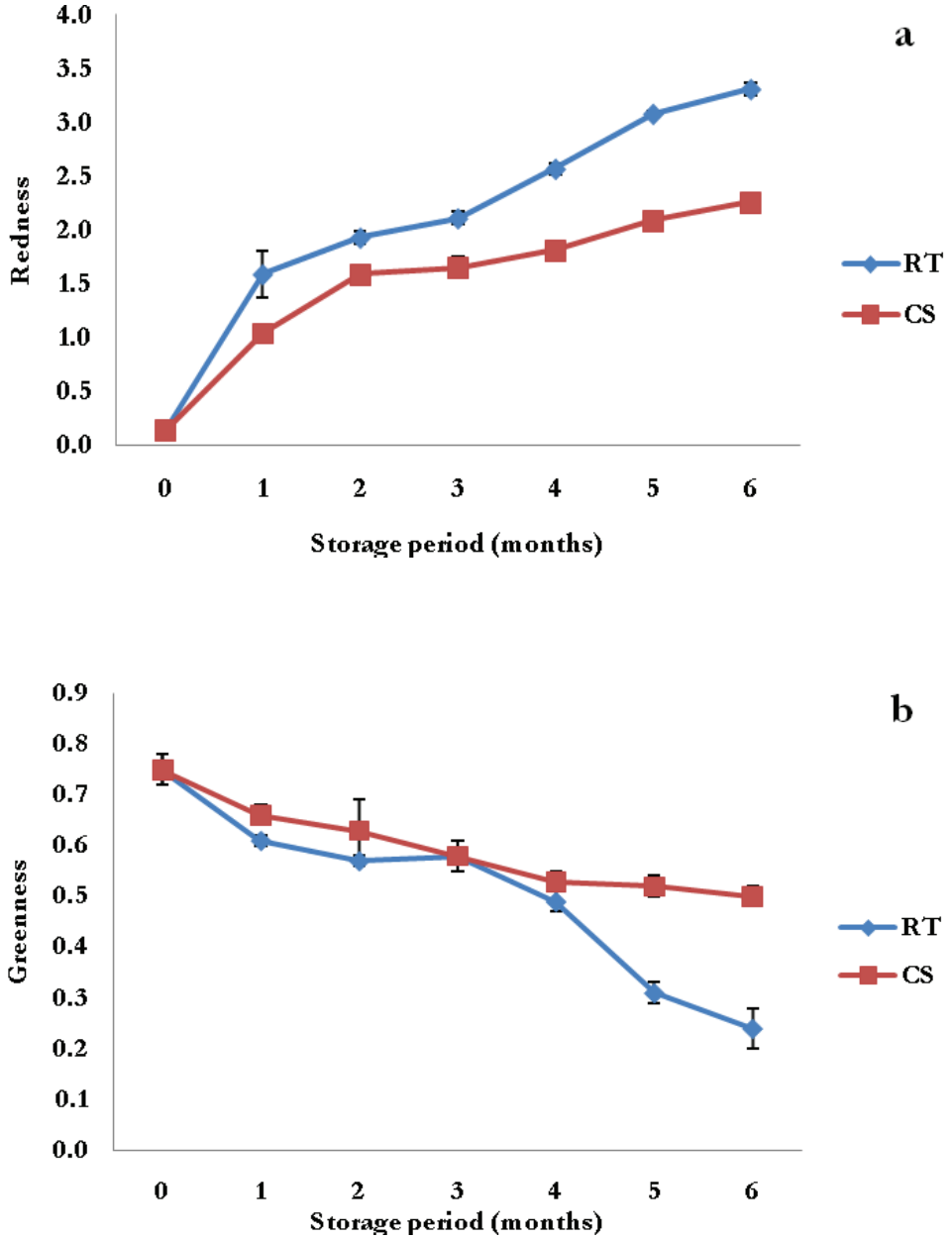


Fig. 6.21 Variations in redness/greenness of optimized tuna protein hydrolysates **a.** FTPH; **b.** ATPH at ambient (28°C) and chilled conditions (4°C)

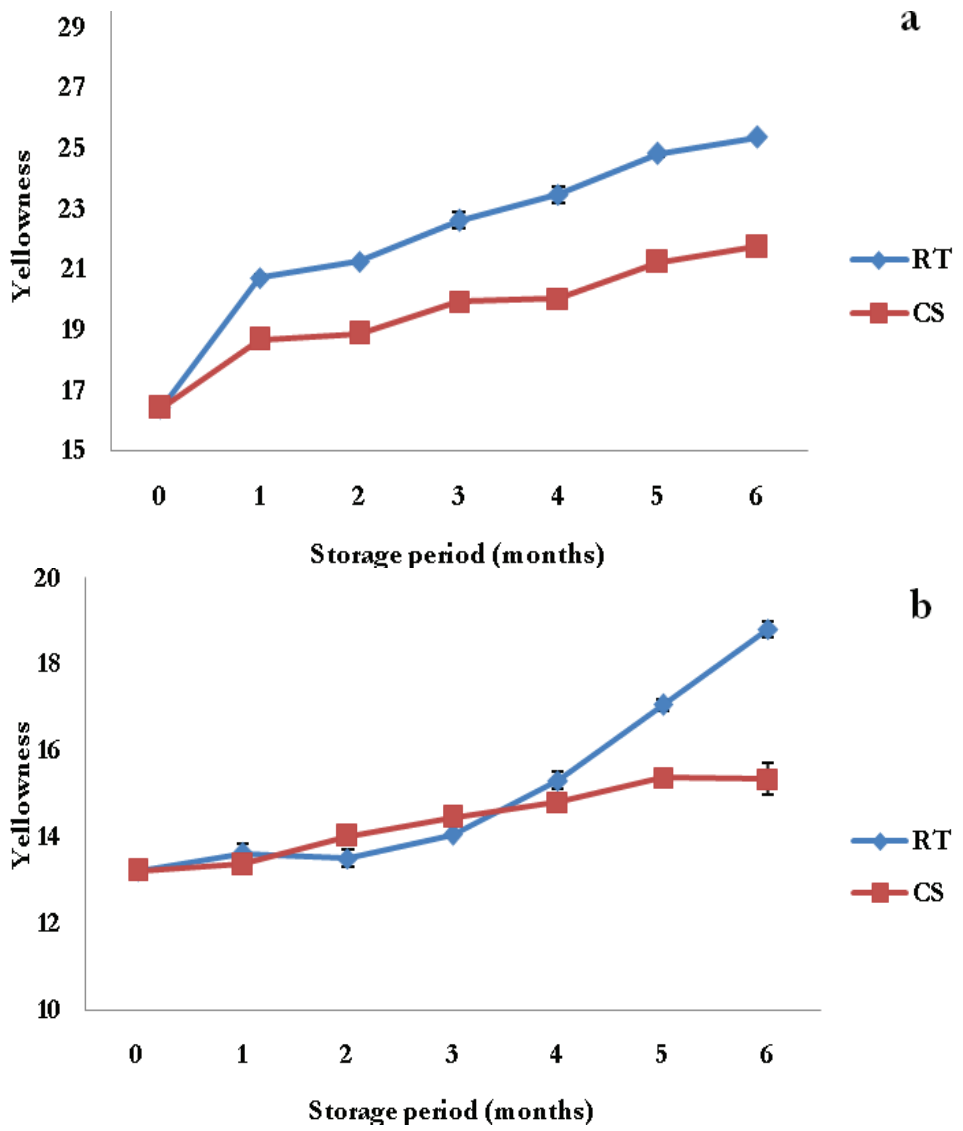


Fig. 6.22 Variations in yellowness of optimized tuna protein hydrolysates **a.** FTPH; **b.** ATPH at ambient (28°C) and chilled conditions (4°C)

6.3.2.4 Solubility

Protein solubility of hydrolysate samples indicated a significant decrease ($p< 0.05$) during storage, more prominent under ambient conditions in comparison to chill storage. On account of the same, the samples viz., FTPH and ATPH showed significant variations between storage period, from second month of storage (Fig. 6.23; Table 6.8). The present solubility results were in concurrence with the reports by Thiansilakul et al. (2007a) who also observed decrease in solubility of round scad

protein hydrolysates during storage. Aggregates, referred to as self-associated state of proteins/peptides, involved in covalent bonding, that is effectively irreversible under the conditions it forms (Weiss et al., 2009) is responsible for the decreased solubility. As observed in various studies, the decrease in solubility might be due to the aggregation of the peptides with the concomitant formation of a larger aggregate with lowered solubility (Thiansilakul et al., 2007a).

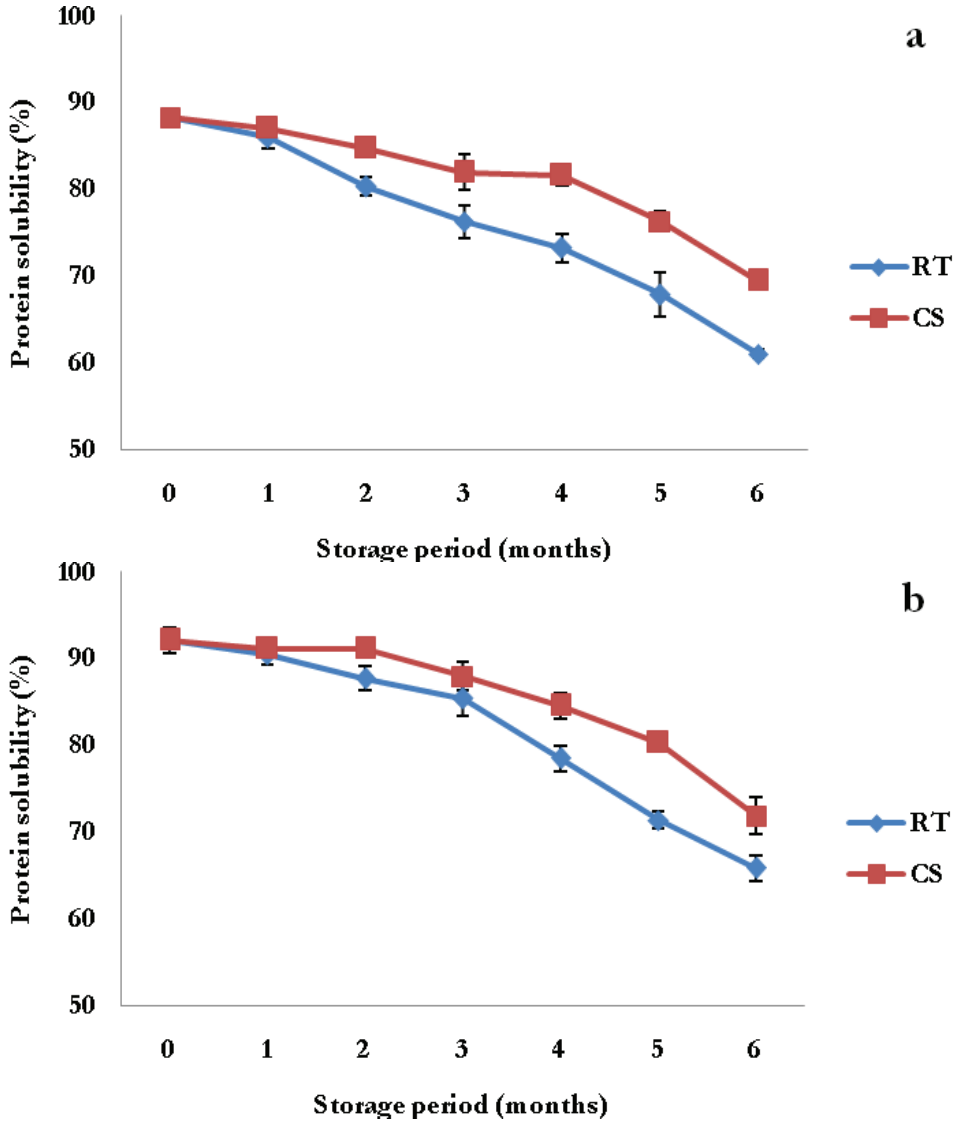


Fig. 6.23 Variations in protein solubility of optimized tuna protein hydrolysates **a.** FTPH; **b.** ATPH at ambient (28°C) and chilled conditions (4°C)

6.3.2.5 TBARS

Evaluation of the variations in TBARS during storage of hydrolysate indicated oxidation to be more prominent and significant ($p < 0.05$) at room temperature in comparison to chilled conditions. For FTPH, the initial TBARS was 0.89 which crossed the acceptability limit of 2 mg malonaldehyde/kg on second month (2.19) when stored at room temperature, while it was extended to three months (2.23) when stored under chilled conditions (Fig. 6.24a; Table 6.8). ATPH had an oxidative stability of three months (2.2) at room temperature and five months (2.2) at chilled conditions. These variations resulted in a significant difference in TBA values between storage period during final period of storage in FTPH. Similar to FTPH, ATPH also indicated variations in TBA which was significant and more prominent ($p< 0.05$) during room temperature storage in comparison to chill storage resulting in significant variations ($p< 0.05$) between the samples stored under these different conditions from the first month of storage (Fig. 6.24b; Table 6.8). Similar results of increase in TBARS were observed in yellow stripe trevally protein hydrolysate stored at room temperature for 12 weeks indicating fat oxidation (Klompong et al., 2012). Studies have reported fish protein hydrolysates prone to oxidation also on account of high content of unsaturated fatty acids (Sohn et al., 2005; Yarnpakdee et al., 2012a; Yarnpakdee et al., 2012b; Rao et al., 2016).

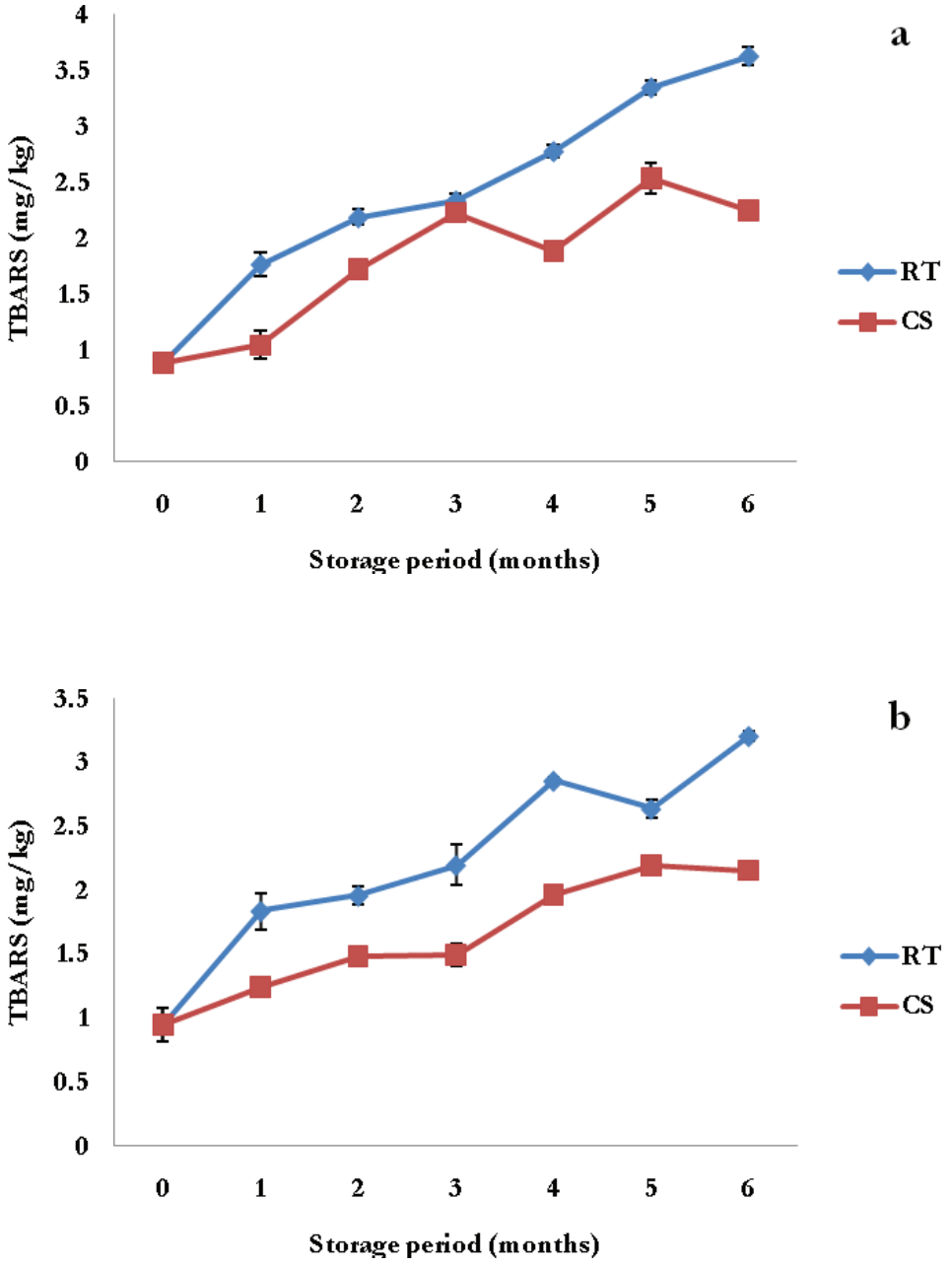
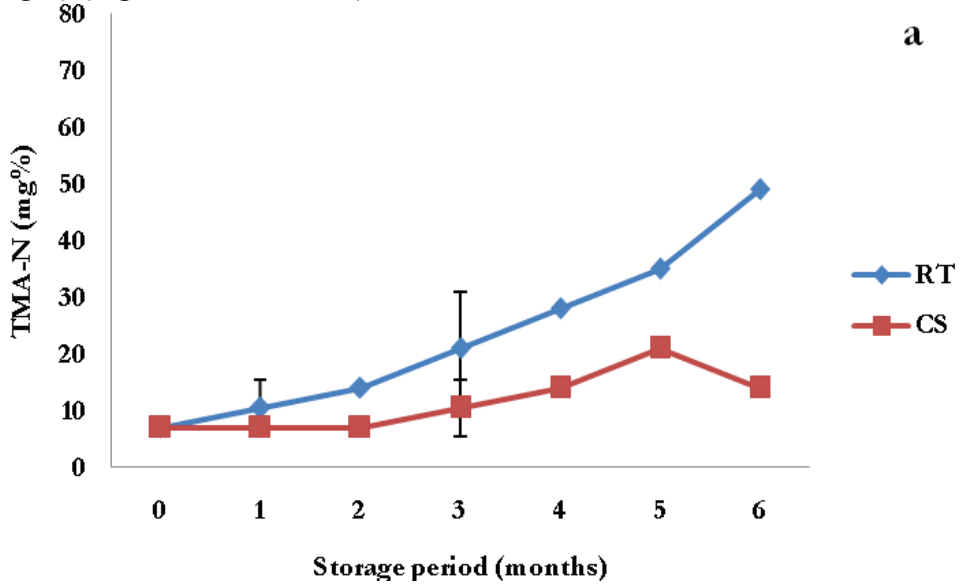


Fig. 6.24 Variations in TBARS of optimized tuna protein hydrolysates **a.** FTPH; **b.** ATPH at ambient (28°C) and chilled conditions (4°C)

6.3.2.6 TMA-N

Similar to the trend observed in other quality parameters during storage, variations in TMA-N were more prominent during room temperature storage in comparison to chilled conditions resulting in significant difference between the samples ($p<0.05$) from third month of storage onwards in FTPH. Variations in TMA-N were less prominent in ATPH in comparison to FTPH. However between the storage temperature, the variations were significant from fourth month at room temperature. During chilled storage, the variations were limited and between the samples a significant difference ($p < 0.05$) was observed from fifth month onwards. Present study indicated an increase in TMA-N during storage and it reached the acceptability limit of 15 mg% during different periods based on the storage condition. In case of FTPH, TMA-N increased prominently at room temperature attaining a value of 21 mg% by third month while it was extended to 6 months (21 mg %) during chilled conditions (Fig. 6.25a; Table 6.8). Similarly for ATPH, the sample was within the TMA-N acceptability limit till four months (21 mg%) while it was within the limit throughout the storage period under chilled conditions (14 mg%) (Fig. 6.25b; Table 6.8).



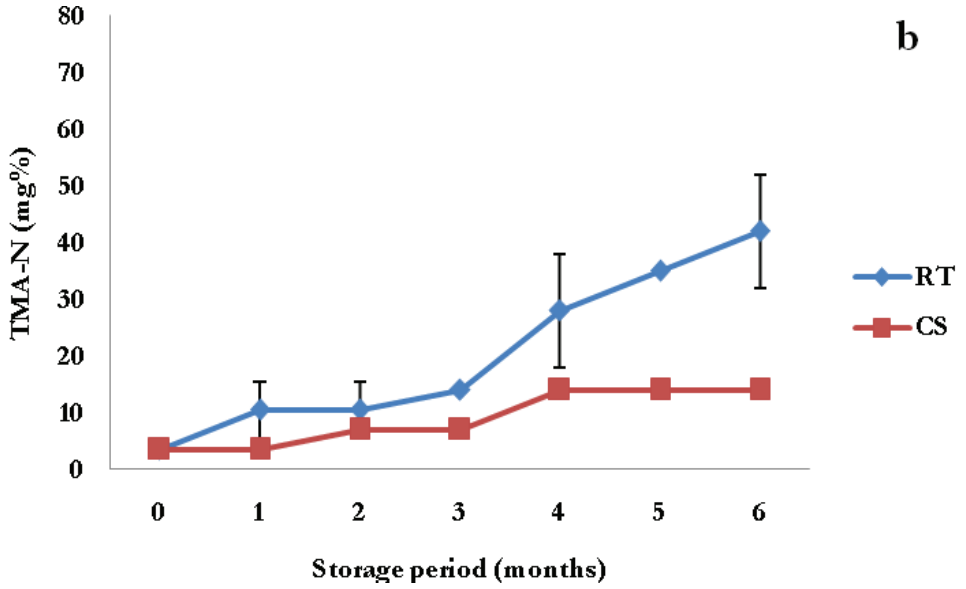


Fig. 6.25 Variations in TMA-N of optimized tuna protein hydrolysates
a. FTPH; **b.** ATPH at ambient (28°C) and chilled conditions (4°C)

6.3.2.7 Sensory indices

There was a significant reduction in sensory score for FTPH stored at room temperature ($p<0.05$) whereas the reduction was not significant upto third month under chilled conditions (Table 6.8). On account of this reduction, a significant difference in the sensory score was noticed between samples stored under the different storage conditions from first month onwards. Sensory scores ranged from 7.0 ± 0.47 to 2.0 ± 1.16 (RT) and 7.0 ± 0.47 to 5.0 ± 0.82 (CS) for FTPH (Fig. 6.26a; Table 6.8) while it varied from 6.0 ± 1.33 to 2.0 ± 0.67 (RT) and 6.0 ± 1.33 to 4.0 ± 1.16 (CS) for ATPH (Fig. 6.26b). Results indicated an acceptability period of two months and five months for FTPH stored under ambient and chilled conditions, respectively. ATPH exhibited an acceptance of one month and three months under ambient and chilled conditions, respectively based on sensory score.

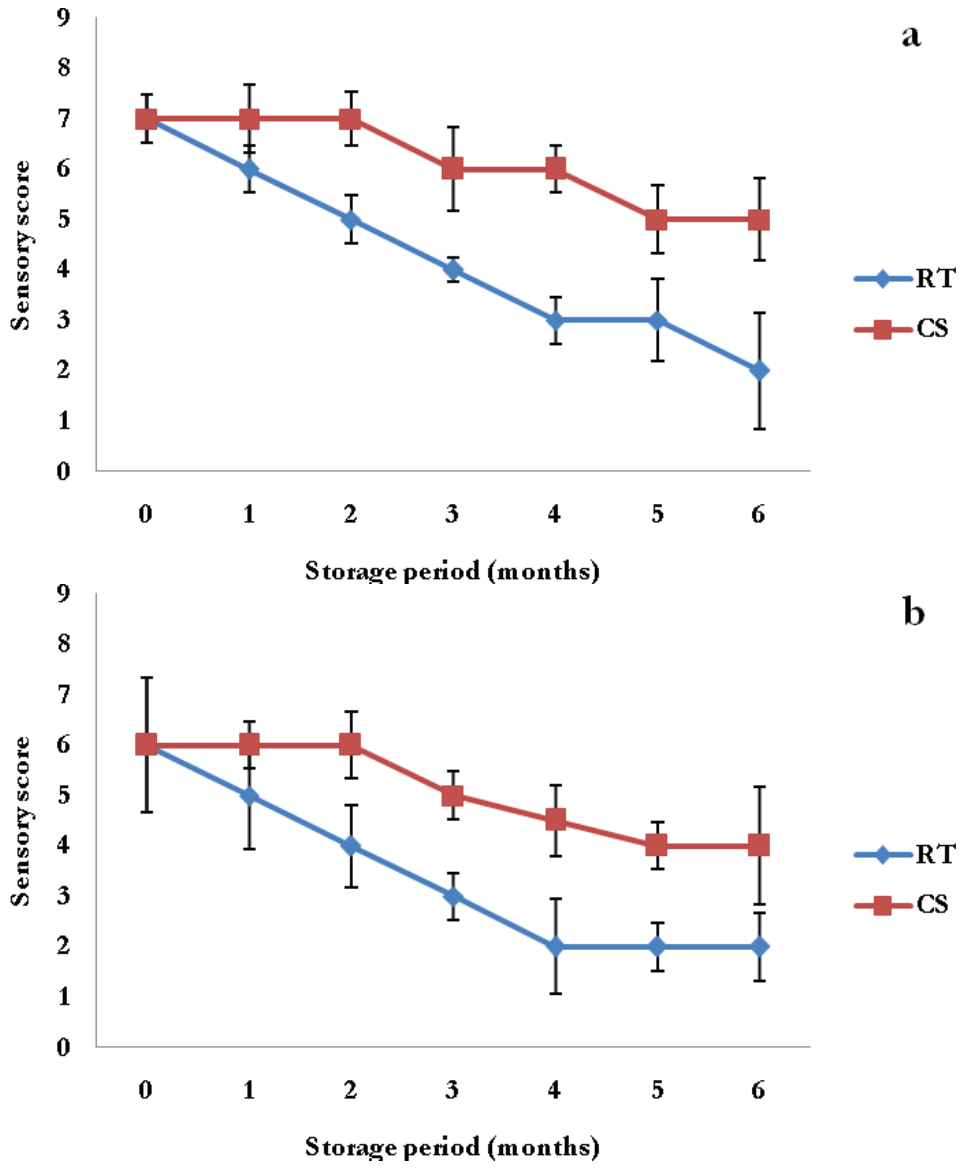


Fig. 6.26 Variations in sensory indices of optimized tuna protein hydrolysates **a.** FTPH; **b.** ATPH at ambient (28°C) and chilled conditions (4°C)

6.3.2.8 Total plate count

TPC values indicated an increasing trend ($p < 0.05$) which was more prominent at room temperature in comparison to chilled storage. During storage, a significant difference ($p < 0.05$) was observed in the microbial count of FTPH, being more prominent at room temperature in comparison to chilled storage (Fig. 6.27a; Table 6.8). In ATPH also an increase in TPC was observed which was significant

towards third month at room temperature whereas it was not so prominent or significant until final storage period in chilled condition (Fig. 6.27b; Table 6.8). In general, an increase by one log cycle was observed for the samples during storage. However the samples were within the microbial limit throughout the period of storage. For FTPH, it increased from an initial value of 4.4 to 5.4 log cfu/g at RT and to 5.1 log cfu/g under chilled condition. ATPH marked an increase from 4.1 to 5.2 log cfu/g and 4.9 log cfu/g at room and chilled conditions, respectively.

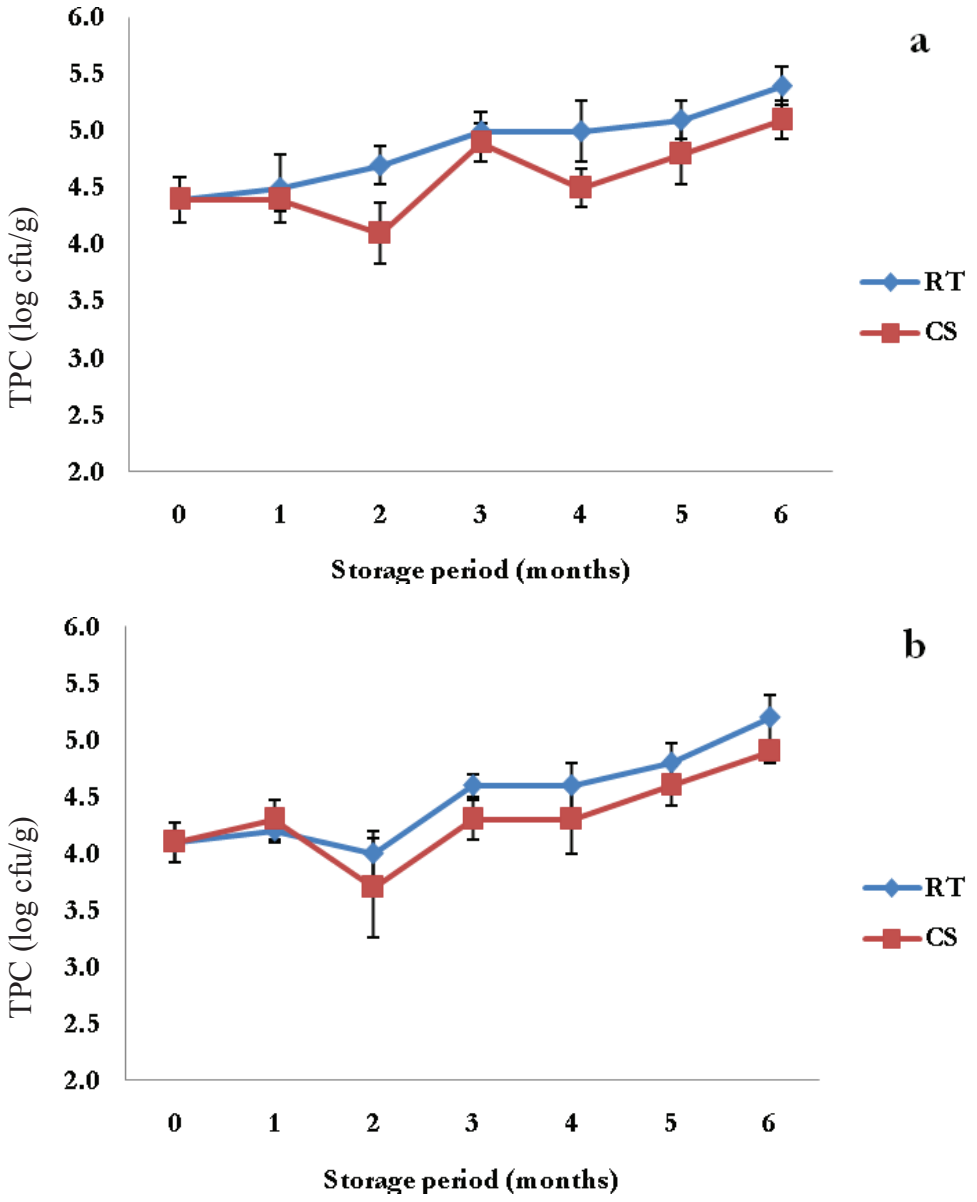


Fig. 6.27 Variations in total plate count of optimized tuna protein hydrolysates **a.** FTPH; **b.** ATPH at ambient (28°C) and chilled conditions (4°C)

Table 6.8 Variations in parameters of optimized tuna protein hydrolysate at ambient & chilled condition.

Parameters	Storage period (months)	FTPH		ATPH	
		Ambient storage	Chilled storage	Ambient storage	Chill storage
Moisture (%)	0	$5.82^{dA} \pm 0.42$	$5.82^{cA} \pm 0.42$	$5.63^{fA} \pm 0.11$	$5.63^{fA} \pm 0.11$
	1	$6.11^{cdA} \pm 0.20$	$5.79^{cA} \pm 0.21$	$5.95^{eA} \pm 0.04$	$5.65^{fB} \pm 0.04$
	2	$6.21^{cdA} \pm 0.26$	$5.98^{cA} \pm 0.18$	$6.18^{dA} \pm 0.07$	$5.89^{eB} \pm 0.07$
	3	$6.54^{cA} \pm 0.54$	$6.01^{cA} \pm 0.14$	$6.19^{dA} \pm 0.05$	$6.11^{dA} \pm 0.07$
	4	$7.89^{bA} \pm 0.32$	$6.33^{bcB} \pm 0.04$	$7.02^{cA} \pm 0.06$	$6.54^{cB} \pm 0.06$
	5	$7.72^{bA} \pm 0.49$	$6.65^{bB} \pm 0.14$	$7.65^{bA} \pm 0.06$	$6.71^{bB} \pm 0.11$
	6	$9.01^{aA} \pm 0.24$	$7.35^{aB} \pm 0.08$	$8.28^{aA} \pm 0.07$	$6.95^{aB} \pm 0.06$
pH	0	$5.75^{aA} \pm 0.02$	$5.75^{aA} \pm 0.02$	$5.71^{dA} \pm 0.04$	$5.71^{cdA} \pm 0.04$
	1	$5.92^{aA} \pm 0.02$	$5.78^{aA} \pm 0.05$	$5.77^{cA} \pm 0.02$	$5.69^{dB} \pm 0.03$
	2	$5.89^{aA} \pm 0.03$	$5.81^{aA} \pm 0.02$	$5.80^{bcA} \pm 0.02$	$5.75^{cB} \pm 0.04$
	3	$5.85^{aA} \pm 0.03$	$5.79^{aA} \pm 0.02$	$5.79^{bcA} \pm 0.03$	$5.71^{cdB} \pm 0.04$
	4	$5.90^{aA} \pm 0.04$	$5.84^{aA} \pm 0.01$	$5.83^{bA} \pm 0.03$	$5.83^{bA} \pm 0.03$
	5	$5.92^{aA} \pm 0.02$	$5.91^{aA} \pm 0.03$	$5.89^{aA} \pm 0.01$	$5.85^{abA} \pm 0.03$
	6	$6.01^{aA} \pm 0.04$	$5.92^{aA} \pm 0.03$	$5.92^{aA} \pm 0.03$	$5.89^{aA} \pm 0.02$

Parameters	Storage period (months)	FTPH		ATPH	
		Ambient storage	Chilled storage	Ambient storage	Chilled storage
Colour L*	0	$89.12^{aA} \pm 0.30$	$89.12^{aA} \pm 0.30$	$89.95^{aA} \pm 0.04$	$89.95^{aA} \pm 0.04$
	1	$86.55^{bB} \pm 0.45$	$87.84^{bA} \pm 0.57$	$89.97^{aA} \pm 0.02$	$89.68^{abA} \pm 0.23$
	2	$84.54^{cB} \pm 0.13$	$85.41^{cA} \pm 0.19$	$89.58^{aA} \pm 0.13$	$89.53^{abA} \pm 0.23$
	3	$83.40^{dB} \pm 0.56$	$85.30^{cA} \pm 0.37$	$88.78^{bB} \pm 0.44$	$89.31^{bA} \pm 0.24$
	4	$82.51^{eB} \pm 0.40$	$84.82^{cdA} \pm 0.12$	$87.43^{cB} \pm 0.26$	$88.83^{cA} \pm 0.30$
	5	$81.03^{fB} \pm 0.50$	$84.12^{deA} \pm 0.12$	$85.39^{dB} \pm 0.30$	$88.10^{dA} \pm 0.45$
	6	$79.72^{gB} \pm 0.80$	$84.00^{eA} \pm 0.33$	$83.94^{eB} \pm 0.19$	$87.06^{eA} \pm 0.30$
a*	0	$0.14^{gA} \pm 0.05$	$0.14^{fA} \pm 0.05$	$-0.75^{eA} \pm 0.03$	$-0.75^{dA} \pm 0.03$
	1	$1.59^{fA} \pm 0.22$	$1.04^{eB} \pm 0.06$	$-0.61^{dA} \pm 0.01$	$-0.66^{cB} \pm 0.02$
	2	$1.93^{eA} \pm 0.06$	$1.59^{dB} \pm 0.08$	$-0.57^{dA} \pm 0.01$	$-0.63^{bcB} \pm 0.06$
	3	$2.11^{dA} \pm 0.06$	$1.65^{dB} \pm 0.10$	$-0.58^{dA} \pm 0.01$	$-0.58^{bA} \pm 0.03$
	4	$2.57^{cA} \pm 0.05$	$1.81^{cB} \pm 0.03$	$-0.49^{cA} \pm 0.02$	$-0.53^{aA} \pm 0.02$
	5	$3.08^{bA} \pm 0.03$	$2.09^{bB} \pm 0.08$	$-0.31^{bA} \pm 0.02$	$-0.52^{aB} \pm 0.02$
	6	$3.31^{aA} \pm 0.06$	$2.26^{aB} \pm 0.04$	$-0.24^{aA} \pm 0.04$	$-0.50^{aB} \pm 0.02$

Parameters	Storage period (months)	FTPH		ATPH	
		Ambient storage	Chilled storage	Ambient storage	Chilled storage
b*	0	$16.41^{gA} \pm 0.09$	$16.41^{eA} \pm 0.09$	$13.23^{fA} \pm 0.03$	$13.23^{eA} \pm 0.03$
	1	$20.73^{fA} \pm 0.10$	$18.67^{dB} \pm 0.11$	$13.63^{eA} \pm 0.23$	$13.37^{eA} \pm 0.12$
	2	$21.27^{eA} \pm 0.04$	$18.89^{dB} \pm 0.06$	$13.52^{efB} \pm 0.21$	$14.03^{dA} \pm 0.17$
	3	$22.63^{dA} \pm 0.26$	$19.95^{cB} \pm 0.05$	$14.07^{dB} \pm 0.03$	$14.47^{cA} \pm 0.18$
	4	$23.49^{cA} \pm 0.27$	$20.04^{cB} \pm 0.20$	$15.32^{cA} \pm 0.19$	$14.82^{bB} \pm 0.04$
	5	$24.83^{bA} \pm 0.09$	$21.24^{bB} \pm 0.30$	$17.07^{bA} \pm 0.13$	$15.38^{aB} \pm 0.14$
	6	$25.38^{aA} \pm 0.04$	$21.75^{aB} \pm 0.21$	$18.82^{aA} \pm 0.18$	$15.35^{aB} \pm 0.37$
Solubility (%)	0	$88.30^{aA} \pm 0.80$	$88.30^{aA} \pm 0.80$	$92.13^{aA} \pm 1.42$	$92.13^{aA} \pm 1.42$
	1	$85.97^{aA} \pm 1.33$	$87.03^{abA} \pm 1.16$	$90.50^{aA} \pm 1.15$	$91.20^{aA} \pm 0.46$
	2	$80.37^{bB} \pm 1.02$	$84.80^{bA} \pm 0.56$	$87.70^{bB} \pm 1.40$	$91.20^{aA} \pm 0.90$
	3	$76.30^{cB} \pm 1.90$	$81.97^{cA} \pm 2.04$	$85.40^{bB} \pm 1.95$	$87.97^{bA} \pm 1.65$
	4	$73.27^{dB} \pm 1.63$	$81.67^{cA} \pm 1.29$	$78.53^{cB} \pm 1.46$	$84.60^{cA} \pm 1.51$
	5	$67.90^{eB} \pm 2.57$	$76.33^{dA} \pm 1.16$	$71.40^{dB} \pm 0.99$	$80.33^{dA} \pm 0.87$
	6	$61.07^{fB} \pm 0.49$	$69.50^{eA} \pm 0.85$	$65.87^{eB} \pm 1.43$	$71.87^{eA} \pm 2.11$

Parameters	Storage period (months)	FTPH		ATPH	
		Ambient storage	Chilled storage	Ambient storage	Chilled storage
TBARS (mg/kg)	0	$0.89^{dA} \pm 0.05$	$0.89^{bA} \pm 0.05$	$0.95^{fA} \pm 0.13$	$0.95^{eA} \pm 0.13$
	1	$1.77^{cdA} \pm 0.11$	$1.05^{bA} \pm 0.12$	$1.84^{eA} \pm 0.14$	$1.25^{dB} \pm 0.04$
	2	$2.19^{cA} \pm 0.07$	$1.73^{abA} \pm 0.06$	$1.96^{eA} \pm 0.07$	$1.49^{cB} \pm 0.07$
	3	$2.34^{bcA} \pm 0.06$	$2.23^{aA} \pm 0.07$	$2.20^{dA} \pm 0.16$	$1.50^{cB} \pm 0.09$
	4	$2.78^{abcA} \pm 0.06$	$1.89^{abA} \pm 0.07$	$2.86^{bA} \pm 0.03$	$1.97^{bB} \pm 0.08$
	5	$3.35^{abA} \pm 0.06$	$2.54^{aA} \pm 0.14$	$2.64^{cA} \pm 0.07$	$2.20^{aB} \pm 0.04$
	6	$3.63^{aA} \pm 0.08$	$2.25^{aB} \pm 0.09$	$3.21^{aA} \pm 0.04$	$2.17^{aB} \pm 0.07$
TMA-N (mg%)	0	$7.00^{eA} \pm 0.00$	$7.00^{cA} \pm 0.00$	$7.00^{cA} \pm 0.00$	$7.00^{aA} \pm 0.00$
	1	$10.50^{eA} \pm 4.95$	$7.00^{cA} \pm 0.00$	$10.50^{cA} \pm 4.95$	$7.00^{aA} \pm 0.00$
	2	$14.00^{deA} \pm 0.00$	$7.00^{cA} \pm 0.00$	$10.50^{cA} \pm 4.95$	$7.00^{aA} \pm 0.00$
	3	$21.00^{cdA} \pm 9.90$	$10.50^{bcB} \pm 4.95$	$14.00^{bcA} \pm 0.00$	$7.00^{aA} \pm 0.00$
	4	$28.00^{bcA} \pm 0.00$	$14.00^{abcB} \pm 0.00$	$21.00^{bA} \pm 9.90$	$14.00^{aA} \pm 0.00$
	5	$35.00^{bA} \pm 0.00$	$14.00^{abB} \pm 0.00$	$35.00^{aA} \pm 0.00$	$14.00^{aB} \pm 0.00$
	6	$49.00^{aA} \pm 0.00$	$21.00^{aB} \pm 0.00$	$42.00^{aA} \pm 9.90$	$14.00^{aB} \pm 0.00$

Parameters	Storage period (months)	FTPH		ATPH	
		Ambient storage	Chilled storage	Ambient storage	Chilled storage
Sensory	0	$7.00^{aA} \pm 0.47$	$7.00^{aA} \pm 0.47$	$6.00^{aA} \pm 1.33$	$6.00^{aA} \pm 1.33$
	1	$6.00^{bB} \pm 0.47$	$7.00^{aA} \pm 0.67$	$5.00^{bB} \pm 1.05$	$6.00^{aA} \pm 0.47$
	2	$5.00^{cB} \pm 0.47$	$7.00^{aA} \pm 0.53$	$4.00^{cB} \pm 0.82$	$6.00^{aA} \pm 0.67$
	3	$4.00^{dB} \pm 0.24$	$6.00^{bA} \pm 0.82$	$3.00^{dB} \pm 0.47$	$5.00^{bA} \pm 0.47$
	4	$3.00^{eB} \pm 0.47$	$6.00^{bA} \pm 0.47$	$2.00^{eB} \pm 0.94$	$4.50^{bcA} \pm 0.71$
	5	$3.00^{eB} \pm 0.82$	$5.00^{cA} \pm 0.67$	$2.00^{eB} \pm 0.47$	$4.00^{cA} \pm 0.47$
	6	$2.00^{fB} \pm 1.16$	$5.00^{cA} \pm 0.82$	$2.00^{eB} \pm 0.67$	$4.00^{cA} \pm 1.16$
TPC (log cfu/g)	0	$4.40^{dA} \pm 0.20$	$4.40^{cdA} \pm 0.20$	$4.10^{cA} \pm 0.17$	$4.10^{cA} \pm 0.17$
	1	$4.50^{dA} \pm 0.30$	$4.40^{cdA} \pm 0.10$	$4.20^{cA} \pm 0.10$	$4.30^{cA} \pm 0.17$
	2	$4.70^{cdA} \pm 0.17$	$4.10^{dB} \pm 0.27$	$4.00^{cA} \pm 0.20$	$3.70^{dA} \pm 0.44$
	3	$5.00^{bcA} \pm 0.17$	$4.90^{aA} \pm 0.17$	$4.60^{bA} \pm 0.10$	$4.30^{bcA} \pm 0.17$
	4	$5.00^{bcA} \pm 0.27$	$4.50^{bcB} \pm 0.17$	$4.60^{bA} \pm 0.20$	$4.30^{bcA} \pm 0.30$
	5	$5.10^{abA} \pm 0.17$	$4.80^{abA} \pm 0.27$	$4.80^{bA} \pm 0.17$	$4.60^{abA} \pm 0.17$
	6	$5.40^{aA} \pm 0.17$	$5.10^{aA} \pm 0.17$	$5.20^{aA} \pm 0.20$	$4.90^{aA} \pm 0.10$

6.3.3 Economic feasibility analysis

The economic feasibility of producing the optimized tuna fish protein hydrolysate (TPH) on an industrial scale was assessed. For this, the enzymatic hydrolytic conditions previously identified to produce TPH with superior functional properties viz., FTPH (0.34 % E/S, 30 min, 60°C, pH 6.5) and TPH with antioxidant properties viz., ATPH (0.98 % E/S, 240 min, 60°C, pH 6.5) in laboratory-scale evaluations, were used to model on large scale production utilizing the pilot facilities available at ICAR-CIFT. Fig. 6.28 outlines a fairly typical process for producing fish protein hydrolysates and Fig. 6.29 picturizes the pilot scale production of optimized TPH.

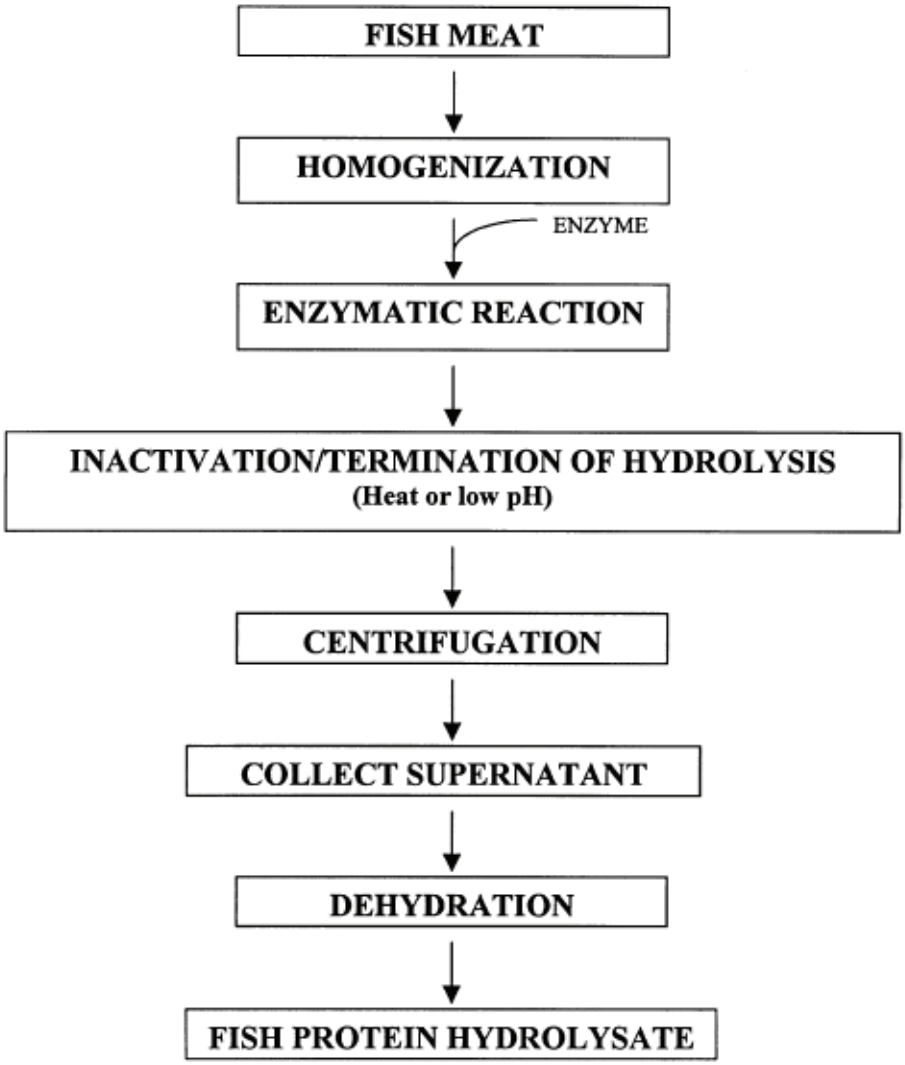


Fig. 6.28 Typical flow diagram for enzymatic hydrolysis of fish protein

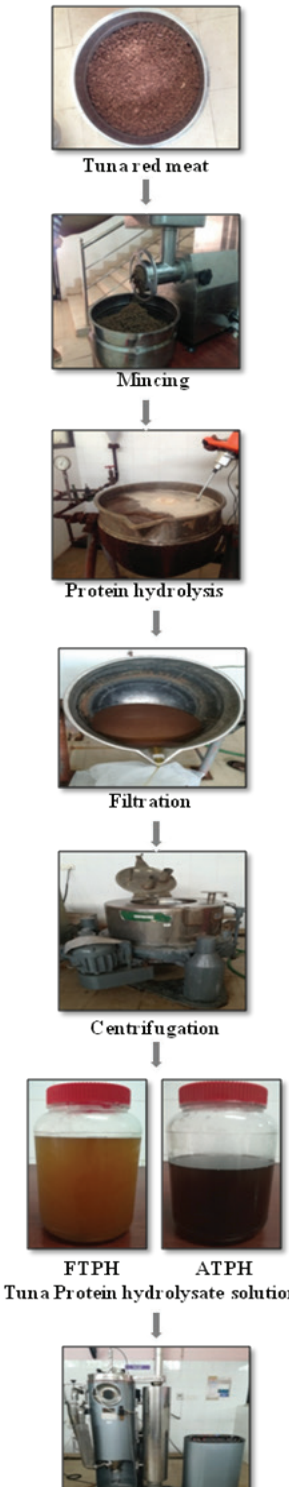


Fig. 6.29 Pilot scale production of optimized tuna protein hydrolysate

The results of the upscaling showed that the yield obtained from raw material after water washing and sodium bicarbonate solution treatment was about 87.5 % and upon further mincing a loss of about 2 – 2.5 % was noted. On an average the protein content of raw material was about 25 % and a recovery of about 48 % was observed on conversion to its hydrolysate (FTPH) whereas the protein recovery was about 56 % in ATPH. The yields obtained on spray drying of the hydrolysate solution were 5.4 % (FTPH) and 7.9 % (ATPH). The final yields obtained from the raw material to their respective hydrolysates were 10.8 % (FTPH) and 12.4 % (ATPH).

Evaluation of purchase cost of cooked tuna red meat showed that currently the Indian seafood industry proposes to sell the sample in bulk at the price of Rs 30/kg. Hence this amount was set as the purchase cost of raw material. Prices of products were assumed at the current market prices of the same products, or similar products as references. The commercial fish protein hydrolysate powders reported a selling price ranging from Rs 250-300/kg for low end crude hydrolysate for fertilizer application on bulk whereas the retail selling price was high upto Rs 1000 /kg. Hydrolysate for high end pharmaceutical applications have an average international market price of Rs. 2000/kg whereas in India the hydrolysate price in domestic market ranged from Rs 500 to 1000/kg. Based on this market survey and our intended application which is meant for food and pharmaceutical sector, an average price of Rs 750/kg was set as the selling price for the end product.

The equipments used in the hydrolysis process included a raw material washing unit, grinder/mincer, chemical reactor for hydrolyzation, decanter, centrifuge, filtration unit, sterilization unit, spray drier and packaging machine. The cost of equipments with reference to the prevailing market price, by adjusting the specific volume or capacity was considered.

The process operation mode was set up as a batch process and the annual operation time was set at 7200 hrs (300 working days per annum), the typical annual operation time for a batch process (He et al., 2015b). The TPH production yield per batch was set based on the raw material intake of one tonne per day yielding final product of about 12.4 %. The economic viability on upscaling the hydrolysate production was assessed (Table 6.9). The net profit ratio was calculated to be 23.72 % with a rate of return of 29.35 %. The breakeven point was assessed to be 50.68 % and an investment payback time of 1.6 years was observed. Return on investment of the scaled up processes were found to be very sensitive to the purchase cost of raw material and selling price of fish protein hydrolysates. The economic feasibility study indicated profitability of producing TPH on an industrial scale, set under proposed set of conditions.

Table 6.9 Economic feasibility analysis of optimized tuna protein hydrolysate

	Particulars	**Amount (Rs)**
A	**Fixed capital**	
1	Land and Building	2000000
2	Machinery and Equipments	15000000
	Total	**17000000**
B	**Working capital (per month)**	
1	Personnel	200000
2	Raw material inc. packaging (Tuna red meat: 1000 Kg @Rs. 30 for 25 days; Papain: 10 Kg @ Rs. 2500 for 25 days; other packaging and miscellaneous charges)	1400000
3	Utilities (Electricity, water, diesel etc.)	150000
4	Other contingency expenses (Repair/maintenance, transportation, publicity, postage, insurance etc.)	100000
	Total	**1850000**
C	**Total Capital Investment (Fixed capital and working capital for three months)**	22550000

	Financial Analysis	
i	**Cost of production per annum** (Working capital for 9 months with depreciation charges on fixed capital (5% for building and 10% for machinery) and interest on total capital investment excluding land and building (15%))	21282500
ii	**Turnover per annum** (Hydrolysate: 124 Kg per day @ Rs. 750 for 300 days	27900000
iii	**Annual Fixed Cost** (All depreciation, interest, 40% of (salary, wages, utilities, contingencies except insurance), insurance)	6799700
iv	**Net Profit** (exclusive tax) (Turnover per annum - Cost of production)	**6617500**
v	**Net Profit Ratio** (Net Profit x 100/Turn over)	**23.72**
vi	**Rate of Return** (Net Profit x 100/Capital Investment)	**29.35**
vii	**Break Even Point** (Annual Fixed Cost × 100)/(Annual Fixed Cost + Profit)	**50.68**
viii	**Payback Period** (Total capital investment + Cost of Production/Turnover) (yrs)	**1.6**

6.4 Conclusion

Present study attempted to characterize the optimized functional hydrolysate and antioxidant hydrolysates from yellowfin tuna cannery waste. Nutritional evaluation indicated its richness in protein with balanced amino acid profile. Similarly element analysis also indicated its richness in minerals like sodium, potassium, calcium, phosphorous and magnesium. Storage stability studies indicated its stability for upto 1 - 2 months at room temperature whereas under chilled condition it was stable upto 3 -5 months, based on the optimization adopted for deriving hydrolysate. Further upscaling of the process marked the production of protein hydrolysate to be economically feasible indicating return on investment of the scaled up processes to be sensitive to the raw material cost as well as selling price of fish protein hydrolysates.

Chapter 7

Tuna protein hydrolysate as fortifying and stabilizing agent in mayonnaise

7.1 Introduction

In the recent years, the preference of consumers for nutritionally rich healthier diet has increased and this scenario has shifted the market demand towards more fortified supplies. Concurrently, the health issues on account of the intake of certain lipid rich foods have diverted the food sector towards the development of low-fat food commodities. Previously, authors like McClements and Demetriades (1998) have reported challenges associated with the development of low fat products, having comparative appearance, texture, stability, and flavor as their full-fat counter parts. Mayonnaise, being a semi-solid oil-in-water emulsion, is extensively studied in this context. Mayonnaise is traditionally prepared from a mixture of egg yolk, vinegar, oil and other optional ingredients (Aluko and McIntosh, 2005; Thomareisa and Chatziantoniou, 2011). Egg yolk is a major ingredient in mayonnaise formulation and they act as an emulsifying and stabilizing agent in this emulsion system. However due to some health concerns like high cholesterol content, allergic problems of some consumers to egg protein as well as easy susceptibility to microbial contamination and spoilage, an urge for partial or total replacement with cholesterol-free ingredients is gaining importance. In this perspective, several plant and animal proteins have been extensively investigated as

egg substitute in mayonnaise emulsion systems (Ghoush et al., 2008; Ma and Boye, 2013; Ghazaei et al., 2015). Alternatively, reports by Binsi et al. (2017a) indicated fish roe (egg) protein powder to have superior emulsifying properties and hence, suggested as an ideal egg substitute in mayonnaise (Sathivel et al., 2009).Similarly, Siripongvutikorn et al.(2016) used tuna roe and inulin as oil replacer in mayonnaise formulation. Likewise, several studies highlighting enzymatic hydrolysis as an effective means for improving the emulsifying properties of fish proteins have been documented (Parvathy et al., 2016; Binsi et al., 2016). Even though many of these reports highlighted the superior surface-active properties of the hydrolysates compared to their native proteins, limited data is available on the application of fish meat hydrolysate as egg substitute in mayonnaise preparations.

Tuna red meat, a major by-product from tuna cannery has currently low commercial value on account of ineffective utilization. Previous studies have established the superior surface active properties of tuna red meat protein including emulsion properties, which can be explored in the stabilization of emulsion based food formulations such as mayonnaise (Sánchez-Zapata et al., 2011; Chi et al., 2015). As an advanced step, in the present work, attempt was made to evaluate the usefulness of tuna red meat hydrolysate as egg substitute in mayonnaise formulation, with emphasis on physico-chemical, rheological, sensory and microbiological indices. Further, an optimized substitution rate was established based on selected product acceptability parameters, and the stability of the selected formulation was monitored under chilled storage condition.

7.2 Materials and methods

7.2.1 Raw Materials and Chemicals

Protein hydrolysate from the red meat of yellow fin tuna optimized for functional properties (chapter 4; table 4.3), hereafter referred to as TPH, was used for the study. Mayonnaise ingredients viz., soyabean oil, sugar, vinegar, mustard and salt were purchased from commercial suppliers. Egg yolk was carefully separated from the egg white after puncturing of the yolk membrane and used. Further all chemicals used for the study were of analytical grade.

7.2.2 Preparation of mayonnaise

Mayonnaise was formulated adopting the procedure described by Gaonkar et al. (2010) with slight modifications, employing the following ingredients viz., soyabean oil (45 %, w/w), fresh egg yolk (15 %, w/w), sugar (20 % w/w), vinegar (17 %, w/w), mustard (2 %, w/w) and salt (1 %, w/w). Egg yolk was thoroughly mixed in a beaker, into which sugar and salt were dissolved with continuous stirring. Further soybean oil was added in small proportions. One minute after the incorporation of oil, vinegar was added and stirring was continued for another minute. The resultant coarse emulsion was further homogenized (230 VAC T-25 digital Ultra-turrax, IKA, India) to get smooth textured mayonnaise which was filled in air tight containers for further analysis. Protein fortified mayonnaise was prepared by replacing egg yolk with TPH in different proportions.

7.2.3 Preliminary product acceptability study

Preliminary trials were carried out to finalise the replacement levels of egg yolk with TPH in mayonnaise for further characterisation and storage analysis. For this, egg yolk (15 %) in mayonnaise was replaced with TPH up to 5 % (1:2::TPH:egg yolk), 7.5 % (1:1) and 10 % (2:1), hereafter referred to as fortified mayonnaise: F_5,

$F_{7.5}$ and F_{10}, respectively. Mayonnaise with egg yolk (no addition of TPH) was kept as control (C).

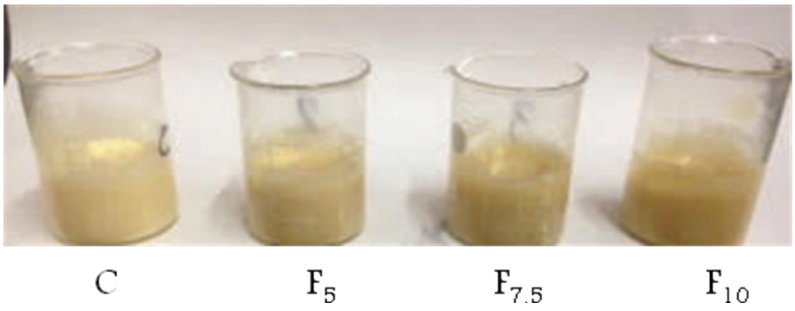


Fig. 7.1 Mayonnaise samples

Sensory evaluation was carried out with ten trained panellists for attributes viz., appearance, odour, flavour, colour, texture and overall acceptability using a 9 point hedonic scale (Meilgaard et al., 2006) (Annexure 3). Other parameters viz.,colour (Colorflex EZ 45/0, Hunter Associates Lab inc., Reston, Virginia, USA) and emulsion stability index (Mun et al., 2009) were also analysed. For the emulsion stability test, about 15 g mayonnaise sample was transferred to a test tube which was tightly sealed with parafilm and stored at 50°C for 48 h. After storage, the emulsion was centrifuged for 10 min at 3000 xg to remove the top oil layer. The emulsion stability was characterised from the proportion of precipitated layer to the initial volume and expressed as percentage. Based on the results of preliminary study, selected fortified mayonnaise combination along with control was taken for proximate analysis, particle analysis, morphological characterisation, rheological properties and storage stability studies.

7.2.4 Characterization of mayonnaise

7.2.4.1 Proximate composition

Evaluation of proximate composition of mayonnaise samples viz., fortified and control were carried out as per AOAC (2012).Total carbohydrate was estimated from the difference in weight of other constituents (protein, fat, water, ash) to the total weight of the sample. Caloric value of the sample was calculated as per Souci et al. (2000) as:

Total calories (kCal) = (4 x protein weight %) + (9 x fat weight %) + 4 x carbohydrate weight %)

7.2.4.2 Emulsion microstructure

Microstructure of the emulsion was analysed by smearing sample directly onto a microscope slide and analysing under an inverted microscope (Leica Microsystems, Wetzlar, Germany) at room temperature (25^{o}C). The images were obtained at 4 x magnification and dimensions were determined using image processing software (LeicaMicrosystems Imaging Solutions, Cambridge, UK) with a CCD camera.

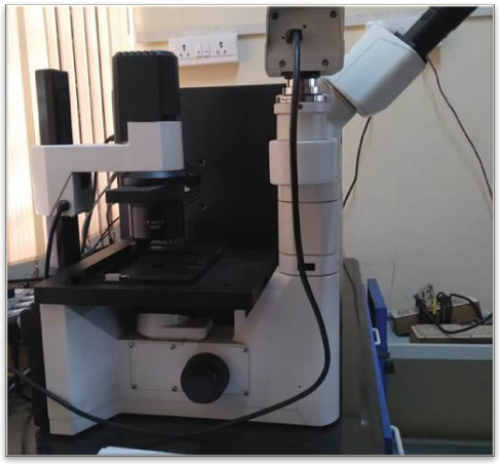


Fig. 7.2 Inverted microscope

7.2.4.3 Particle size analysis

The mean particle size of the mayonnaise samples were determined by particle size analyzer using DLS principle (Particle Sizing Systems, Inc. Santa Barbara, Calif., USA).The principle involves the sample of interest being illuminated by a laser beam and the fluctuations of the scattered light are detected at a known scattering angle θ by a fast photon detector. Using this technique of light scattering, particle sizing down to 1 nm diameter is facilitated.

Fig. 7.3 Particle size analyser

7.2.4.4 Rheological properties

Linear dynamic viscoelasticity measurements, including strain sweep, frequency sweep, temperature ramp, were analyzed using a Controlled-Stress Rheometer (Physica MCR 101, TruGapTM system, Anton Paar GmbH, Austria) in oscillatory mode in the range of 1 to 200 Pa at 25^0C. A 20 mm parallel-plate geometry was used with a gap setting of 1 mm between peltier plate and geometry. Sample was loaded onto the temperature-controlled peltier plate, equilibrated to 25^oC and the plate geometry was lowered to the gap previously adjusted. Shear stress was measured at varying shear rates from 0 to 100 s^{-1}. The elastic modulus (G') and viscous modulus (G") were measured as a function of frequency. Frequency was varied from 1 Hz to 10 Hz and storage and loss modulus were obtained as a function of frequency. The slope of the regression of G' and G" (on log scale)

with change in frequency was obtained in order to assess the viscoelastic nature of the sample. Oscillation stress sweep was plotted for storage modulus against oscillation stress from 0.2 to 100 Pa. Temperature sweep of the sample was carried at temperatures ranging from 0 to 100 ^{o}C (heating rate - 5 ^{o}C/min) at a constant shear of 100 s^{-1}. Changes in viscosity with shear rates ranging from 0 to 100 s^{-1} at constant temperature of 25^{o}C was carried out and the flow curve was obtained by plotting log shear rate with log viscosity values. Among the various rheological models, Herschel-Bulkley model was found to be most appropriate for explaining the flow behavior of fish mayonnaise with the following equation:

$$\tau = \tau_o + k\,\gamma^{n}$$

where, $\tau$is the shear stress (Pa), τ_o the yield stress (Pa), γ is the shear rate (s^{-1}), k is the consistency coefficient (Pa s^{n}) and n is the flow behavior index (dimensionless).

Fig. 7.4 Controlled-Stress Rheometer

7.2.5 Storage stability studies

Selected fortified mayonnaise and control mayonnaise were packed in airtight plastic bottles, stored at 4^{o}C and subjected to weekly analysis for indices

viz., pH (ECPH S1042S, Eutech Instruments, Singapore), emulsion stability index (Mun et al., 2009) (described in section 7.2.3), viscosity (DV-E Brookfield digital viscometer), PV and FFA (AOAC, 2012), sensory (Meilgaard et al., 2006) (described in section 7.2.3) and microbiological parameters (USFDA, 2001) for a period of four weeks.

Fig. 7.5 Viscometer

PV was determined iodimetrically after proper sample dehydration followed by fat extraction using chloroform.A 20 ml chloroform extract (for fat) was taken directly and dissolved in 30 ml glacial acetic acid into a clean 250 ml Iodine flask. 5 ml of saturated potassium iodide was added to the flask and kept in dark for 30 min. 50 ml distilled water was added and titrated against 0.01 N sodium thiosulphate solution using starch as an indicator. From the titre value, PV of the sample was calculated as follows and expressed as milli equivalent of peroxide per kg of fat.

$$PV = \frac{V \times N \text{ of } Na_2S_2O_3 \times 1000}{\text{Weight of sample}}$$

For estimation of FFA, suitable quantity of the sample was blended with anhydrous sodium sulphate in a mortar. The blend was shaken with chloroform,

and filtered. Twenty millilitre of extract was taken in a clean beaker. Chloroform was evaporated on a water bath and weight of fat determined. Another 20 ml of the extract was transferred to conical flask. Chloroform evaporated off and to this 10 ml of neutral alcohol was added. This was titrated against 0.01 N NaOH using phenolphthalein indicator. Percentage FFA was calculated (1 ml of 1N NaOH = 0.28 g of oleic acid in 1 L).

$$\text{FFA} = \frac{\text{Volume of NaOH used X 0.01 X 0.28 X 100}}{\text{Weight of fat}}$$

For aerobic plate count, serial dilution of blended sample using pour plate technique was adopted.Phosphate buffer was used for dilution in ratio (1: 9 :: sample : buffer (w/v)) and the homogenized sample was serially diluted. Three consecutive dilutions were pipetted into clean dry petri dishes. About 12-15 ml plate count agar (cooled to 45 ± 1°C) was added to each plate and mixed thoroughly and uniformly. Plates were then incubated at 35°C ± 2°C for 48 ± 2 h for evaluating aerobic plate count and results expressed as log cfu/g.

7.2.6 Statistical analysis

The analytical data obtained in triplicate were subjected to analysis of variance (ANOVA) and the differences between means were evaluated by duncan's multiple range test. SPSS statistic programme (SPSS 16.0 for Windows, SPSS Inc., Chicago, IL) was used for result interpretation.

7.3 Results and discussion

7.3.1 Preliminary product acceptability study

To comprehend the levels of TPH incorporation in mayonnaise as an egg substitute without affecting the quality as well as stability attributes, initial trials were carried out. Fortified mayonnaises viz., F_5, $F_{7.5}$, F_{10} and control sample were mainly subjected to sensory acceptability and evaluation of selected physico-chemical properties which are generally considered as crucial in determining the acceptability and stability of mayonnaise viz., colour and emulsion stability. The results indicated a significant decrease ($p < 0.05$) in overall sensory acceptability of the product with higher levels of protein hydrolysate (at and beyond 50 %) incorporation, as inferred from sensory evaluation scores. This must be on account of the fish flavor as well as slight bitterness imparted by protein hydrolysate when incorporated at higher concentrations (Table 7.1). The development of bitterness in hydrolysate is generally associated with the levels of hydrophobic amino acids. During hydrolysis of protein the buried hydrophobic peptides get exposed resulting in detection of bitter taste by human taste buds. There are previous studies reporting the release of bitter tasting peptides during hydrolysis creating acceptability issues during food applications (Yarnpakdee et al., 2015).

Colour is one of the major perceptive attributes that influences the overall acceptability of a product. Instrumental colour characteristics of the mayonnaise formulations were evaluated (Table 7.1). Incorporation of TPH as egg yolk substitute in mayonnaise formulation resulted in significant changes ($p < 0.05$) to the product colour with higher levels of substitution imparting reddish-brown colour to the mayonnaise. The colour variations are bound to occur on account of the compositional variations in the samples. Normally, a product appeal is affected when it deviates from the standard colour range and this variation also had an

influence on the overall acceptability of the fortified samples.

Emulsion stability refers to the ability of an emulsion to resist changes in its physicochemical properties over time and is influenced by various mechanisms viz., gravitational separation, flocculation, coalescence, partial coalescence, ostwald ripening and phase inversion (McClements, 2005). The protein hydrolysate used in the present study possessed comparatively superior functional properties (Table 4.3) than previously reported studies (Taheri et al., 2013; Chi et al., 2014), and hence could be considered ideal for using as egg substitutes. Emulsion stability index (ESI) of mayonnaise samples also indicated similar higher values with narrow range of variations between control and fortified samples (98.67 - 99.89 %). Similar range of ESI between 98.77 - 99.80 % was reported by Siripongvutikorn et al. (2016) in both control mayonnaise and samples containing different levels of tuna roe with inulin gel. Based on the preliminary analysis, fortified mayonnaise: F_5 (containing 10 : 5 :: egg yolk : TPH (g/100g mayonnaise)) was considered having superior properties and hence selected for further storage stability studies.

Table 7.1 Variations in parameters viz., colour, emulsion stability index and overall sensory score of mayonnaise samples

Parameters	C	F_5	$F_{7.5}$	F_{10}
L*(Lightness)	$80.87^{d}\pm 0.14$	$67.98^{c}\pm 0.06$	$62.78^{b}\pm 0.03$	$59.34^{a}\pm 0.02$
a* (Redness)	$1.78^{a}\pm 0.00$	$3.26^{b}\pm 0.01$	$3.34^{c}\pm 0.01$	$5.15^{d}\pm 0.01$
b* (Yellowness)	$28.55^{a}\pm 0.02$	$30.69^{c}\pm 0.02$	$29.32^{b}\pm 0.01$	$33.87^{d}\pm 0.03$
Emulsion Stability Index (%)	$99.89^{c}\pm 0.04$	$98.67^{a}\pm 0.5$	$99.17^{ab}\pm 0.16$	$99.75^{bc}\pm 0.06$
Overall sensory score	$8.3^{c}\pm 0.5$	$7.6^{c}\pm 0.7$	$5.9^{b}\pm 0.6$	$4.8^{a}\pm 0.6$

Values are expressed as Mean ± SD; n = 3; Different superscript within the row indicate significant difference ($p < 0.05$); C: Control (15 egg yolk :0 protein hydrolysate); F_5 (10 egg yolk :5 protein hydrolysate); $F_{7.5}$ (7.5 egg yolk : 7.5 protein hydrolysate); F_{10} (5 egg yolk : 10 protein hydrolysate) in 100 g mayonnaise formulation

7.3.2 Characterization of selected mayonnaise formulation

7.3.2.1 Proximate composition

Presently, food industry has made it mandatory for almost all food commodities to have standardized nutritional labels so as to ensure awareness among consumers on the nutritional status of the foods they choose. In addition, they serve as a means to build proper conditions for fair marketing among competing food companies.The direct effect of egg yolk substitution with TPH was a statistically significant ($p < 0.05$) increase in protein content(by 2.58 %) and reduction in fat content (by 4.93 %) in fortified mayonnaise (F_5) compared to control (Table 7.2). Previously, El-Bostany et al.(2011) reported a reduction in total fat content in emulsion based products on account of their fat components being replaced with non-fat ingredients. Moisture content in fortified sample was significantly lower than control ($p < 0.05$) which might be on account of the replacement of egg yolk with protein hydrolysate which is a dry powder with negligibly low moisture content. Rashed et al. (2017) reported a broad range of moisture content values, ranging between 16.63- 59.93 % in selected commercially available mayonnaise samples in Malaysia. As per USDA/NASS (2005), traditional mayonnaises present a caloric value of 717 kcal/100 g while the light versions contain 324 kcal/100 g. The present study indicated a value in medium range with control sample having a caloric value of 534.54 kcal/100g while it was slightly lower for fortified one (524.05 kcal/100g).

Table 7.2 Proximate composition of mayonnaise samples

Sample	Proximate composition (%)					Calories (kcal)
	Moisture	Protein	Fat	Ash	Carbohydrate	
Control	$28.24^{a}\pm 0.87$	$3.58^{a}\pm 0.09$	$50.22^{a}\pm 0.97$	$0.9^{a}\pm 0.02$	$17.06^{a}\pm 1.91$	534.54
Fortified	$24.56^{b}\pm 0.11$	$6.16^{b}\pm 0.38$	$45.29^{b}\pm 0.37$	$1.04^{b}\pm 0.05$	$22.95^{b}\pm 0.84$	524.05

Values are expressed as Mean ± SD; n = 3; Different superscript in the same column indicate significant difference ($p < 0.05$)

7.3.2.2 Emulsion microstructure

Mayonnaise represents emulsions which are kinetically stabilised mixtures of two immiscible fluids. Their droplet size distribution as well as surfactant packing at the oil/water interface was monitored microscopically. Observation of microstructure of emulsion under an inverted microscope indicated stabilized emulsion droplets with adequate protection by aqueous phase around core oil molecules indicating the high emulsifying capacity of yolk protein as well as protein hydrolysate (Fig. 7.6).The emulsions showed particles in the size range of 40 - 120µ in control while the particle size was comparatively lower (5 – 100 µm) in fortified one indicating the better interfacial stability provided by smaller peptides. Reports indicate emulsion based foods to have droplet diameters generally ranging between 0.1 and 100 µm (Walstra, 1996; Dickinson and Rodriguez, 1999). Studies suggested that emulsifiers with superior capacity are capable of reducing the average oil droplet size as well as increase the droplet interaction forming an extensive structural network in emulsion influencing the appearance, texture as well as mouth feel of the product (Ma and Boye, 2013).

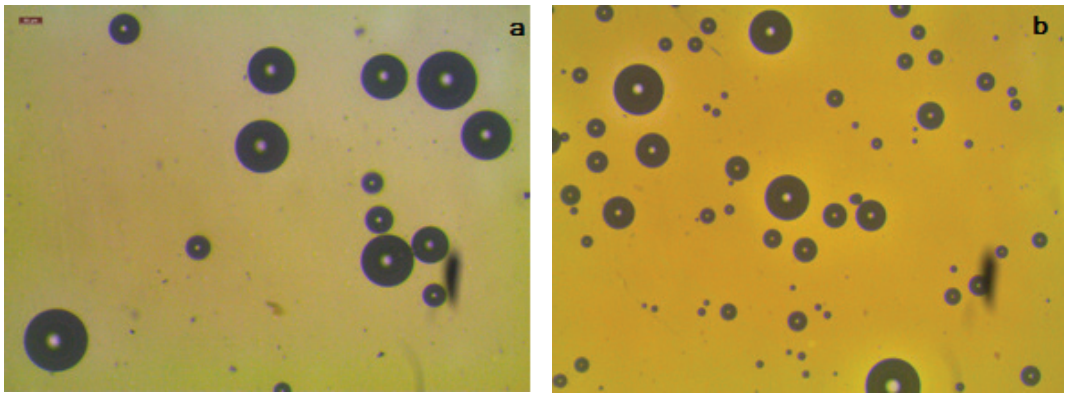


Fig. 7.6 Inverted microscopic image of **a.** Control mayonnaise **b.** Fortified mayonnaise

7.3.2.3 Particle size analysis

Measurement of particle size within an emulsion is important as it has strong impact on its stability, optical properties, rheology as well as its sensory attributes (McClements, 2007). The three most commonly used mean particle size values are the number-weighted mean diameter (d_{10}), the surface-weighted mean diameter (d_{32}) and the volume-weighted mean diameter (d_{43}). Generally, a significant difference between these values indicates broad particle size distribution.

In the present study, the average particle size (d_{32}) of control mayonnaise was about 476.1 µm (Fig. 7.7a) and that of fortified mayonnaise was 406.6 µm (Fig. 7.7b). Lower particle size, indicative of higher emulsion quality was observed for fortified mayonnaise which must be on account of superior emulsifying properties of TPH used to partially replace egg yolk. Previous studies suggest better emulsification activity for hydrolyzed proteins than their parent source due to the fact that enzymatic hydrolysis results in unfolding of the globular structure and increase hydrophobicity of proteins enhancing their interaction with the oil droplets (Klompong et al., 2007). The variations observed in the particle size values were 999.3 µm (d_{43}) and 70.6 µm ((d_{10}) for control and 849.6 µm (d_{43}) and 84.2 µm ((d_{10}) for fortified one implying the wide distribution in particle size within the samples viz., both control as well as fortified one. The particle size distribution curves (Fig. 7.7) also revealed that samples in the present study contained droplets of different size range referred to as poly disperse curves. However the range of particle size were comparatively more in control sample (about 6 particle size range) whereas it was limited to three size range in fortified one confirming the superior emulsifying properties of protein hydrolysate to generate more uniform sized emulsion droplets compared to control.

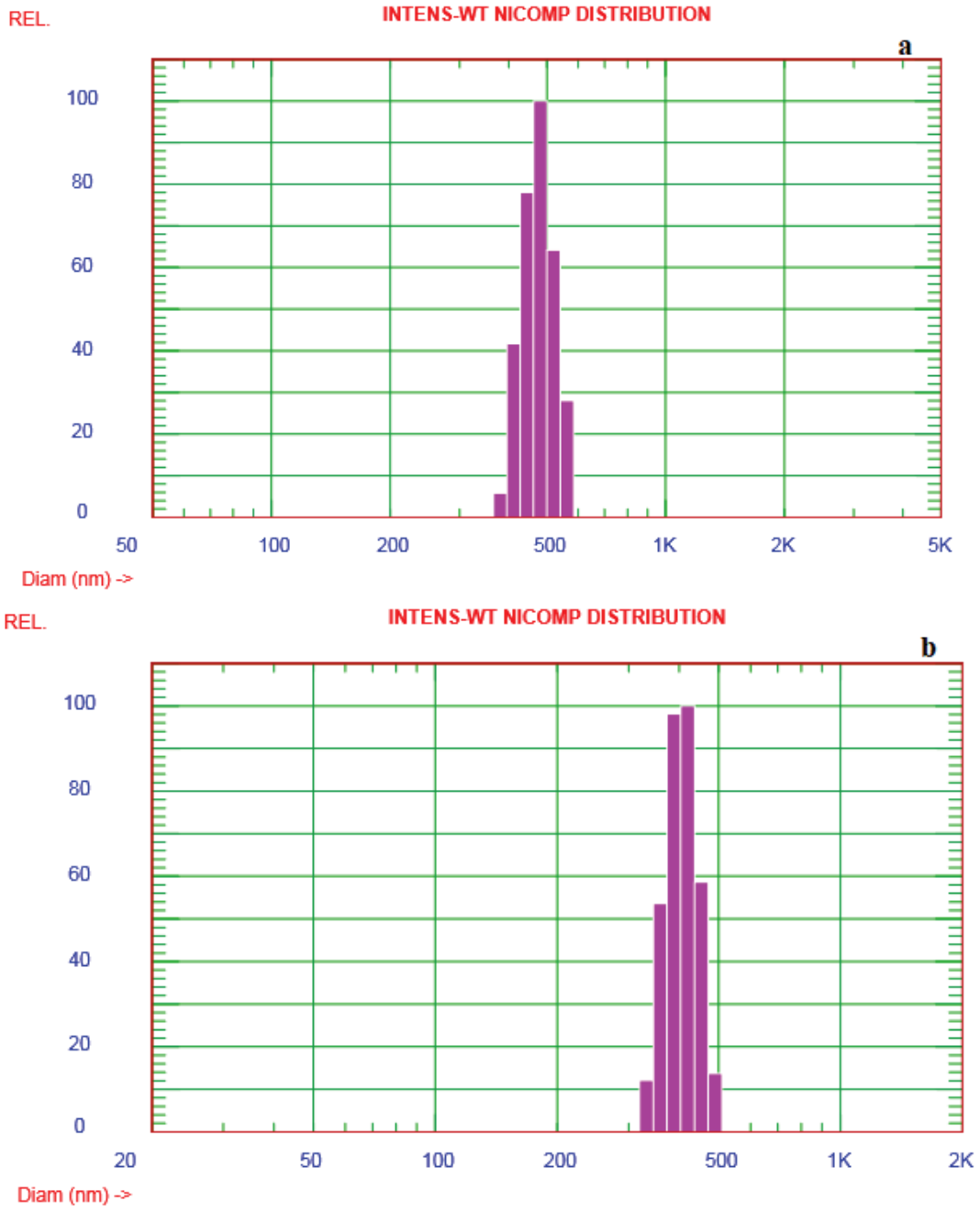


Fig. 7.7 Particle size distribution of **a.** Control mayonnaise **b.** Fortified mayonnaise

7.3.2.4 Rheological properties

7.3.2.4.1 Frequency sweep

In order to assess the stability of mayonnaise emulsion, frequency sweep was carried out at constant temperature of 25°C, in the frequency range of 1-10 Hz. The rheological behavior of fortified and control samples showed distinct differences as a function of frequency, with the fortified sample having significantly higher storage and loss modulus values compared to that of control sample (Fig. 7.8). Slope indicated the strength of the network with the applied frequency (Binsi et al., 2006). The significantly higher G' values of fortified samples suggests the superior elastic behavior of fortified sample compared to that of control samples which may be due to the formation of strong intermolecular network in the fortified samples. The viscoelastic profile of the mayonnaise samples obtained by the frequency sweep measurement classifies both the samples as 'weak gels' as G' values were higher than G'' values; however strength of fortified sample gel is considered as comparatively stronger, as the recorded G' values were much higher than that of control sample. Previously, the weak gelling characteristics of mayonnaise were reported by Aslanzadeh et al. (2012) in low fat mayonnaise containing modified wheat bran. The slope of the G' curve as a function of frequency was determined as 0.832 for control against a much lower value of 0.237 for fortified samples, indicating the superior stability of the fortified sample against the application of stress. The frequency sweep curve gives a good rheological description of how the product will behave during storage and application as well as helps to characterize or classify dispersion. Gallegos et al. (1992) observed that higher oil content produces a significant increase in the elastic characteristics of mayonnaise. However, in the present study such a correlation was not evident, which might be due to the presence of peptides carrying exposed hydrophilic and hydrophobic groups which further limited the diffusion of oil and water phase.

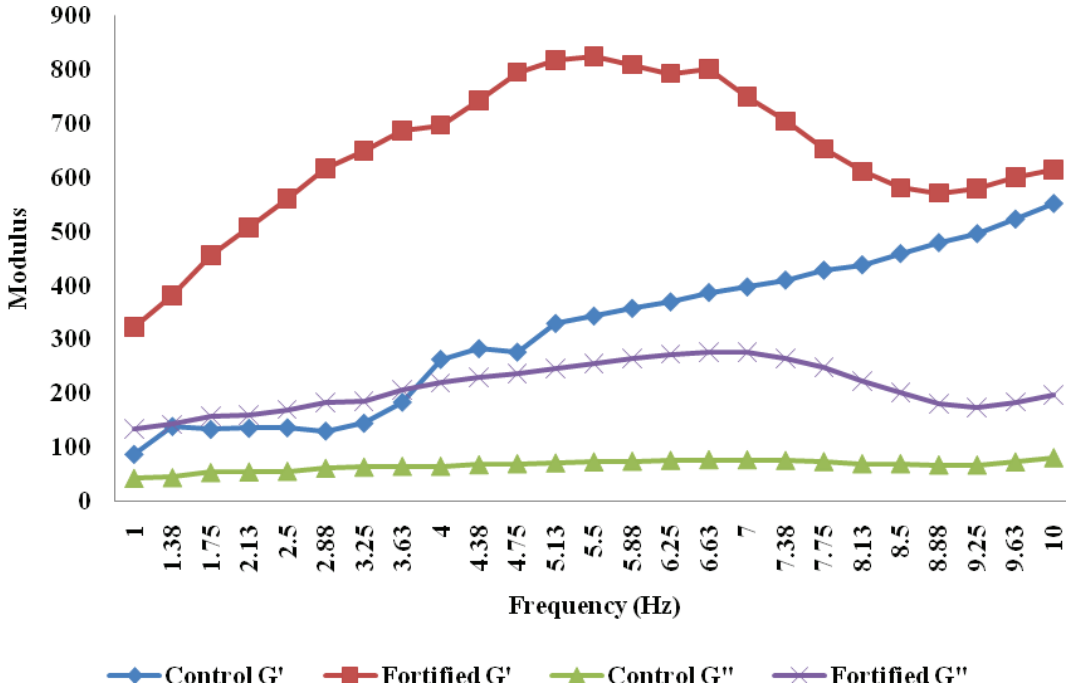


Fig. 7.8 Frequency sweep curve of mayonnaise samples

7.3.2.4.2 Strain sweep

The G'values of mayonnaise samples as a function of strain (%) values indicated distinctly higher values for fortified sample compared to that of control sample, suggesting the higher consistency of fortified sample. A structure build-up and break down phenomenon was observed in fortified sample, as indicated by a maximum G' value of 825.3 Pa at 1 % strain value, and thereafter showing a decreasing trend (Fig. 7.9). On the other hand, control sample showed much lower, still gradually increasing values throughout the given range of strain values. The G'' values of fortified sample also followed a similar trend as that of G', whereas the G'' values of control samples were independent of strain values within linear viscoelastic region, and remained nearly constant with increase in % strain (Fig. 7.9).

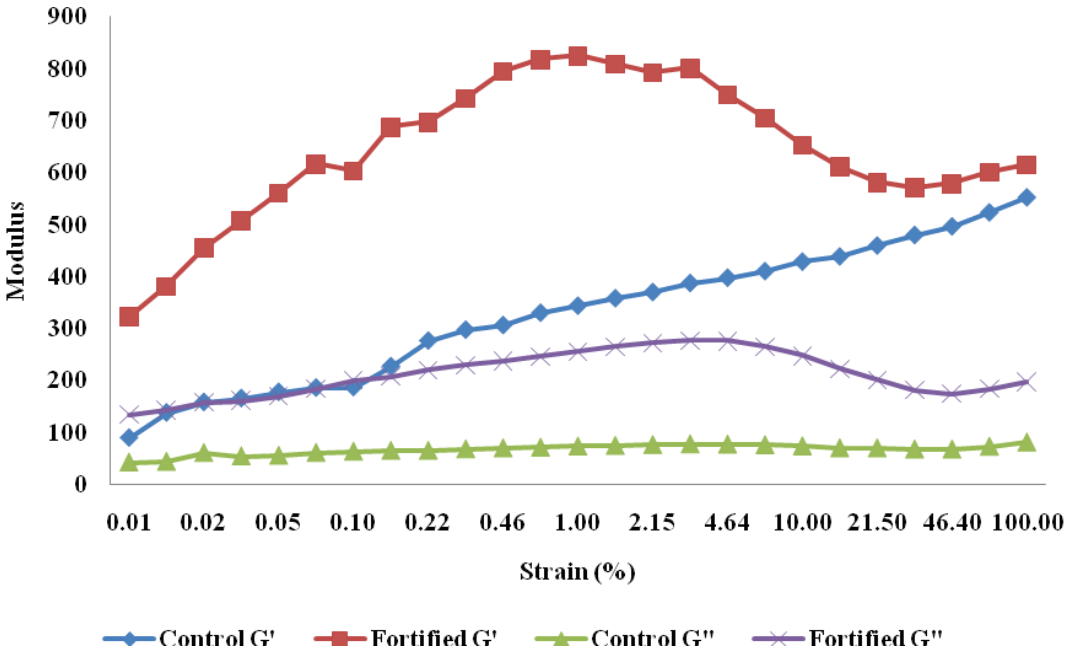


Fig. 7.9 Strain sweep curve of mayonnaise samples

The changes in damping factor also followed a concurrent trend with a minimum value at the intermediary frequency in fortified sample, whereas control sample followed a gradually decreasing pattern till the end of strain sweep (Fig. 7.10). The fortified mayonnaise may be considered as a concentrated emulsion as the moisture content is much less than that of control mayonnaise, and hence more inter-droplet interactions may be expected leading to the formation of an ordered three-dimensional network of aggregated droplets. As the strain crosses a critical limit, aggregates tend to deform and eventually disrupt the ordered network once formed (McClements, 1999).

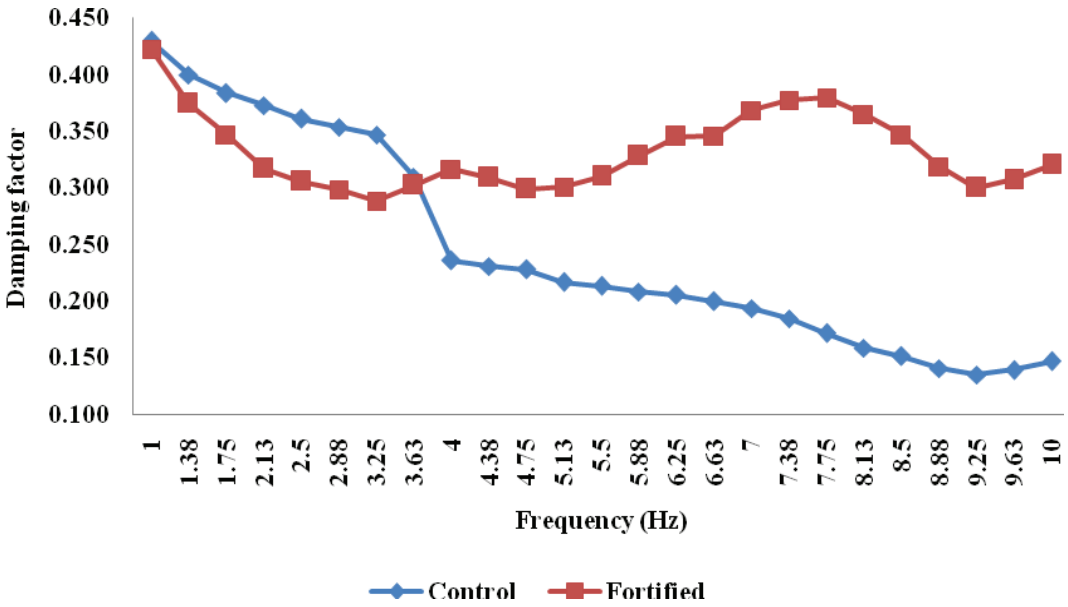


Fig. 7.10 Damping factor curve of mayonnaise samples

7.3.2.4.3 Flow profile

Variations in viscosity with shear rates (0 - 100 s^{-1}) at constant temperature of 25 °C (Fig.7.11) indicated non-Newtonian behavior with shear thinning characteristics for the control sample, whereas distinct shear thickening behavior was observed for fortified samples. The apparent viscosity of the fortified mayonnaise sample was significantly higher (94400 cP) than control sample (33433 cP) at any given shear rate.

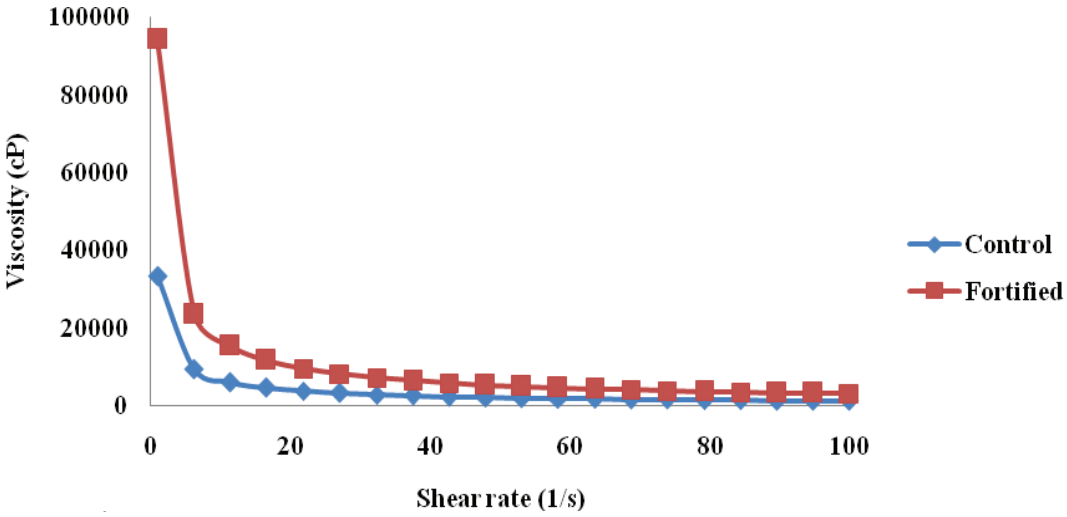


Fig. 7.11 Flow properties of mayonnaise samples

Shear stress–shear rate data of mayonnaise samples were fitted to various rheological models using the software provided along with the rheometer. Based on standard error data obtained, Herschel-Bulkley model was found to be most appropriate in explaining the flow behavior of mayonnaise samples. Both control and fortified samples showed distinct yield stress values, with almost five fold higher value for fortified sample (Table 7.3).This might be on account of higher non-specific interactions by the lipophilic and hydrophilic side chains of shorter peptideslimiting the fluidity of the emulsion. Concurrent to that, the consistency

coefficient (k) of fortified sample was nearly quintuple times higher than that of control sample. Similarly, the flow behavior index 'n' of fortified was lower than that of control sample, indicating the viscous nature of fortified sample at ambient temperature. The farther the flow behavior index from 1, the more the deviation from Newtonian behavior (Lewis, 1990). A high value of 'n' tends to impart slimy mouth feel to the food preparations. Hence, a lower n value is desirable for the preparations, when a thicker solution with good mouth feel characteristics are desired. From the viscosity data, it is apparent that addition of fish protein improved the mouth feel of mayonnaise. Reports indicate a flow behavior index values (n) ranging from 0.13 to 0.91 for some commercial emulsions and model emulsion systems (Dickie and Kokini, 1983; Steffe, 1992).

Table 7.3 Herschel – Bulkley model parameters for mayonnaise samples

Samples	Yield stress (τo)	Consistency coefficient (k)	Flow behavior index (n)	Regression coefficient (R^2)
Control	2.133	37.37	0.27	0.99
Fortified	9.896	178.64	0.18	0.99

The flow properties of the samples were evaluated from shear-stress sweep curves which provided information on resistance to shearing as well as any structural impairment (Dileep et al., 2012). Variations in shear stress with shear rates ranging from 0 to 100 s^{-1} indicated an increasing trend in stress upto shear rate of 6 s^{-1}, more prominent in control than fortified one, reaching a constant on further increase in shear rate (Fig. 7.12). The significantly higher stress value registered for fortified sample (94.5 Pa) compared to that of control (33.5 Pa) indicated higher intermolecular interactions resulting in greater resistance to flow.

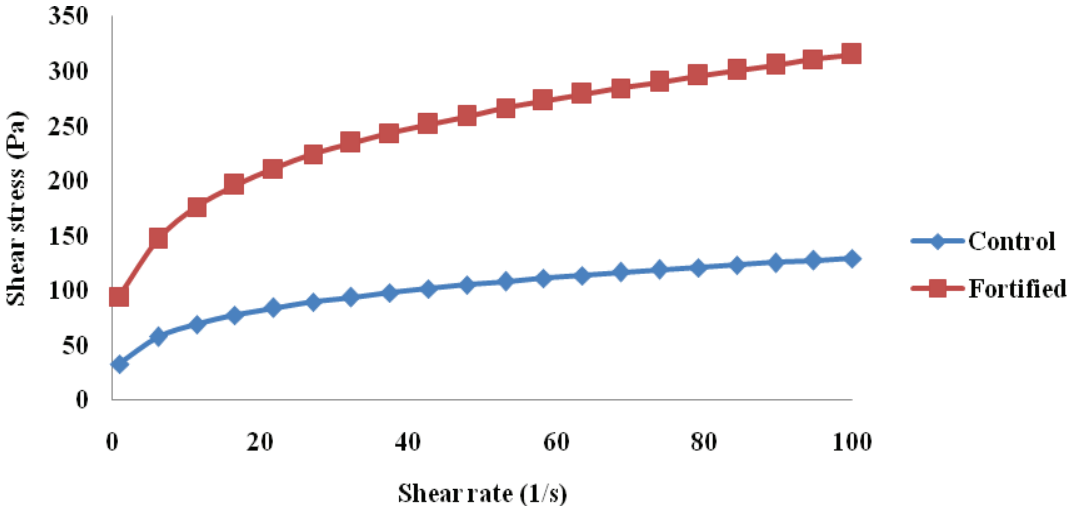


Fig. 7.12 Shear stress-rate curve of mayonnaise samples

Viscosity as influenced by variations in temperature (0 to 100^{0}C) at constant shear rate of 100 s^{-1} indicated decreasing trend for both the samples, however the reduction was more prominent upto 40^{0}C, and thereafter followed by a nearly constant rate of change (Fig. 7.13). Among the two samples, the rate of decrease was higher in fortified sample compared to control sample, indicating a structural alteration in fortified sample initiated by temperature fluctuations. However, the absolute value was higher in fortified sample throughout the range of temperature. Viscosity ranged from 1810 cP (0 ^{o}C) to 234 cP (100^{o}C) for control sample whereas for fortified one, it ranged from 2797 cP (0 ^{o}C) to 318 cP (100^{o}C). The higher viscosity values coupled with the structural alterations induced by heating suggest the presence of an originally structurally ordered emulsion formation in the fortified mayonnaise. At higher temperatures, unfolded proteins and peptides have the tendency to dissociate from the miscellar structure which ultimately lead to phase separation.

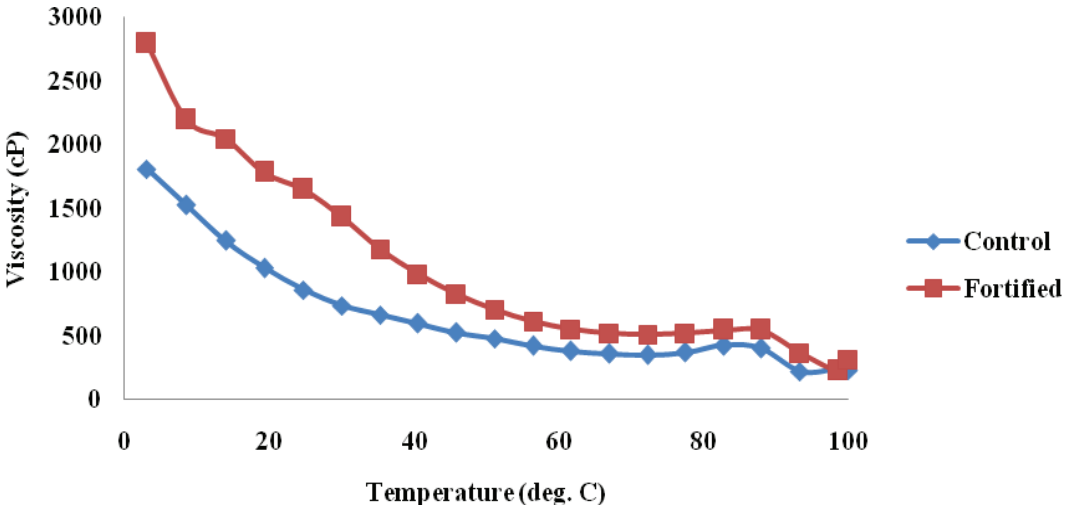


Fig. 7.13 Temperature sweep of mayonnaise samples

7.3.3. Storage stability analysis

7.3.3.1 pH

The pH of mayonnaise can have a dramatic effect on the structure of the emulsion. Studies suggest viscoelasticity and stability of the mayonnaise to be highest when the pH is close to the isoelectric point of the egg yolk proteins which range between 4 to 5 (Depree and Savage, 2001). In the present study, pH of the samples was influenced by protein hydrolysate incorporation with significantly higher values for fortified one (4.14 ± 0.03) compared to control (3.48 ± 0.01). The reason behind higher pH of fortified mayonnaise may be the partial replacement of egg yolk which normally have a pH of 6.0 with protein hydrolysate that had a pH of 6.24. During storage, pH was observed to remain nearly steady, with deviation within a range of not more than 0.2 pH units, for fortified samples as well as control (Fig. 7.14) giving the interpretation that both samples were moreover stable during the study period.

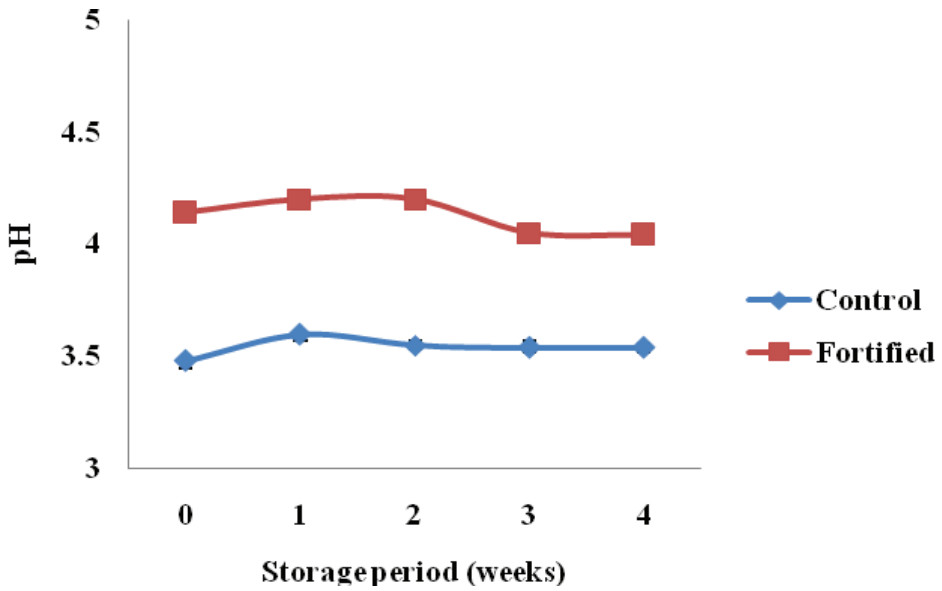


Fig. 7.14 Variations in pH of mayonnaise samples during storage at 4°C

7.3.3.2 Emulsion stability index

During storage, a significant decrease ($p < 0.05$) in ESI values was observed in control sample, by about 11 % of initial value, whereas fortified sample showed minimum reduction of about 6 % from the initial value (Fig. 7.15). The result confirms thatTPH acted as an effective emulsion stabilizer in mayonnaise sample. Previously, Mun et al. (2009) reported higher stability for mayonnaise samples with low fat compared to full fat samples on account of increased viscosity of the aqueous phase reducing the oil droplet movement. In the present study, the superior stability observed in fortified sample may be on account of more exposed hydrophilic and hydrophobic peptides, which in turn acted as an interphase stabilizer between the aqueous and oil phase. Even though initially both the samples had almost similar ESI values, incorporation of TPH improved the stability of the mayonnaise during storage.

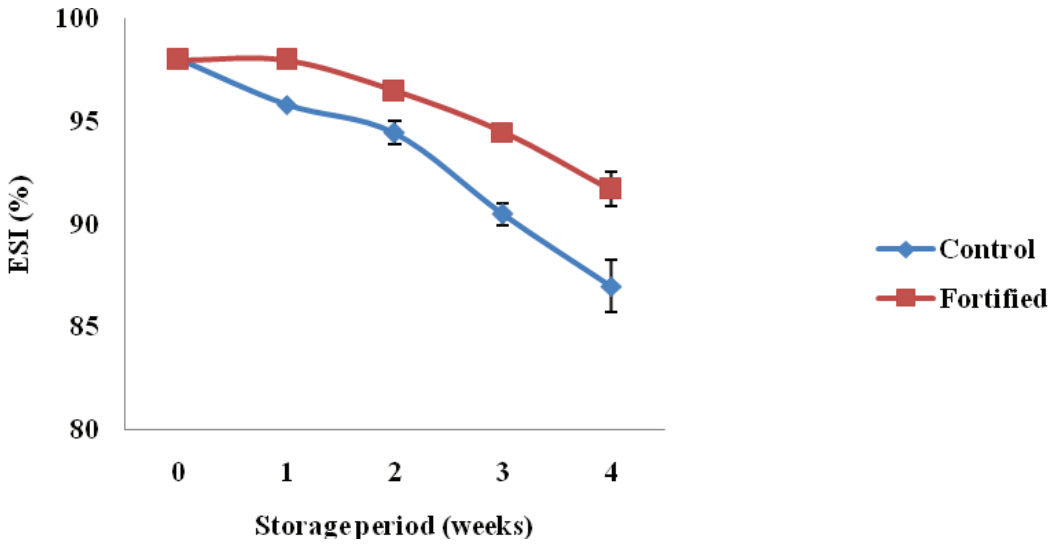


Fig. 7.15 Variations in emulsion stability index of mayonnaise samples during storage at 4°C

7.3.3.3 Viscosity

The viscosity of fortified sample registered higher values ($p < 0.05$) during chilled storage compared to control which was substantiated from the rheological properties of the samples (Fig. 7.16). The higher viscosity observed in the case of fortified sample may be due to the higher total protein content as well as its higher water binding ability thereby inhibiting its continuous phase mobility. Gaonkar et al. (2010) also observed higher viscosity and yield stress values for mayonnaise samples prepared using whey protein concentrate and whey protein isolate, compared to that of control (egg component). A gradual decrease in viscosity was observed during storage,which was comparatively predominant in fortified sample. Reduction in viscosity must probably be associated with the reduction in ESI, which largely affects the homogeneity of the sample by releasing the oil phase from the emulsion, and thereby decreasing the viscosity of the sample.

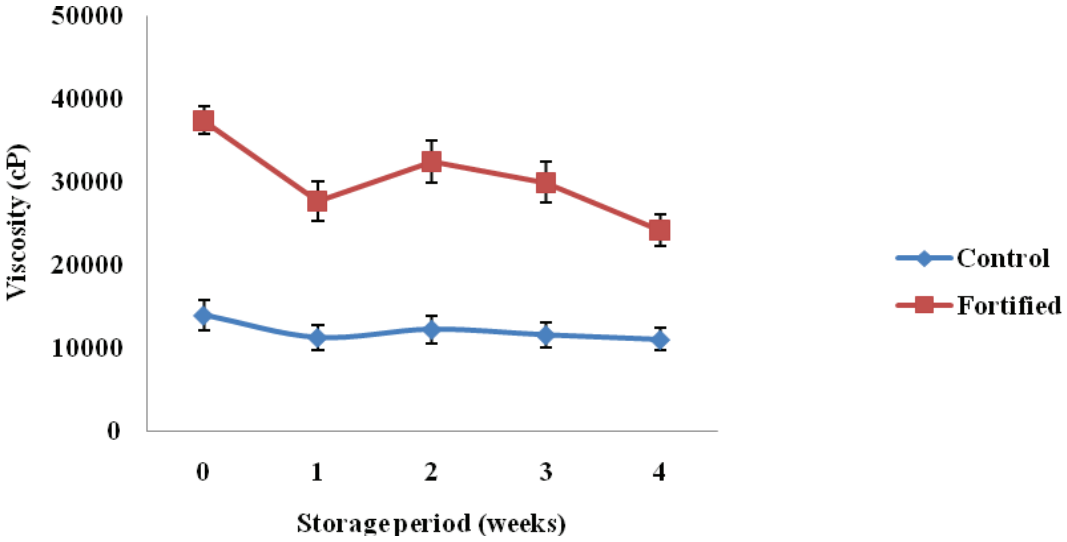


Fig. 7.16 Variations in viscosity of mayonnaise samples during storage at 4°C

7.3.3.4 Free fatty acid

Lipids in food may undergo hydrolysis resulting in the formation of FFA during storage which results in textural changes, enhanced oxidation of lipids, and development of off flavors in the food (Sequeira-Munoz et al., 2006). FFA also exhibited a gradual but significant increase ($p< 0.05$) in both the samples during storage (Fig. 7.17). Though initially the FFA levels were comparable between samples, further on storage a significant variation was observed between them with higher oxidation exhibited by control than fortified sample. Free fatty acids are produced by the oxidation of double bonds of unsaturated fatty acid esters.This oxidation could have occurred by the action of oxidative enzymes in the presence of a proportion of atmospheric oxygen in the headspace (Abu-Salem and Abou-Arab, 2008).

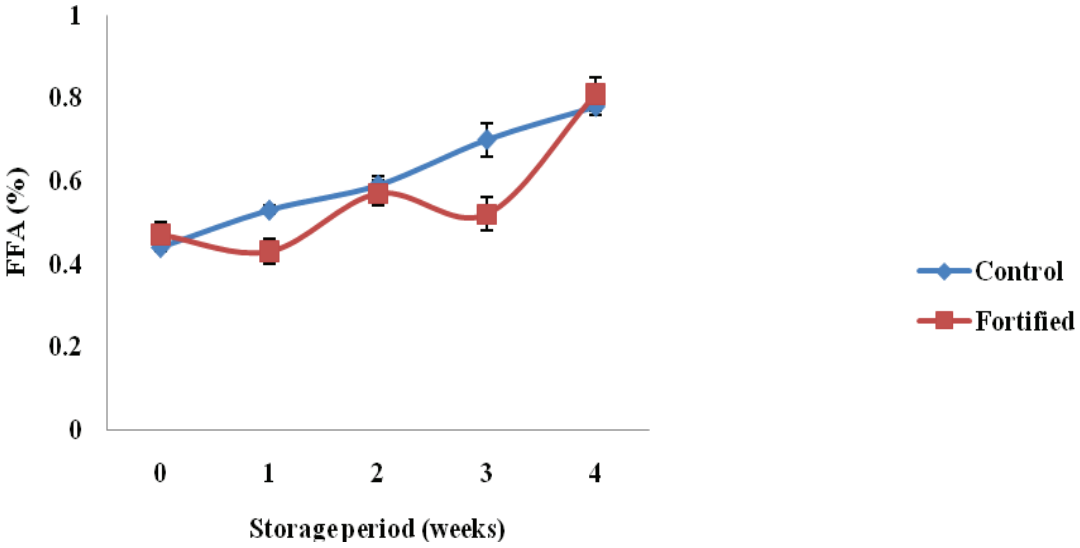


Fig. 7.17 Variations in free fatty acid of mayonnaise samples during storage at 4°C

7.3.3.5 Peroxide value

Oxidation of unsaturated fatty acids is regarded as one of the main reasons behind chemical instability of emulsions. Lipid oxidation in mayonnaise leads to the development of potentially toxic reaction products, undesirable off-flavours and phase separation (Alemán et al., 2015). Peroxide value, indicative of primary oxidation of fat content in the sample indicated almost similar initial values 4.08 ± 0.01 and 4.34 ± 0.07 mEq O_2/kg, respectively in control and fortified one which on storage ($p < 0.05$) showed distinctly higher values for control sample(Fig. 7.18). This may be partly due to the compositional variation with higher fat content in control from egg yolk than fortified one which was partially replaced with TPH. Further, bioactive peptides present in the hydrolysate might have imparted oxidative protection on account of their antioxidant properties as reported previously in literature (Klompong et al., 2007). The *in vitro* antioxidant activity of tuna red meat protein hydrolysates was established in our earlier studies as well (Parvathy et al., 2018c).

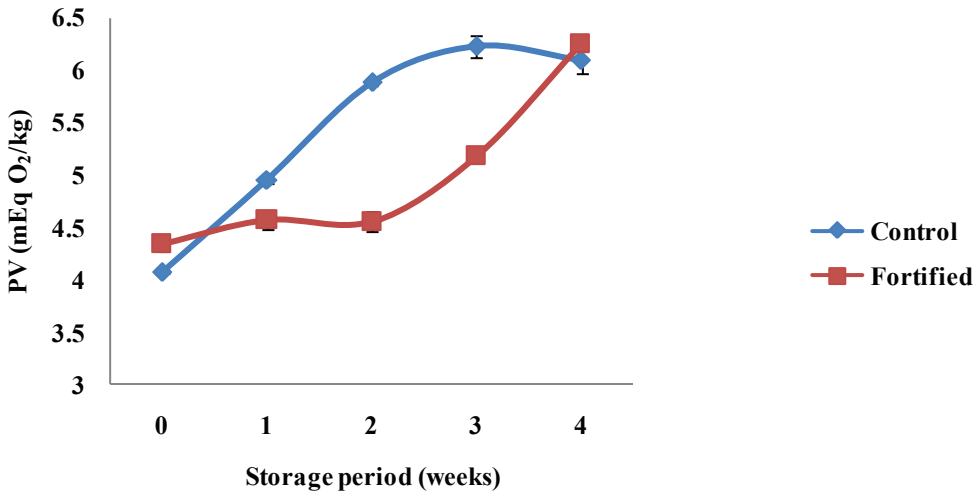


Fig. 7.18 Variations in peroxide value of mayonnaise samples during storage at 4°C

7.3.3.6 Sensory evaluation

Sensory evaluation is regarded as one of the major quality attribute decisive in determining the storage stability of a commodity. In the present study, control sample had a better sensory acceptance with an initial score of 8.3 ± 0.5 compared to fortified one (7.6 ± 0.7). This score demarcation may be on account of the slight bitterness and fish flavor imparted by TPH. Further, similar lower score was obtained for fortified sample throughout the storage period, reaching 7.2 ± 0.5 and 6.8 ± 0.4 for control and fortified sample, respectively towards fourth week of storage (Fig. 7.19). However, both the samples were acceptable till the end of storage study.

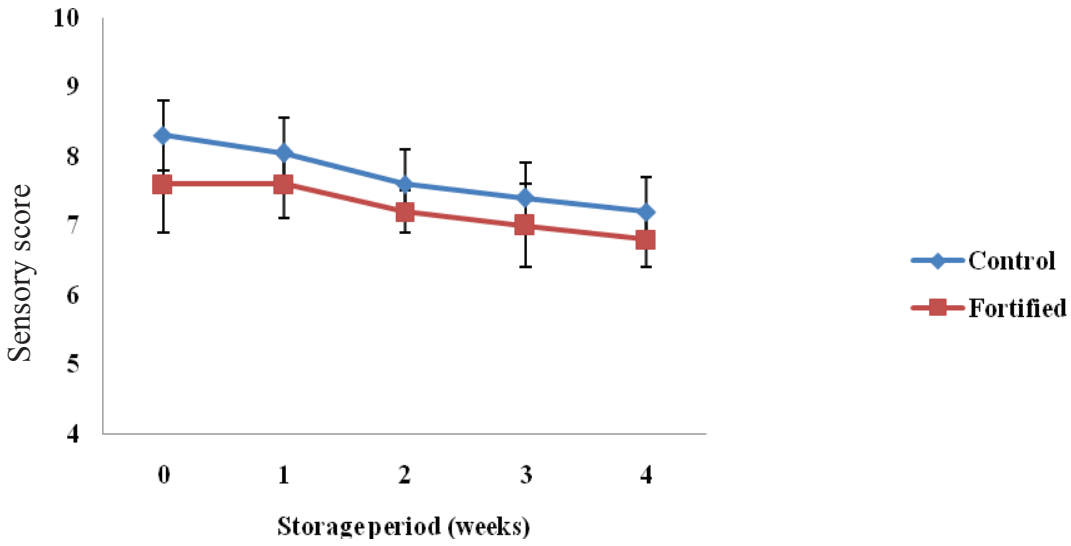


Fig. 7.19 Variations in sensory score of mayonnaise samples during storage at 4°C

7.3.3.7 Microbiological studies

Microbial stability of food products is a major attribute of consideration, especially in ready to eat products like mayonnaise. Initial TPC values of both the samples were close to 2.6 logs, indicating the product to be microbiologically stable.However during storage, markedly higher values were registered by control sample, even during the first week of chilled storage. The microbial growth was more prominent in control sample during the entire period of storage, approaching

a value of 3.24 log (1735 cfu/g) and 2.89 log (785 cfu/g), respectively in control and fortified sample, at the end of 4 weeks of chilled storage (Fig. 7.20). The results further suggests the possible antimicrobial property of TPH, similar observations were reported previously for hydrolysates from various protein sources (Da Rocha et al., 2018). Further, it is also possible that the higher fat content in control mayonnaise had a protective effect on microorganisms to reside safely in the oil phase without having an effect of change in the microenvironment of the aqueous phase (Pourkomailian, 2000). Studies by Karas et al. (2002) also suggested chances of more bacterial contamination on account of higher moisture content in control, which was dominated by lactic acid bacteria.

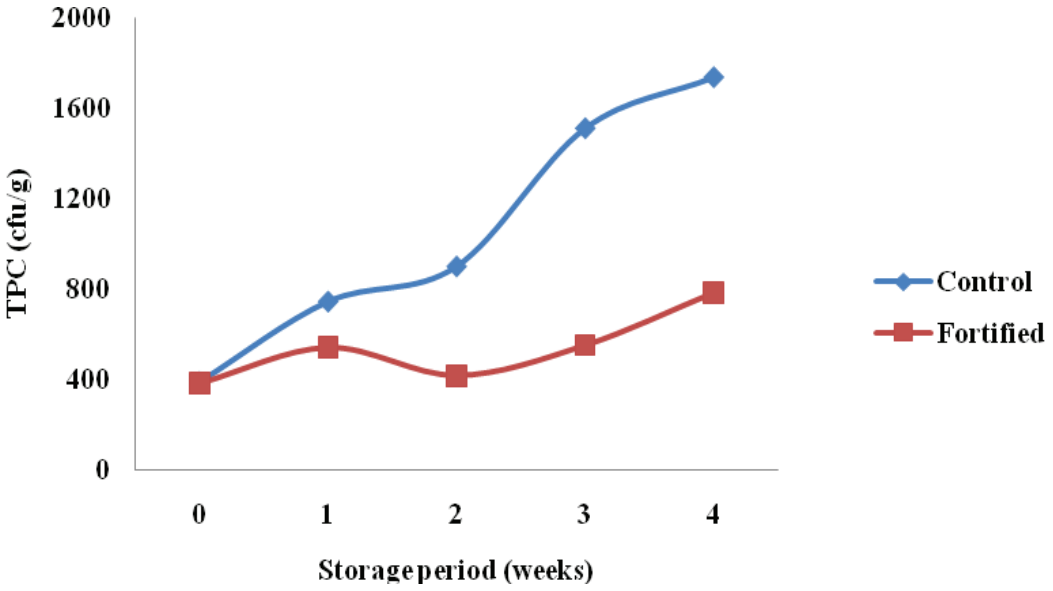


Fig. 7.20 Variations in microbiological indices of mayonnaise samples during storage at 4°C

7.4 Conclusion

Fortified mayonnaise was attempted by partial replacement of egg yolk with functionally optimized tuna protein hydrolysate. Incorporation of tuna protein hydrolysate improved the nutritional status of the mayonnaise formulation. A reduction in particle size and increased viscosity was observed in fortified sample. Incorporation of TPH in mayonnaise modified the sample's colour profile with increased yellowness and redness in fortified formulation. Storage studies indicated better oxidative stability for fortified samples compared to control during storage at 4^{o}C. Present study explores the potentiality of protein hydrolysates in mayonnaise preparations for fortification as well as better stability.

Chapter 8

Utilization of Tuna red meat hydrolysate for fish oil encapsulation and encapsulate acceptability studies in selected food products

8.1 Introduction

Encapsulation may be defined as a method which facilitates the entrapment of one substance, being the compound of interest, also referred to as the active agent within another substance viz., wall material. The encapsulated substance can also be called as the core, fill, active, internal or payload phase while the substance that is encapsulating is often referred to as the coating, membrane, shell, capsule, carrier material, external phase, or matrix. It is an effective technique to improve the stability and delivery of sensitive bioactive components like pigments, minerals, vitamins, fatty acids, phytosterols, enzymes etc. as well as living cells like probiotics into foods (Wandrey et al., 2010). These bioactive components are completely enveloped and protected by a physical barrier/wallcoating without any protrusion of these bioactive components, which in turn facilitates the controlled and extended release of the core contents under specific conditions. Among the different encapsulation techniques followed, spray drying is one of the oldest and most common method adopted in the food industry on account of its flexible, continuous, economic operations and it produces particles of micro and nano scale (< 40 μm) (Zuidam and Heinrich, 2010).However, one of the major constraint

associated with oil encapsulation is the elevated operational temperature and the mechanical shearing during atomisation which destabilises the emulsion, leading to capsule collapse and oxidation of fish oil (Binsi et al., 2017a).

Several authors like Serfert et al. (2009); Morales-Medina et al. (2016)have reported that microencapsulation by spray drying in the presence of antioxidants can enhance the storage stability of fish oil. It is also thought that the stabilization efficiency of antioxidants may not be the same when they are used as wall material component and as core material along with the oil. In addition, the ordered orientation of wall and core polymers in capsular alignment is a major aspect affecting the stability of emulsions during homogenisation and atomisation, thereby the encapsulation efficiency and the integrity of the microcapsules. In this regard, employing polymers exhibiting dual properties viz., emulsifiers with antioxidative properties are desirable, which can physically stabilize the oil-in-water emulsions during atomisation and protect oil encapsulates from oxidation during storage.

Protein hydrolysates are breakdown products of proteins into smaller peptides obtained by the enzymatic/chemical process. These peptides, based on the extent of hydrolysis possess superior bioactivity compared to their parent proteins. Recent studies have suggested the applicability of fish protein hydrolysate as antioxidants in microencapsulated fish oil stabilization when used along with core material (Morales-Medina et al., 2016).As previously researched, this observed stability was mainly ascribed to the antioxidant properties of hydrolysates with their superior free radical scavenging and metal chelating activities (Qian et al., 2008; Sheriff et al., 2014). A second probable mechanism of use in microencapsulation is the strengthening of capsular wall matrix by these peptides by acting as co-wall polymers or rather as fillers, owing to their smaller size. In addition, the superior emulsifying properties of protein hydrolysates are also thought to be helpful in the

structured orientation of core and wall polymers in the emulsion droplets, thereby minimising capsular collapse during atomisation.

Tuna red meat which serves as a cheap and high-quality protein source, was ideally utilized by converting to hydrolysates with desirable functional and bioactive properties with the assumption that tuna protein hydrolysate significantly improve the structural and oxidative stability of fish oil during both spray drying and further storage. In the present work, a comparison was made between the efficiency of yellowfin tuna (*Thunnus albacares*) red meat hydrolysate as wall and core polymer for encapsulating sardine oil. Further, the storage stability of the encapsulates under accelerated atmosphere (60^{o}C), ambient temperature (28^{o}C) and chilled temperature (4^{o}C) were evaluated.

8.2 Materials and methods

8.2.1 Raw materials, enzymes and chemicals

Fish oil extracted from Indian oil sardine (*Sardinella longiceps*) was procured from a local seafood processing factory, analysed for the fatty acid composition and was used for microencapsulate core preparation. Protein hydrolysate from the red meat of yellow fin tuna optimized for antioxidant properties (chapter 4; table 4.3) was used for the study. Sodium caseinate (SRL Pvt. Ltd., Mumbai, India), maltodextrin,gum Arabic, pepsin (activity 1 Anson unit/gm of protein), pancreatin 4 X (3 NF/USP from porcine pancreas) (Hi Media Pvt Ltd., Mumbai, India) were used for the study. All chemicals of analytical grade were used for the study.

8.2.2 Fatty acid profiling

Fatty acid composition of sardine oil and sardine oil encapsulates were determined in the present investigation. Total lipid was extracted from the encapsulates by the method of Folch et al. (1957). Known quantity of sample was

triturated with 15 times the sample volume of ice cold chloroform: methanol (2:1) and allowed to stand for five minutes for lipid extraction. It was then filtered and transferred to a separating funnel to which water was added to a level of 20 % of the filtrate. The flask was mixed well, released off vapour and allowed for phase separation, overnight. The lower chloroform phase was passed through anhydrous sodium sulphate and collected in a flat bottom flask. Solvent was completely evaporated using a rotary evaporator and the lipid residue was dissolved in known quantity of chloroform (about 10 ml). About one ml of the chloroform extract was evaporated off the solvent under nitrogen or in case of oil or fat 25-100 mg sample was taken in a 30 ml teflon lined screw capped test tube. For the purpose of saponification, about 1.5 ml of 0.5 M methanolic NaOH was added, blanketed with nitrogen gas, closed tightly and heated at 100^{o}C for five mins. Further the tube was cooled and 2 ml of boron trifluoride methanol was added, blanketed with nitrogen gas, tightly closed and heated at 100^{o}C for 30 mins. The tube was further cooled and one ml of n-hexane was added and mixed thoroughly followed by the addition of 5 ml saturated sodium chloride solution. The test tube cap was loosened and allowed for phase separation for 5 to 10 mins. From the mixture, about 0.8 ml of the upper hexane phase was carefully transferred into a test tube. The process was repeated one more time to extract with 1 ml of hexane and the extract was pooled. The solvent was evaporated to concentrate fatty acid methyl esters (FAME) which was injected to gas chromatogram. Fatty acid composition analysis was performed using gas chromatography (Varian, Guindy, Chennai; Model no: CP-3800) with a Cpsil 88FAME column (100 m length x 0.25 mm internal diameter; 0.20 μm film thicknesses. Nitrogen was used as the carrier gas. Injector and detector temperatures were set at 260^{o}C and 270^{o}C, respectively. Injection was performed in split mode (1:50). The column temperature was programmed initially at 140^{o}C for 2 min and then to increase at a uniform rate to reach a final temperature of 240^{o}C in one

hour time. A flame ionization detector was employed and peaks were identified by comparing the mass spectra with the mass spectral database.

Fig. 8.1 Gas chromatograph

8.2.3 Preparation of emulsion and spray drying

The composition of the emulsion was prepared with fish oil (core material) to wall material in the ratio 1:5. The wall materials consisted of maltodextrin, gum Arabic and sodium caseinate (2:2:1) in control (SO). Sodium caseinate was partially (1:1) and completely replaced with protein hydrolysate for comparison in which hydrolysate acted as a wall material component. Hereafter the encapsulate where sodium caseinate was partially replaced is referred to as SPO and completely replaced with protein hydrolysate is coded as PO. Another emulsion combination consisted of fish oil containing 1 % protein hydrolysate (w/w of fish oil), where hydrolysate acted as core material component, hereafter referred to as SOP. This particular concentration of hydrolysate was selected based on ABTS IC_{50} value of hydrolysate from previous studies which was observed to be 7.42 mg/ml. For emulsion, wall materials were dissolved in distilled water and kept overnight at chilled condition (4^{o}C) to ensure full saturation of the polymer molecules. To the saturated mixture, fish oil was added dropwise with continuous stirring at 1000 rpm for 10 min using magnetic stirrer. The mixture was then homogenized with a high

speed homogenizer (Poly system PT 2100, Kinematica, AG) at 25,000 rpm for 5 min. Prior to spray drying, the emulsions were allowed to stabilize at 4°C for 1 h.

Spray drying was done using a pilot-scale spray dryer (Hemraj Pvt. Ltd, Mumbai) equipped with a two-fluid nozzle atomizer under the following experimental conditions viz., inlet temperature 160°C; outlet temperature 80°C; spray flow feed nozzle diameter 1.5 mm; air pressure 2.5 bar. Fish oil encapsulates prepared by spray drying were stored in air tight containers for further analysis.

8.2.4 Characterization of emulsion

8.2.4.1 Emulsion stability index

Emulsions were prepared employing sardine oil and wall material in 1:5 ratio in the previously described combinations (Section 8.2.3). Aliquot of prepared emulsions (150 ml) were transferred to graduated test tubes and kept at 4°C for 24 h. After this time period, bulk unseparated phase volume was measured and the stability was expressed as:

$$\text{Emulsion Stability Index (\%)} = \left(\frac{H1}{H0}\right) \times 100$$

where H_0 represented the initial emulsion volume and H_1, the unseparated phase volume.

8.2.5 Characterization of microencapsulates

8.2.5.1 Scanning electron microscopy

The surface appearance and morphology of sardine oil encapsulates was monitored by employing SEM (Philips XL 30, The Netherlands). Samples were fixed onto double-sided adhesive carbon tabs mounted on SEM stubs, coated with gold in vacuum using sputter coater, and examined by SEM at 10kV with magnification of 800 x.

Fig. 8.2 Scanning electron microscope

8.2.5.2 Differential scanning colorimetry

Thermal characteristics of the sample were determined by employing differential scanning calorimeter (described in chapter 6; section 6.2.2.5.2).

Fig. 8.3 Differential scanning colorimeter

8.2.5.3 Fourier-transform infrared spectroscopic analysis

The FTIR spectra of encapsulates were recorded using a FTIR spectrometer (Shimadzu IR-Prestige-21) (described in chapter 6; section 6.2.2.5.3).

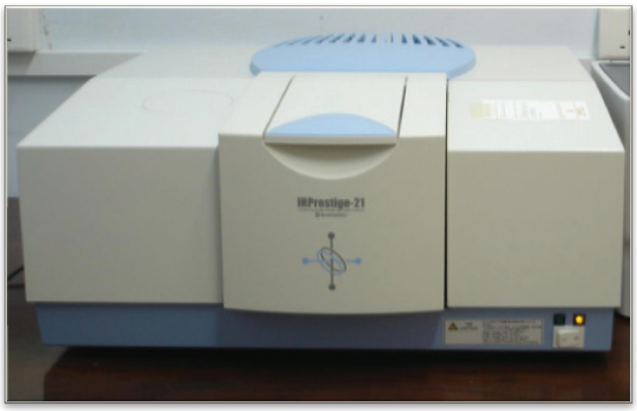


Fig. 8.4 Fourier-transform infrared spectroscope

8.2.5.4 Encapsulation efficiency

Encapsulation efficiency (EE) throws idea regarding the efficacy of encapsulation adopted. This is accounted by determining the relationship between total oil and surface oil/free oil of the sample. Soxhlet method (AOAC, 2012) was adopted to determine the total oil and free oil was determined as per the methodology described by Sankarikutty et al. (1988). About 2.5 g of oil encapsulate was mixed with 100 ml n-hexane with continuous stirring at ambient temperature for 15 min and then passed through Whatmann No. 4 filter paper. The solvent was evaporated in a rotary evaporator and the extracted oil was dried to constant weight by using a stream of nitrogen.

$$\text{EE (\%)} = \frac{\text{Total oil} - \text{Surface oil}}{\text{Total Oil}} \; x \; 100$$

8.2.6 Physical properties of microencapsulates

8.2.6.1 Moisture content and hygroscopicity

Moisture content was evaluated from the percentage loss in sample weight after oven drying at 105°C until it reached a constant weight in comparison to the initial sample weight (AOAC, 2012).

Sample hygroscopicity was determined adopting the methodology of Cai and Corke (2000) with slight modifications (described in chapter 6; section 6.2.2.6.1).

8.2.6.2 Bulk density and tapped density

Methodology described by Chinta et al. (2009) was followed for determining bulk density (ρB) and tapped densities (ρT) of the samples to understand the flow properties exhibited by them (described in chapter 6; section 6.2.2.6.2).

8.2.6.3 Colour

Colour of oil encapsulates were measured using Hunter- Lab scan XE – Spectrocolorimeter (Color Flex, Hunter Associates Laboratory, Reston, USA.) (described in chapter 6; section 6.2.2.6.3).

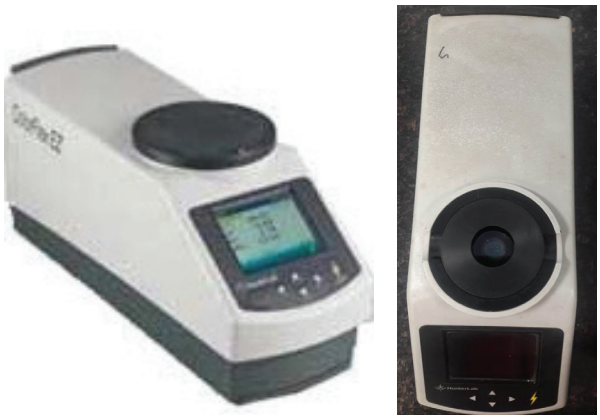


Fig. 8.5 Hunterlab colorimeter

8.2.7 In vitro oil release kinetics

In vitro oil release kinetics using simulated gastro-intestinal fluids were studied by the methodology described by Burgar et al. (2009). The method in the US Pharmacopeia (2000) was adapted for the preparation of simulated gastrointestinal fluids. For making simulated gastric fluid (SGF), sodium chloride (2.0 g) and 36 % HCl (7.0 ml) were dissolved in 900 ml of deionised water. Pepsin (3.2 g) was then added and the pH of the solution was adjusted to 1.2. The final volume was made up to one litre and the solution stored at 4 °C until further use. Simulated intestinal fluid (SIF) was prepared by dissolving potassium hydrogen phosphate (6.8 g) in 900 ml of deionised water. Sodium hydroxide (0.2 M, 77 ml) and 100.0 g of pancreatin were then added and the solution was left stirring overnight at 4 °C. The pH was adjusted to 6.8 with 1 M sodium hydroxide or with 1 M HCl and the final volume was made up to one litre with deionised water. Both the solutions were stored at 4 °C until further use.

A known quantity of oil encapsulates (about 5.0 g) were initially subjected to gastric digestion using SGF (50 ml) containing pepsin at 37°C for 2 h under acidic condition (pH 1.2) in a shaking water bath. Further, pH was adjusted to 6.8 using 1 M sodium hydroxide and SIF (50 ml) was added and intestinal digestion was continued under similar temperature conditions for another 3 h. The quantity of oil released from the microcapsules after exposure to SGF and SIF was determined separately by the method given by Millqvist-Fureby (2003). Solvent extraction method with petroleum ether was adopted for the released oil estimation. The whole sample, following exposure to gastric or gastric and intestinal fluids, was transferred into a stoppered separating funnel. Petroleum ether (75 ml) was added and the solution was mixed thoroughly. This was followed by the aqueous and organic solvent phase separation. The extraction process was repeated twice, using

25 ml petroleum ether each time. The solvent phases were pooled together and petroleum ether was removed at 60°C using a rotary evaporator and further dried in a hot air oven at 100 ± 2°C for 1 h and cooled in a dessicator to room temperature. From the weight of the extracted oil, the percentage of released oil in the sample was calculated.

8.2.8 Storage stability of sardine oil encapsulates

Oxidative stability of fish oil encapsulates stored under accelerated temperature of 60ºC was determined for one week on a daily basis by hot air oven method. At ambient (28°C) and refrigerated (4°C) temperature, they were analysed for upto 4 weeks at weekly interval. The sardine oil encapsulates were stored in sealed and aluminium foil wrapped glass bottles to avoid exposure to oxygen and light.

Peroxide value was determined following the methodology of Shantha and Decker (1994). About 0.3 g of sample was mixed with 1.5 ml ofisooctane/2-propanol (3:1, v/v) and vortexed at high speed. The organic phase was separated bycentrifugation at 10,000 rpm for 2 min. To the organic solvent phase (0.2 ml), 2.8 ml of methanol/1-butanol(2:1, v/v) was added followed by addition of 30 µl of ammonium thiocyanate and ferrous solution mixture and mixed well. The mixture was incubated for 20 min and the absorbance measured at 510 nm. Hydroperoxide concentration in the sample was determined using a standard curve (in triplicate) made from cumene hydroperoxide and expressed in mmol of oxygen per kg.

Thiobarbituric acid reactive substances (TBARS) were assessed as per McDonald and Hultin (1987). About 2 ml of thiobarbituric acid reagent was taken in test tube into which 1 ml of the sample (5 mg of fish oil encapsulate in 1 ml of acetate buffer) was added. For blank, sample was replaced with distilled water. Test tubes were closed and vortexed thoroughly followed by incubation in boiling water

bath (100°C) for 15 min. Further it was cooled for 10 min and centrifuged at 1,000 rpm for 15 min. The absorbance of the supernatant was measured at 532 nm and TBARS was expressed in mg of malonaldehyde/kg.

The variations in colour of microencapsulates were determined by Hunter-Lab scan XE– Spectrocolorimeter (Color Flex, Hunter Associates Laboratory, Reston, USA.) as described previously.

8.2.9 Product acceptability studies

The encapsulate selected with respect to its efficiency and stability was subjected to acceptability studies for determining the encapsulate concentration applicable in different food products without significantly affecting its sensory parameters.Four different products viz.,milk, juice,corn flakes and noodles which vary in ingredient composition and preparation methods were chosen. For the study, a semi-trained panel consisting of 10 members was constituted for testing the acceptability (Annexure 3). Encapsulate was added @ 2.5, 5.0, 7.5 and 10 % levels in the selected food products and one was kept as control (no added encapsulate). Sensory acceptability was conducted based on appearance, flavor, odour, taste, colour and overall acceptability to determine the maximum permissible concentration of encapsulate in these products. Further evaluation of the nutrient enrichment by incorporation of the acceptable concentration of encapsulate in these four products was also performed.

8.2.10 Statistical analysis

SPSS software version 16.0 (SPSS Inc, Chicago, Illinois, USA) was used to conduct one way analysis of variance (ANOVA) on the data obtained. Duncan's multiple range test was adopted to determine the differences between the means and were considered significant at 5 % levels ($p<0.05$).

8.3 Results and discussion

8.3.1 Fatty acid profiling

Fatty acid profiling of the sardine oil used for encapsulation indicated palmitic acid to be the major contributor with 25.81% of the total fatty acids. A high proportion of DHA (11.94%) and EPA (9.18%) were also observed in the sample (Table 8.1) which was in agreement with previously reported values (Som and Radhakrishnan, 2013; Binsi et al., 2017a). The fatty acid profile of the sardine oil encapsulates also showed prominence of these components. Among the various encapsulates, SOP samples showed comparable fatty acid profile as that of fresh sardine oil. However, the content of unsaturated fatty acids, in particular the omega-3 fatty acids were markedly lower in PO samples compared to fresh sardine oil and other oil encapsulates indicating the loss of these fatty acids to greater extend in PO sample. This was assumed to be on account of severe oxidation of core oil during homogenisation and subsequent atomisation.

Table 8.1 Fatty acid profile of sardine oil and sardine oil encapsulates

Major Fatty acids	Sardine oil	SO	SPO	PO	SOP
Myristic acid C14:0	$6.46^{a} \pm 0.05$	$6.95^{c} \pm 0.07$	$6.73^{b} \pm 0.06$	$7.96^{d} \pm 0.08$	$6.80^{b} \pm 0.07$
Palmitic acid C16:0	$25.81^{a} \pm 0.08$	$27.13^{d} \pm 0.07$	$26.23^{b} \pm 0.11$	$31.33^{e} \pm 0.12$	$26.85^{c} \pm 0.09$
Palmitoleic acid C16:1	$8.78^{a} \pm 0.10$	$9.07^{b} \pm 0.09$	$9.02^{b} \pm 0.08$	$10.54^{c} \pm 0.13$	$8.80^{a} \pm 0.10$
Stearic acid C18:0	$8.22^{a} \pm 0.04$	$8.58^{b} \pm 0.14$	$8.30^{a} \pm 0.13$	$9.99^{c} \pm 0.11$	$8.60^{b} \pm 0.05$
Oleic acid C18:1	$8.68^{a} \pm 0.08$	$8.95^{b} \pm 0.07$	$8.76^{a} \pm 0.05$	$10.19^{d} \pm 0.09$	$9.60^{c} \pm 0.08$
Arachidonic acid C20:4	$3.36^{b} \pm 0.05$	$3.58^{cd} \pm 0.06$	$3.63^{d} \pm 0.07$	$2.72^{a} \pm 0.10$	$3.48^{bc} \pm 0.04$
Eicosapentaenoic acid C20:5	$9.18^{b} \pm 0.08$	$9.50^{c} \pm 0.09$	$9.80^{d} \pm 0.04$	$6.13^{a} \pm 0.10$	$9.19^{b} \pm 0.08$
Docosahexaenoic acid C22:6	$11.94^{b} \pm 0.12$	$12.17^{c} \pm 0.07$	$12.39^{d} \pm 0.03$	$7.25^{a} \pm 0.03$	$12.06^{bc} \pm 0.06$
Saturated fatty acids	$40.49^{a} \pm 0.15$	$42.66^{d} \pm 0.15$	$41.26^{b} \pm 0.07$	$49.28^{e} \pm 0.17$	$42.25^{c} \pm 0.04$
Unsaturated fatty acids	$41.94^{b} \pm 0.15$	$43.27^{c} \pm 0.15$	$43.6^{d} \pm 0.01$	$36.83^{a} \pm 0.07$	$43.13^{c} \pm 0.07$
Omega-3 fatty acids	$21.12^{b} \pm 0.17$	$21.67^{c} \pm 0.08$	$22.19^{d} \pm 0.07$	$13.38^{a} \pm 0.07$	$21.25^{b} \pm 0.13$

Values are expressed as Mean ±SD; n = 3; Different superscripts in the same row indicates significant difference ($p < 0.05$)

8.3.2 Characterization of emulsion

8.3.2.1 Emulsion stability index

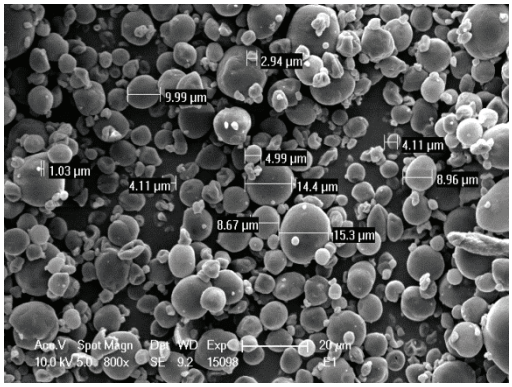
A stable liquid emulsion is regarded as a prerequisite for proper encapsulation in spray-dried powders. The stability of the atomized emulsion is one of the key parameter determining the resultant stability and associated physico-chemical characteristics of its oil encapsulate during storage. Concurrently the stability of emulsions depends primarily on the composition and combinations of the ingredients used as wall and core material. All the emulsions prepared in this study were kinetically stable at rest without any visible phase separation, even 24 h after homogenization which indicated its suitability for spray drying. In general, proteins and polypeptides are considered to have superior emulsion properties, as they easily get adsorbed at the oil-water interface to stabilise both stearic and electrostatic forces (Lam and Nickerson, 2013). The observed stability of the emulsions indicated that the matrix proteins used in the present study viz., sodium caseinate as well as protein hydrolysate were efficient in forming a well-structured emulsion, which remained stable at rest under chilled condition. However, this static stability does not offer stability during atomisation under elevated temperature and shearing, as there can be alteration in emulsion structure under severe conditions. Microstructural analysis of encapsulates can facilitate further confirmation in this regard.

8.3.3 Characterization of microencapsulates

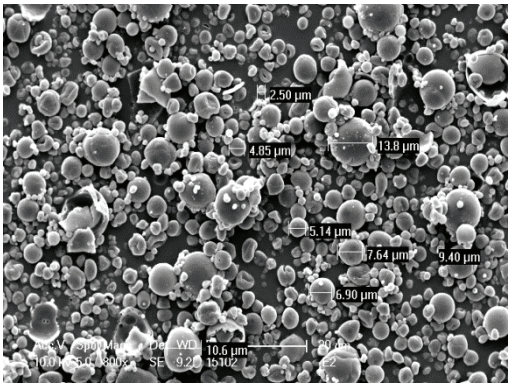
8.3.3.1 Scanning electron microscopy

Surface morphological analysis of sardine oil encapsulates revealed distinct differences in size and surface morphology both within and between the samples (Fig. 8.6). Globular capsules with diverse degrees of concavities were observed with a visible difference between the control caseinate encapsulates (SO) and those containing hydrolysates either as core or wall material, with reduction in the average

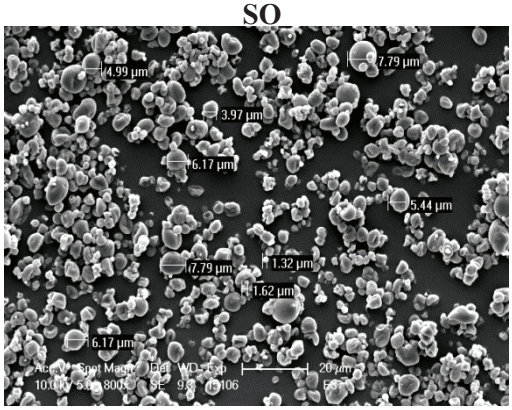
dimension of encapsulates in the latter. The encapsulates having protein hydrolysate alone as wall material (PO) exhibited a distinct reduction in particle size compared to samples containing sodium caseinate (SO, SOP and SPO). This might be due to variations in the micelle size formed during emulsification process, on account of the superior surface-active properties possessed by protein hydrolysates. However, the SEM image of hydrolysate containing samples showed the presence of several shrunken capsules compared to that of SO encapsulates. A probable reason may be that in these samples the emulsion might have contained several intact shorter peptides which could not be fit into the miscellar structure, and hence might have appeared as shrunken capsules in the final desiccated product. On comparing the three hydrolysate incorporated samples, better capsular uniformity was exhibited by encapsulate where hydrolysate was used as co-wall material (SPO) compared to that as core material (SOP), or as the sole wall material (PO).



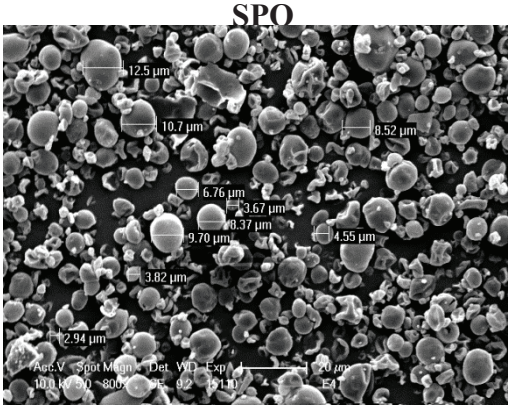
SO



SPO



PO

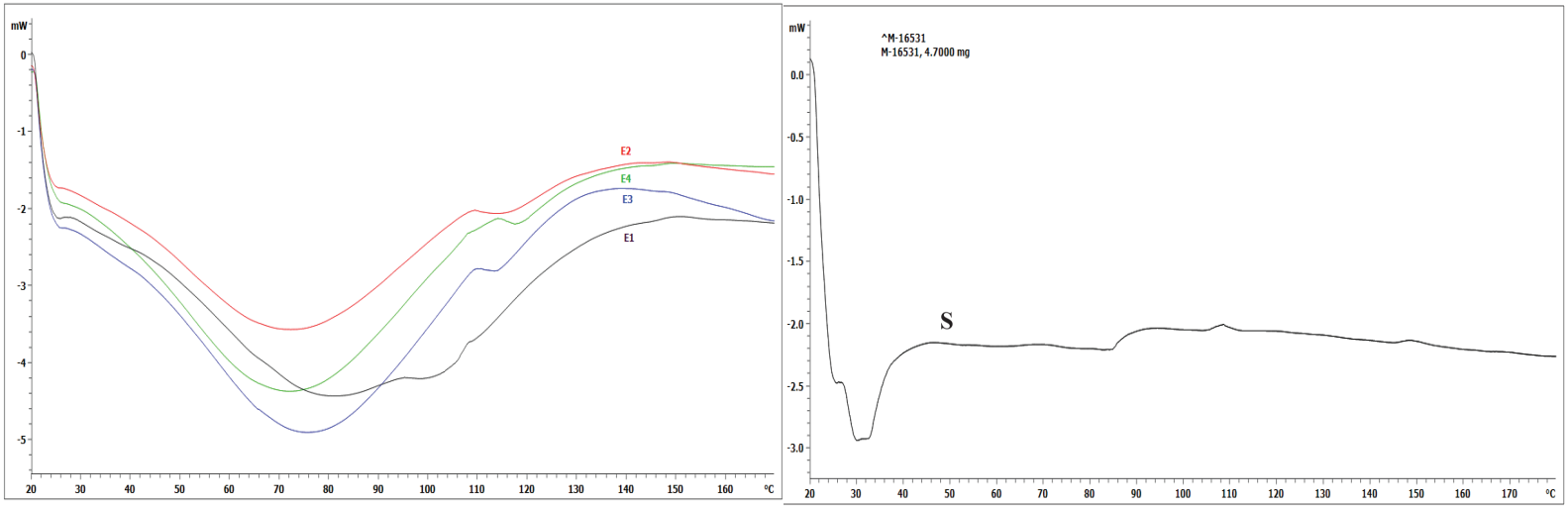


SOP

Fig. 8.6 SEM images of sardine oil encapsulates

8.3.3.2 Differential scanning colorimetry

Thermal analysis of encapsulates were carried out to predict the crystallinity and thermal behaviour of fish oil and microcapsules (Fig. 8.7). The DSC analysis of pure sardine oil revealed three endothermic transitions corresponding to initial peak temperature of 25.58^{o}C and a second well defined melting curve with peak transition at 30.83^{o}C and the third minor transition at 84.27^{o}C. The oil encapsulates, on the other hand, showed distinctly different pattern with three different endothermic transitions. The control SO encapsulate showed a glass transition at 25.33^{o}C, closer to that of pure fish oil. The incorporation of hydrolysate in the wall material slightly reduced the glass transition point of encapsulates, whereas addition to core material slightly increased the glass transition point. The well-defined melting transition observed at 30.83^{o}C of pure fish oil could not be detected in the thermogram of any of the encapsulates. Instead, a distinct endothermic transition was observed at 71-78^{o}C, with the lowest temperature for SOP and the maximum for SO.This second transition is more likely associated with the destabilisation of wall polymers owing to helix-coil transformation of protein components. This was further confirmed by the higher enthalpy of transition evidenced in hydrolysate containing samples, with the highest value for PO followed by SOP and SPO. The endothermic transition observed in pure fish oil at 84.27^{o}C could not be detected in any of the encapsulates, as it was merged with the second transition. However, a third endothermic transition was evident at 102 -118^{o}C with the lowest temperature for SO and the highest for SOP. This point can be related to be the critical limit beyond which the amorphous powders are prone to unfavourable changes like capsular collapse, oil release, caking and stickiness further affecting their quality.



Sample	Notation	Glass transition		Phase transition I		Phase transition II	
		Transition temperature (°C)	Enthalpy (J/g)	Transition temperature (°C)	Enthalpy (J/g)	Transition temperature (°C)	Enthalpy (J/g)
Sardine oil	S	25.58	0.078	30.83	-0.065	84.27	-0.28
SO	E1	25.33	0.16	77.26	-0.51	102.82	-0.90
SPO	E2	24.94	0.55	72.17	-1.38	115.72	-0.54
PO	E3	25.31	0.068	75.36	-3.71	113.42	-0.39
SOP	E4	26.88	0.29	71.07	-2.48	117.92	-0.59

Fig. 8.7 Thermal characteristics of sardine oil and sardine oil encapsulates

8.3.3.3 Fourier-transform infrared spectroscopic analysis

The encapsulates in the region of 4500 to 400 cm^{-1} were analysed for FTIR pattern to figure out the interaction behaviour of wall materials as well as the oxidation pattern of encapsulated fish oil. The amide-I band observed at 1649.14 cm^{-1} in SO which represents C=O stretching vibrations of amide groups coupled with in-plane NH bending underwent a slight shift in band width toward higher wave number in SPO and PO, and towards a lower wavenumber in SOP (Fig. 8.8). However, the Amide-II band that appeared at 1535.34 cm^{-1} in SO was much compressed in SPO and PO, but shifted towards a higher wave number in SOP. Moreover, an additional band at 1411.89 cm^{-1}appeared in SPO and PO. Kong and Yu (2007) mentioned that the bands between 1654 $cm^{-1}$and 1658 cm^{-1}were assigned to alpha-helix while those between 1642 $cm^{-1}$and 1624cm^{-1} to beta-pleated components. The characteristic band for random coil conformation was assigned to the band located at 1648 ± 2 cm^{-1}. In the current study, the major structural rearrangement was evident with the incorporation of hydrolysate as wall material, from random coil to helical confirmation. However predominance of beta-pleated conformation was observed when used along with core material. The amide-A band, which represents NH stretching vibrations appeared at 3392.79 cm^{-1}in SO shifted towards lower band width in all the other samples with major shift in SOP. Binsi et al. (2017c) reported the shifting of amide-A band towards lower frequency, when the N-H group of a peptide is involved in a hydrogen bond. The major shift in this band in SOP indicates the interaction between oil and the hydrolysate through hydrogen bond, as expected during emulsion formation. However, amide-B band, which also represent the NH stretching vibrations remained at the similar wave length in all the samples.

The progress of oil oxidation was monitored by following the shift in the characteristic groups of secondary and tertiary oxidation products. The band at 2852.72 cm^{-1} in SO characteristic of the symmetric stretching vibration of the aliphatic CH_2, underwent slight shift to lower frequency in PO. Moreover, the band corresponding to symmetric stretching vibration of aliphatic CH_2 group present in pure fish oil disappeared totally in all the encapsulates. The band corresponding to ester carbonyl group of triglycerides observed at 1742.76 cm^{-1} in pure oil was shifted to 1745.58 in SO, SPO and SOP, however, underwent minimum shift to 1743.65 in PO. This band is characteristic of the axial deformations of carbonyl bonds, present in the majority of the oxidation products, and the broadening of this band indicates the incidence of oxidation. Accordingly, in the present study, the area under ester band followed the order of SOP<SO<SPO<PO, indicating extended rate of oxidation in PO and SPO and a significantly lower value in SOP. The band at 1147.69 assigned to the vibration of the C-O ester groups in pure sardine oil shifted to 1149.57 cm^{-1} in all the encapsulates. However, the well pronounced C–H stretching vibration of the cis-double bond observed in pure fish oil at 3011 cm^{-1} completely disappeared in all the encapsulates.Similarly, the bending vibrations of the CH_2 /CH_3 observed at 1464 cm^{-1} in pure fish oil underwent major shift towards lower frequency and was much condensed in all the encapsulates, indicating that all the encapsulates underwent certain extent of oxidation during spray drying process.

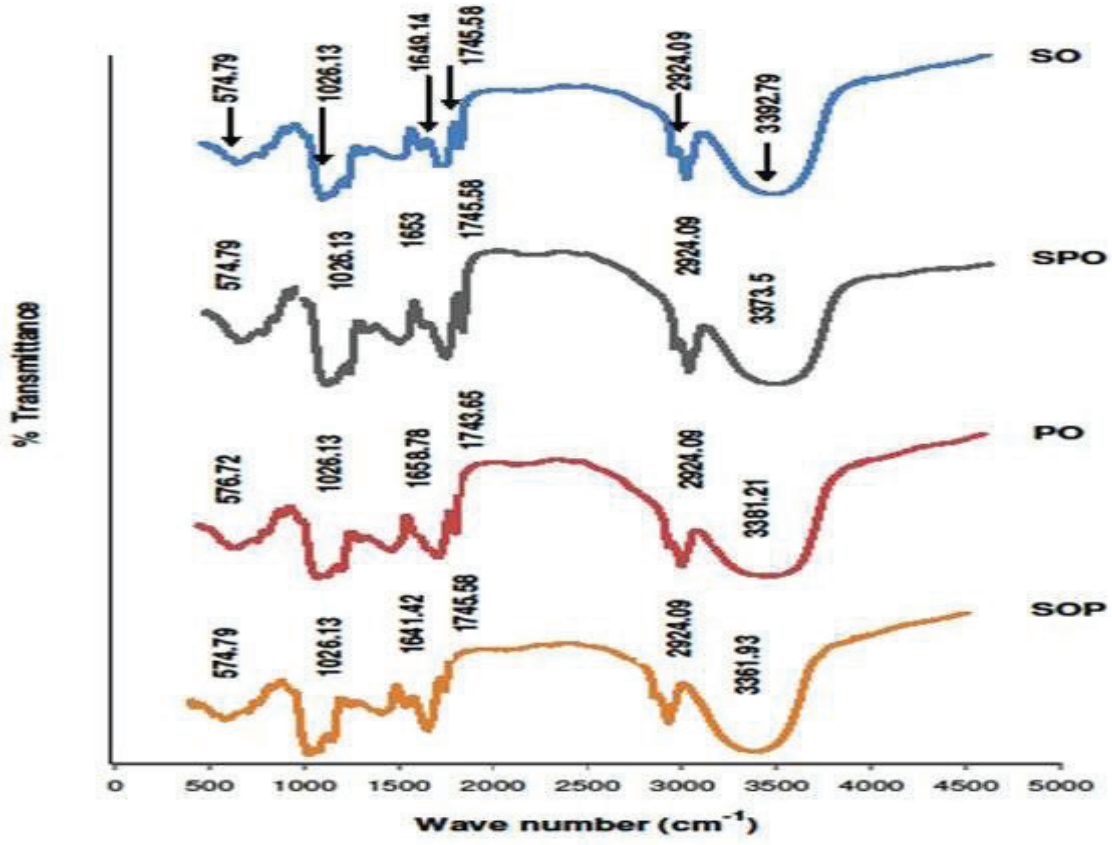


Fig. 8.8 Infra-red spectral characteristics of sardine oil encapsulates

8.3.3.4 Encapsulation efficiency

The encapsulation efficiency defines the degree of protection offered by the wall material to the embedded core component within the matrix. The choice of encapsulation materials and method must ensure stability of core molecule with maximum retention of its biological activity, which is directly related to the encapsulation efficiency achieved by the process (da Rosa Zavareze et al., 2014). In the current investigation, the encapsulation efficiency was associated to the levels of protein hydrolysate in emulsion composition with higher efficiency for SPO and SOP in comparison to SO and PO (Table 8.2). The superior emulsifying properties of hydrolysates must have facilitated the formation of a strong and stable emulsion capable to withstand the shearing forces during homogenisation and atomization,

resulting in higher EE. However, the complete replacement of wall polymer with hydrolysate significantly reduced the EE. This further suggests the possibility of the formation of a weak capsule wall in PO encapsulates, supporting the hypothesis that hydrolysate primarily act as filling polymers rather than as wall polymer.

8.3.4 Physical properties of microencapsulates

8.3.4.1 Moisture content

A major factor influencing the storage stability of dry powders is the moisture content, which enhances oxidative and microbial deterioration during storage.In the present study, the moisture content of the encapsulates ranged from 4.3 – 6.0 % (Table 8.2). It was observed that replacement of sodium caseinate with protein hydrolysate as wall material resulted in a decrease in the moisture content. SO encapsulates exhibited higher moisture content which might be due to larger capsular size, hindering rapid diffusion and escapement of moisture during the spray drying process. On the other hand, the hydrolysate rich in hydrophilic shorter peptides reoriented the water molecules towards the capsular surface facilitating quick release of moisture during atomization.

8.3.4.2 Hygroscopicity

One of the major determinant factors influencing the reconstitution property of powder is its water absorption characteristics or hygroscopicity (Fernandes et al., 2013). Simultaneously, hygroscopic powders easily absorb water from the surrounding environment, developing stickiness and caking during storage affecting the powder dispersibility, which is not desirable. Hence, moderate hygroscopic nature is preferrable for spray-dried powders having desirable dispersibility and storage stability.In the present study, incorporation of protein hydrolysate in the emulsion increased the hygroscopicity of encapsulates(Table 8.2), which might be due to the hygroscopic nature of hydrolysate itself (Mohan et al., 2015). In addition, the smaller particle size of hydrolysate encapsulates as confirmed by microscopic

analysis, might have increased its surface area promoting more uptake of moisture from the storage environment. The hygroscopicity of PO encapsulates was almost double of that of SO samples, whereas SOP and SPO showed moderate values.

8.3.4.3 Bulk density and tapped density

Bulk density and tapped density are important parameters related to packing, transport as well as distribution of powders. Though, high bulk density is preferred from economic point of view, desirable flow properties demand low bulk density to the powders. Both, bulk and tapped densities showed significantly higher values when protein hydrolysate was used as wall material (SPO and PO), while showing a lower value when used as core material along with fish oil (SOP) (Table 8.2).

The bulk density includes the contribution of the inter-particulate void volume apart from true density of powder particles, hence also depends on size and shape regularity of particles, and the spatial arrangement of particles in powder bed(Binsi et al., 2017b). The increase in both bulk and tapped density values associated with incorporation of hydrolysate in the wall material indicates the formation of spherical microcapsules of smaller sizes in SPO and PO, as also confirmed by SEM images, which might have facilitated the compact packing of microencapsulates in a specific volume. Similarly, the lower bulk density coupled with almost similar tapped density values observed in SOP encapsulates indicates microcapsules of larger dimensions and lesser uniformity.

The carr index and hausner ratio are used to evaluate the flow properties of powders (Fitzpatrick and Ahrné, 2005). In the present study, the flow property indices revealed passable nature {1.26-1.34 (hausner ratio); 21-25 (carr index)} for encapsulates, viz., SO (1.3; 23.21) and PO (1.31; 23.36), whereas the flow properties were superior with fair flowability {1.19-1.25 (hausner ratio); 16-20 (carr index)} for SPO (1.23; 18.67) and SOP (1.25; 19.84).

Table 8.2 Physico-chemical properties of sardine oil encapsulates

Parameters		SO	SPO	PO	SOP
Moisture content (%)		$6.02^{b} \pm 0.26$	$5.80^{b} \pm 0.21$	$4.59^{a} \pm 0.24$	$4.30^{a} \pm 0.88$
Hygroscopicity (%)		$4.92^{a} \pm 0.26$	$6.98^{b} \pm 0.29$	$8.31^{c} \pm 0.71$	$6.56^{b} \pm 0.39$
Bulk Density (g/cc)		$0.095^{b} \pm 0.003$	$0.143^{c} \pm 0.003$	$0.175^{d} \pm 0.008$	$0.085^{a} \pm 0.002$
Tapped Density (g/cc)		$0.123^{a} \pm 0.006$	$0.176^{b} \pm 0.008$	$0.229^{c} \pm 0.025$	$0.106^{a} \pm 0.006$
Colour	**L***	$85.74^{c} \pm 0.04$	$83.02^{b} \pm 0.08$	$81.93^{a} \pm 0.09$	$86.45^{d} \pm 0.11$
	a*	$1.07^{b} \pm 0.01$	$1.87^{d} \pm 0.01$	$0.68^{a} \pm 0.01$	$1.18^{c} \pm 0.02$
	b*	$10.56^{b} \pm 0.06$	$13.09^{d} \pm 0.04$	$11.48^{c} \pm 0.04$	$8.47^{a} \pm 0.02$
Encapsulation efficiency (%)		$73.89^{a} \pm 1.53$	$78.29^{b} \pm 1.06$	$76.64^{ab} \pm 1.17$	$78.73^{b} \pm 1.94$
In vitro oil Release (%)	**1hr**	68.2	32.1	53.1	70.4
	2hr	77.7	51.2	69.7	82.4
	3hr	78.7	56.4	71.8	87.31
	4hr	81.5	69.0	75.8	90.8
	5hr	88.7	78.0	76.3	93.6

Values are expressed as Mean ±SD; n = 3; Different superscripts in the same row indicates significant difference ($p < 0.05$)

The lower hausner ratio and carr index value exhibited for SPO and SOP signifies the free flowing and less cohesive nature of the powders on incorporation of hydrolysate in wall as well as core material. However, absolute replacement of caseinate with hydrolysate might have resulted in the aggregation and lumping of powder in PO, due to higher hygroscopicity of the powder. Variations in the wall material composition was reported as a major influential factor determining the flow properties of oil encapsulate (Jeyakumari et al., 2015).

8.3.5 In vitro oil release kinetics

Simulated digestion technique facilitates to understand whether the intended form and quantity of encapsulated oil is released in a controlled manner to the specific parts of the gastro-intestinal tract during digestion process (Kosaraju et al., 2009). In the current work, hydrolysate incorporation in the wall matrix significantly retarded the release of encapsulated oil during gastric phase of digestion, with a steady state of release during intestinal phase (Table 8.2; Fig. 8.9). The SO sample released about 78 % of total oil after gastric digestion against the lowest value of 51 % in SPO and 70 % in PO. Conversely, almost 82 % of total loaded oil was released after gastric digestion in SOP, with a cumulative release of 94 % of total loaded oil after gastro-intestinal digestion. Moreover, a spurt release of about 70 % of total loaded oil was observed in the case of SO and SOP within 1 hr of gastric digestion, whereas only 32 % was released by SPO sample during the same duration. The higher rate of gastric release coupled with lower surface oil content, confirmed the poor structural integrity of capsular wall in SO and SOP samples. Similarly, the lower extent of oil released during gastric digestion in SPO indicated the structural stabilisation of capsular wall in the presence of hydrolysate. However, in PO samples the fish oil was tightly held by the hydrolysate owing to the superior fat binding and emulsifying properties of the hydrolysate (Parvathy et al., 2016). The smaller capsular dimensions revealed by microscopic images in PO sample also suggested a similar inference.

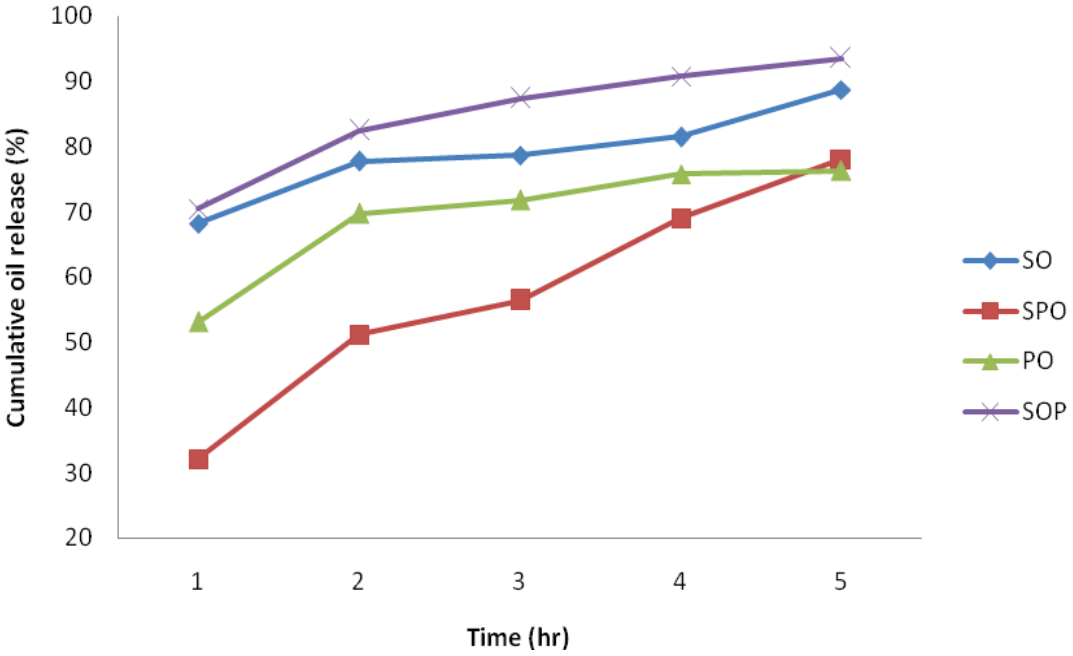


Fig. 8.9 Cumulative oil release pattern of fish oil encapsulates in simulated gastro-intestinal conditions

8.3.6 Storage stability of oil and encapsulates

8.3.6.1 Changes in peroxide value

The protective effect of protein hydrolysate against lipid oxidation was assessed by comparative evaluation of the oxidation pattern of sardine oil and oil encapsulates under different storage conditions (Fig. 8.10a,b,c). Peroxide value of the encapsulates were higher initially in comparison to pure sardine oil. However it was not significantly different between the encapsulate samples, except for PO. The higher PV of encapsulates observed immediately after spray drying might be on account of the high temperature and mechanical shearing subjected during encapsulation process which in turn triggered fat oxidation. Previously, Horn et al. (2012) reported high shear during the homogenization process and the incorporation of oxygen during emulsification to be accelerators of oxidation. This was substantiated by initial high peroxide values for emulsions in studies carried out by

García-Moreno et al. (2016). However, during accelerated storage for seven days, distinctly different values were observed ($p < 0.05$), with highest rate of increase in PO, followed by pure sardine oil. The SPO and SO samples showed almost similar rate of increase, whereas SOP exhibited a gradual and lowest rate of increase in PV till the last day of analysis. Similar profile was observed during storage at ambient atmosphere (Fig. 8.10b) and chilled conditions (Fig. 8.10c) as well, with higher absolute values at ambient atmosphere compared to chilled atmosphere.

The trends in PV of samples under varying storage conditions suggested that the highest rate of protection against oxidation was observed when hydrolysate was used along with fish oil as core material. However, protein hydrolysate failed to form proper wall matrix, when used as the sole wall material giving higher PV values irrespective of storage conditions. Similarly, partial replacement of sodium caseinate did not impart additional oxidative protection to the encapsulates, compared to that with sodium caseinate alone as wall material.

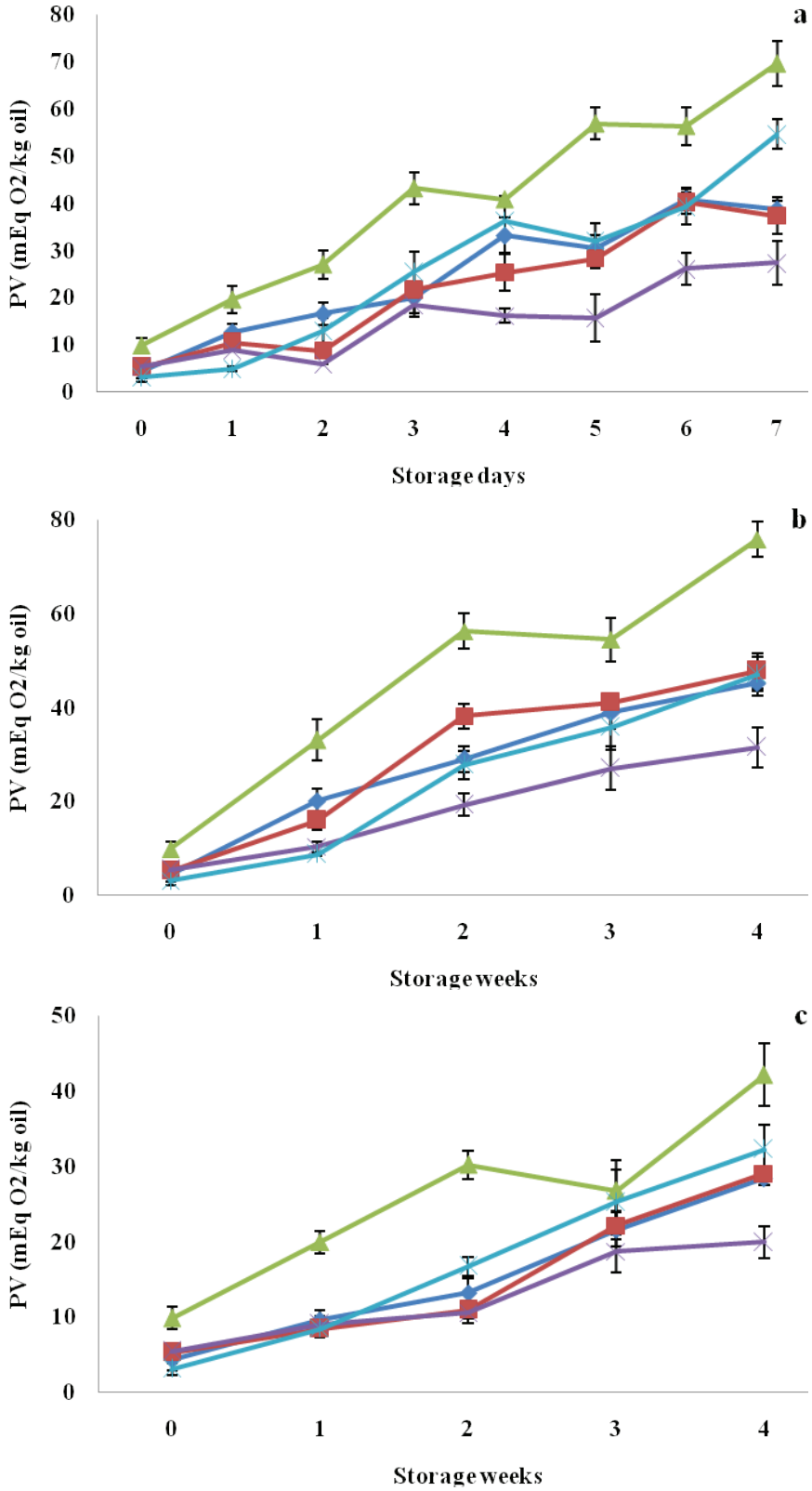


Fig. 8.10 Variations in peroxide value of sardine oil and sardine oil encapsulates at **a.**accelerated, **b.**ambient and **c.**chilled storage conditions

8.3.6.2 Changes in TBARS

During accelerated storage, significant variation ($p < 0.05$) was observed in the TBARS values of pure fish oil and the different encapsulates (Fig. 8.11a,b,c). The initial TBARS value of 0.03 ± 0.001 mg malonaldehyde/kg of fresh sardine oil increased drastically and significantly ($p< 0.05$) during storage at accelerated atmosphere (Fig. 8.11a). Similar trend was observed at ambient (Fig. 8.10b) and chilled conditions too (Fig. 8.11c). However oxidation rate was comparatively less in chilled conditions in comparison to other storage conditions. Similar to PV, the encapsulates also showed higher initial TBARS value compared to that of pure fish oil. Among the encapsulates, PO samples showed significantly higher ($p < 0.05$) TBARS values throughout the storage period, irrespective of storage conditions, whose pattern was closely followed by pure fish oil. Though initially similar, a marked difference in oxidative pattern was observed between SO, SPO and SOP from first week of storage under chilled conditions (Fig.8.11c) with rate of oxidation at a decreased rate in SOP whereas, a sharp increase in values were observed for SO and SPO from 2nd week onwards under ambient atmosphere (Fig. 8.11b). Irrespective of storage conditions, SO and SPO exhibited almost similar pattern of increase in TBARS values, whereas SOP showed much lower absolute values. This variation was more prominent during accelerated atmosphere with similar rate of increase ($p < 0.05$) for SO and SPO, whereas the TBARS values of SOP samples did not show much variations during the first 7 days of storage (Fig. 8.11a).

The overall oxidative pattern of encapsulates during different storage conditions revealed the lowest TBARS formation in SOP followed by SPO and SO. Protein hydrolysates alone as wall material (PO) indicated higher PV and TBARS values as pure fish oil, suggesting least oxidation protection during storage.

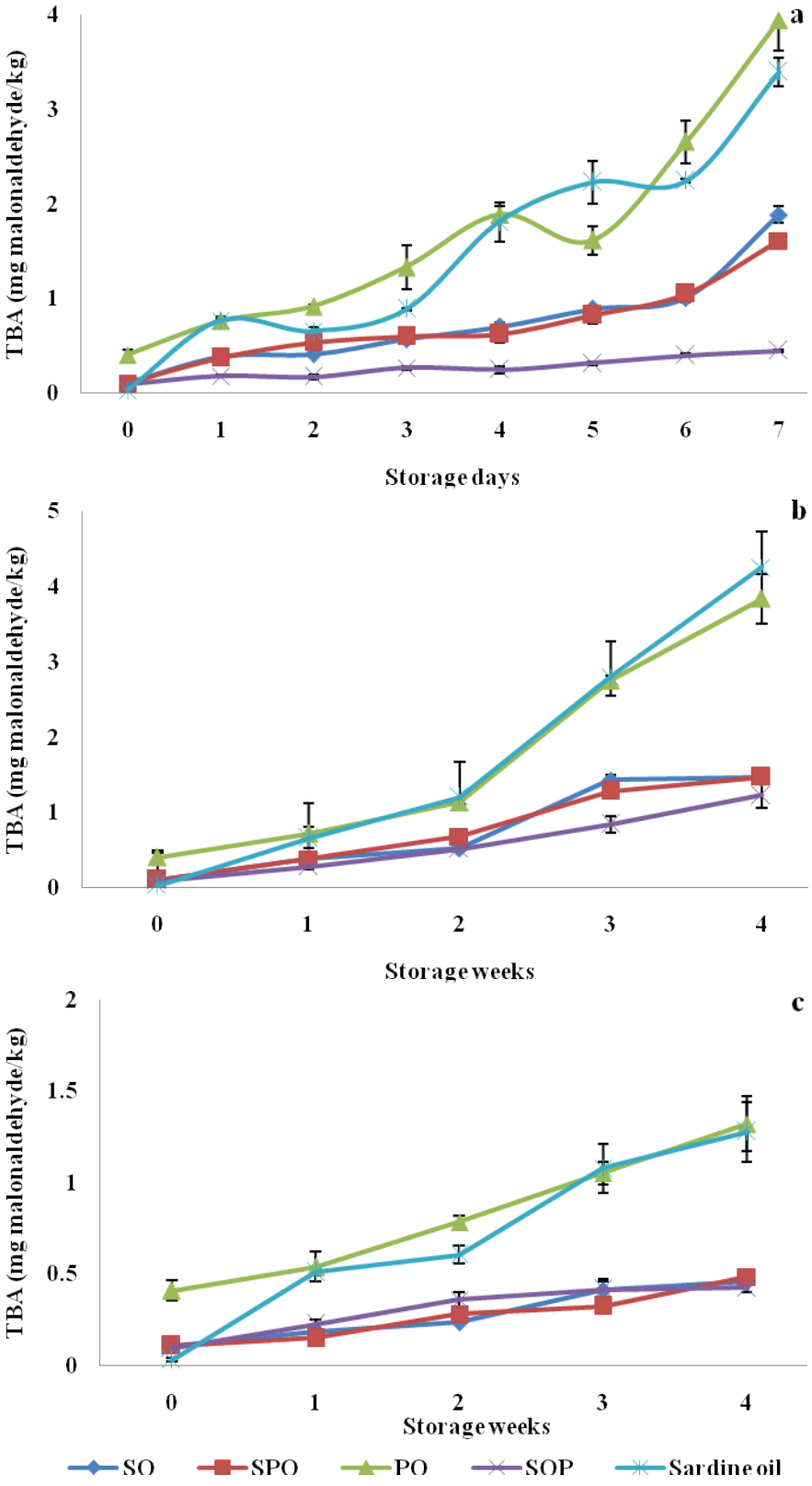


Fig. 8.11 Variations in TBARS of sardine oil and sardine oilencapsulates at **a.** accelerated, **b.** ambient and **c.** chilled storage conditions

8.3.6.3 Changes in colour parameters

High lipid foods undergo oxidative deterioration and associated colour changes viz., brown-colored polymers in the presence of other compounds such as amines, amino acids and antioxidants during storage(Zamora and Hidalgo, 2005; Augustin et al., 2006).A sharp increase in L* values were observed in pure sardine oil after 48 h of storage under accelerated conditions (Fig. 8.11a) and the difference was significant during further storage ($p < 0.05$). Similarly, a sharp increase in lightness was noted after one week storage of sardine oil under ambient (Fig. 8.12b)and chilled condition (Fig. 8.12c), which varied significantly ($p < 0.05$) during storage. However like sardine oil, the variations in lightness values were not so distinct in the encapsulates under accelerated, ambient and chill storage conditions (Fig. 8.13a, 8.14a, 8.15a, respectively). Due to compositional variations in wall material, a significant difference ($p <0.05$) in lightness value was observed between the encapsulate samples. In the present study, the freshly prepared sardine oil encapsulates were in general, creamish white in appearance with variations in their intensity between the samples. It was observed that samples with higher proportion of sodium caseinate had lighter colour which could be attributed to the lighter colour of the polymer itself. The colour of sample was lighter with less yellowish tint when protein hydrolysate was used as core material (SOP) which could be due to the masking of yellowish brown colour of the sardine oil used as core, by the protein hydrolysate.

The chroma parameters such as a* (redness) and b* (yellowness) values of sardine oil and oil encapsulates showed marked changes during different storage conditions. Fresh sardine oil showed a marked decrease in redness after 48 h of accelerated storage condition (Fig. 8.13b) whereas an abrupt decrease in redness was noticed after one week of storage under ambient and chilled conditions (Fig. 8.14b and 8.15b, respectively). However the rate of change in redness was slightly lower

for oil stored under chilled conditions. The freshly encapsulated samples indicated their nearness towards red chroma with significant variations ($p < 0.05$) between the encapsulates and PO had a comparatively lower redness value which might be due to compositional variations. A significant decrease ($p < 0.05$) in redness value was observed for all the encapsulates during accelerated (Fig. 8.13b) and ambient storage conditions (Fig. 8.14b). However the rate of decrease in redness was comparatively less prominent in the encapsulates during chilled storage conditions (Fig. 8.15b).

Variations in yellowness also exhibited similar trends to that of lightness with a marked increase in yellowness noticed in sardine oil after 48 h of accelerated storage (Fig. 8.13c) and after one week of storage at ambient (Fig. 8.14c) and chilled (Fig. 8.15c) conditions. These variations were prominent and significant ($p < 0.05$) under accelerated conditions while it was not significant at ambient and chill conditions, except during initial storage period. The b* values indicating yellowness of samples were observed to vary significantly ($p <0.05$) between the encapsulates and it increased significantly ($p < 0.05$) during accelerated, ambient and chill storage conditions (Fig. 8.13c, 8.14c, 8.15c, respectively). Though initially SPO exhibited higher yellowness due to compositional variation, during accelerated storage on account of higher oxidation, the b* values increased at a higher rate in PO which led to the formation of a homogenous group towards the end of storage and were significantly different from other samples (SO and SOP). Similar observations were made by Binsi et al. (2017a) where the oil encapsulates exhibited an increase in yellowness during storage which indicated the rapid oxidation of surface oil yielding coloured secondary and tertiary oxidation products.Studies by Carneiro et al. (2013) reported whey protein concentrate incorporated flax seed oil encapsulate to exhibit better oxidative stability and associated colour changes being influenced by it.

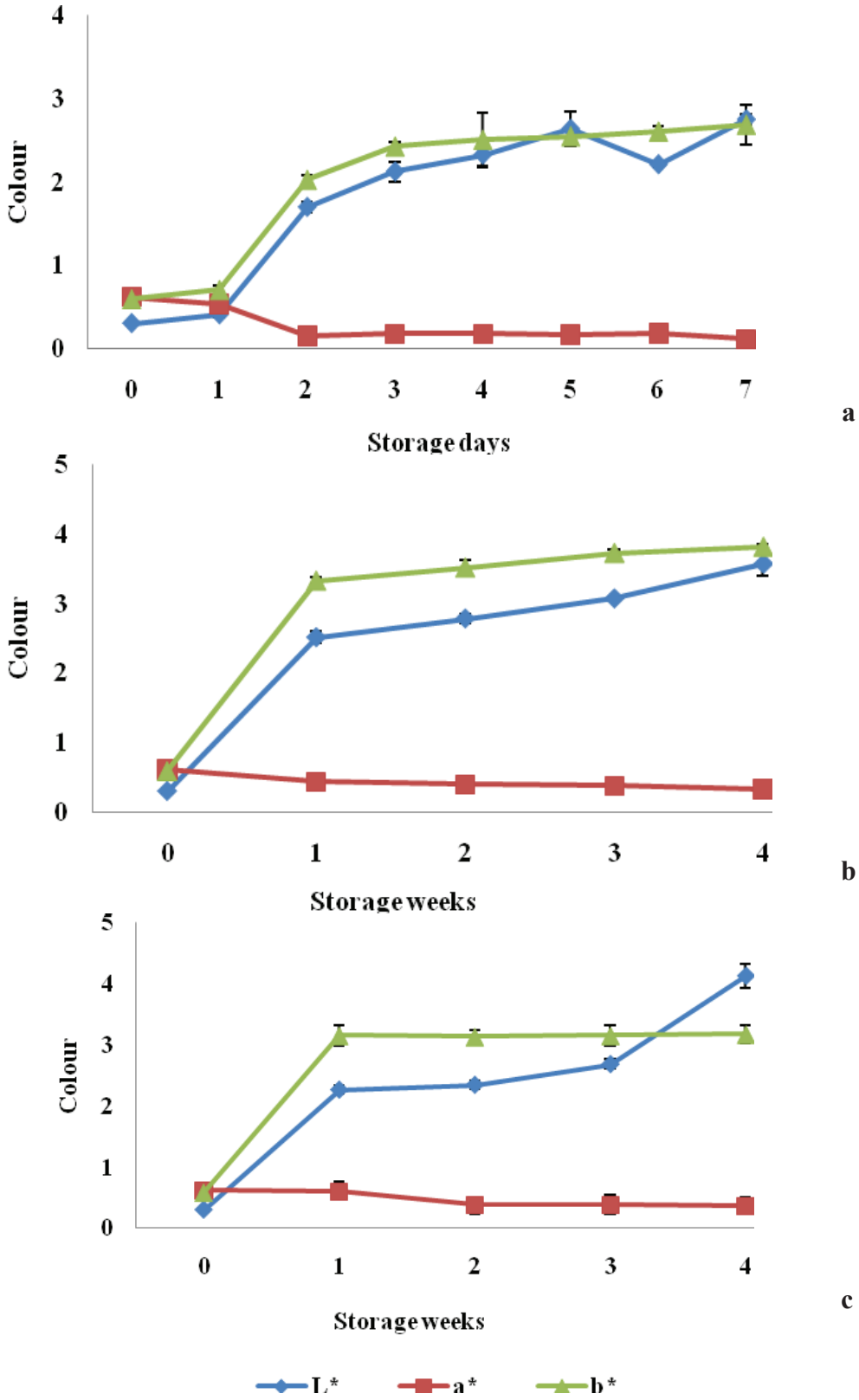


Fig. 8.12 Variations in colour indices of sardine oil during **a.** accelerated (60°C), **b.** ambient (28 °C) and **c.** chilled storage (4°C)

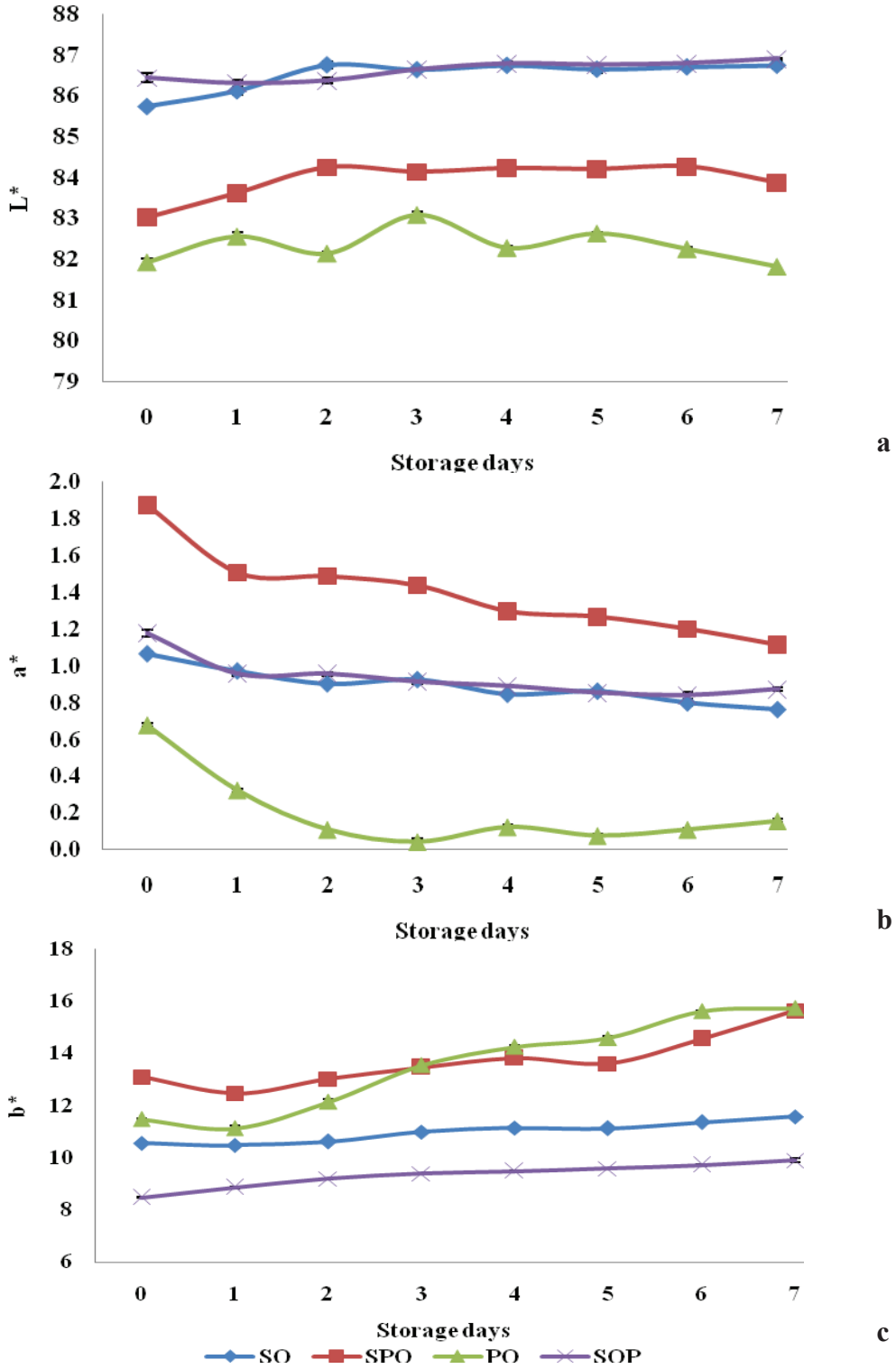


Fig. 8.13 Variations in colour indices viz., **a.** Lightness; **b.** redness; **c.** yellowness of sardine oil encapsulates during accelerated storage (60°C)

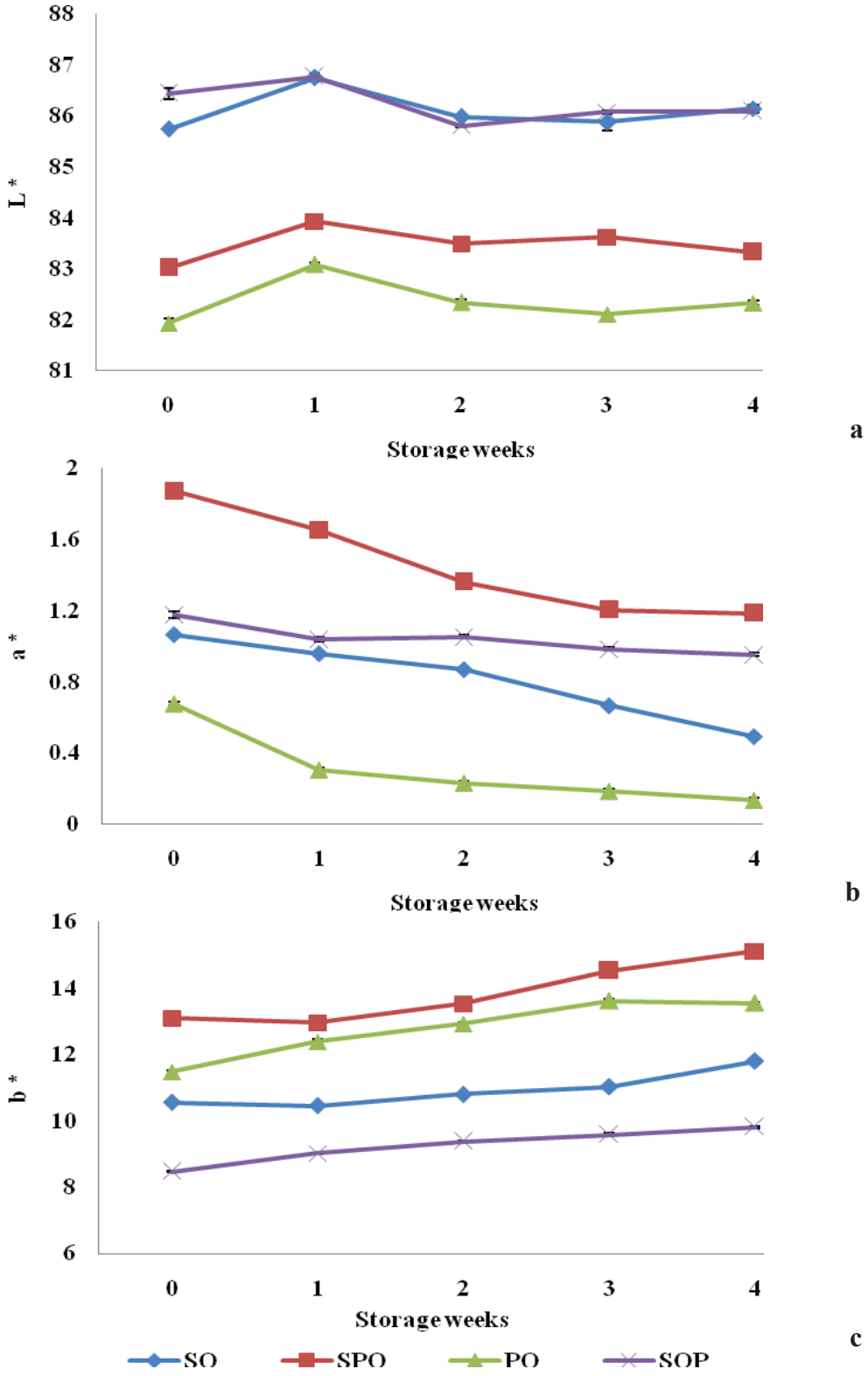


Fig. 8.14 Variations in colour indices viz., **a.** Lightness; **b.** redness; **c.** yellowness of sardine oil encapsulates during ambient storage (28 °C)

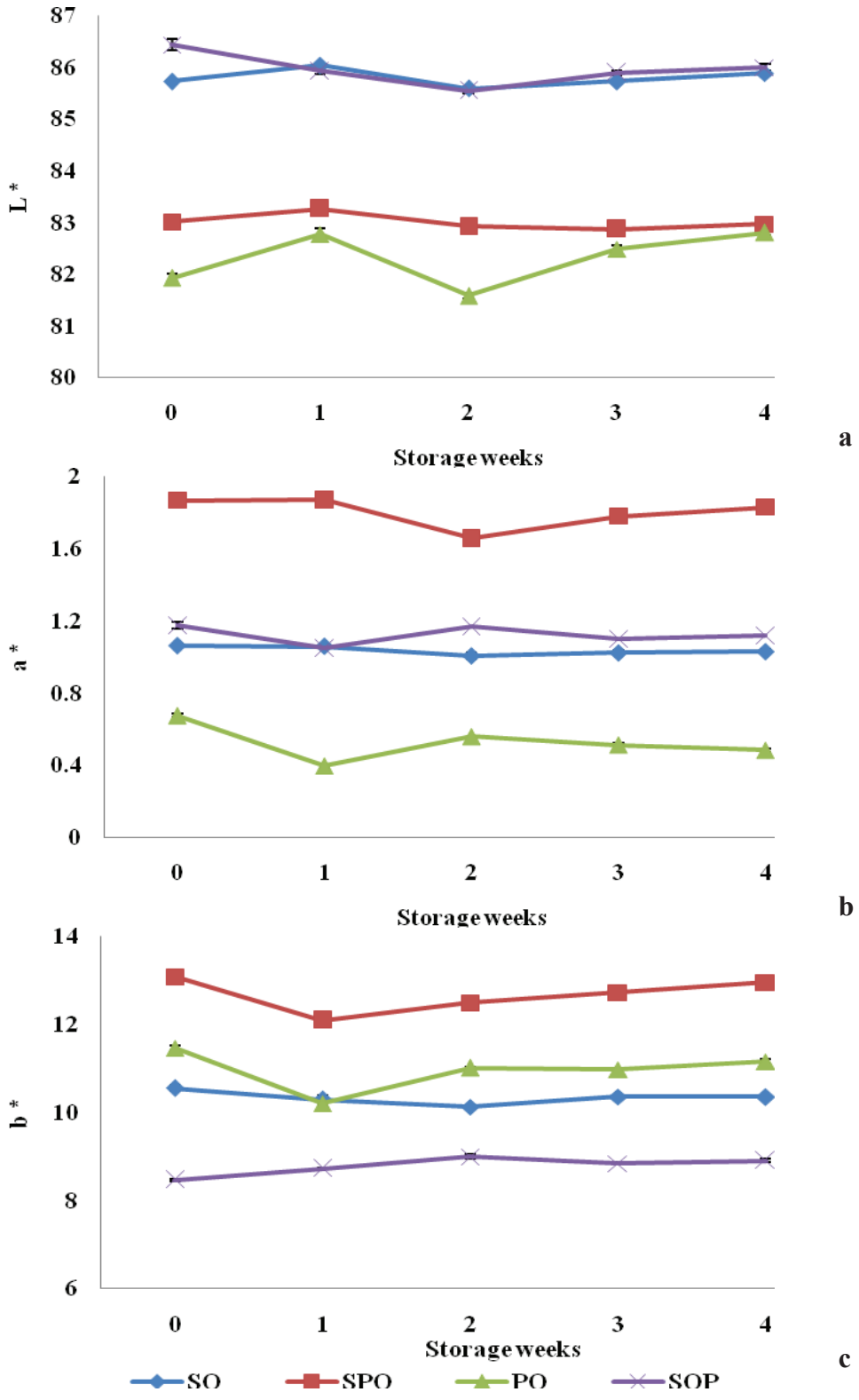


Fig. 8.15 Variations in colour indices viz., **a.** Lightness; **b.** redness; **c.** yellowness of sardine oil encapsulates during chilled storage (4°C)

8.3.7 Product acceptability study

Fortification or enrichment usually refers to the addition of nutrients to food in which they are absent or present in negligible amounts. Enrichment of foods with omega-3 PUFA is regarded as a way of increasing dietary intake of these fatty acids for health promoting effects as well as to reduce the risk associated with coronary diseases (Kolanowski, 2005). However, Drusch and Mannino (2009) reported the major challenge associated with the development of enriched food products to beits acceptance criteria, including addition of active ingredient, product freshness, sensory characteristics, appearance, storage conditions, ease of preparation and safety standards. Several studies have reported development of fish oil fortified products viz., dairy products (Kolanowski and Laufenberg, 2006; Kolanowski and Weiβbrodt, 2007; Kwak et al., 2014), bakery products (Masur et al., 2009; Jeyakumari et al., 2016), mayonnaise (Jacobsen et al. 1999), spaghetti (Verardo et al., 2009), juices (Ilyasoglu and El, 2014) etc. There is an increased demand for foods fortifiedby omega-3 fatty acids and hence is now globally available (Bakry et al., 2016).

Evaluating and improving the acceptability of a product is a critical step as it determines the future market of a new commodity. In the present study, the encapsulate selected with respect to its efficiency and stability viz., SOP, was subjected to acceptability studies with the major objective of determining the encapsulate concentration applicable in different food products without significantly affecting its sensory parameters while simultaneously improving its nutritional value.Four different food products viz., milk, juice, corn flakes and noodles which vary in ingredient composition and preparation methods were chosen. Fish oil encapsulate was enriched with omega 3 fatty acid as well as protein due to its composition viz., presence of fish oil and tuna protein hydrolysate as core material. As per the

composition of fish oil encapsulate (SOP), one gram sample contained 0.16 g oil which in turn had 0.032 g EPA and DHA (Table 8.1). The recommended daily intake (RDI) of EPA and DHA is indicated in Table 8.3, was considered for understanding the nutritional enrichment brought about in the selected food products on account of fish oil encapsulate addition.

Table 8.3 Recommendations for the intake of EPA and DHA

Organization	**Recommendation (g/day)**
American Heart Association	0.5-1.0
US Food and Drug Administration	0.3-0.5
British Nutrition Foundation Task Force	1.0-1.5
UK Department of Health	0.2
World Health Organization	0.7
Institues of Medicine Dietary Reference Intakes	0.11-0.16

Source: *https://foodinsight.org/functional-foods-fact-sheet-omega-3-fatty-acids/*

The acceptability of encapsulate in milk with added sugar (4.5 %) was 2.5 %. As per USFDA, the recommended level of calcium is 1000-1200 mg/day for adults, which can be satisfied from 750 ml of milk (250 ml/glass). Hence a daily intake of three glasses of milk can satisfy the recommended calcium level. As per the acceptability level, 18.75 g encapsulate could get incorporated on consuming 750 ml milk, which could satisfy 0.6 g EPA/DHA per day (Table 8.4; Fig. 8.16).

The acceptability of encapsulate in juice containing 5 % sugar was 7.5 %.The sensory analysis carried out indicated no marked variation with respect to fortified and unfortified samples up to 7.5 % level of incorporation. By daily consumption of 250 ml quantity of juice, an intake of 18.75 g encapsulate can be met, satisfying 0.6 g EPA/DHA per day.

In corn flakes, with a recommended serving of 100 g in 500 ml milk and 3 % added sugar, the acceptability level of SOP was 5.0 %. This concentration satisfied an intake of 5 g encapsulate and hence 0.16 g EPA/DHA per day.

Acceptability of encapsulate in noodles with masala was 7.5 %. With a daily serving of 100 g, about 7.5 g encapsulate gets consumed which is equivalent to an intake of 0.24 g EPA/DHA per day.

In the present study it was found that the encapsulate added into the products had no clear effects with respect to the perceived bitterness, fish taste and odor up to the acceptable levels of encapsulate concentration. Results from the study revealed the possibility of incorporating oil encapsulated with protein hydrolysate in different products for fortification without significant modifications in their sensory characteristics.

Table 8.4 Sensory scores for product acceptance

Products	**Acceptability concentration (%)**	**Average consumption (per day)**	**Omega 3 intake (g)**
Milk	2.5	750 ml	0.60
Juice	7.5	250 ml	0.60
Corn flakes	5.0	100 g	0.16
Noodles	7.5	100 g	0.24

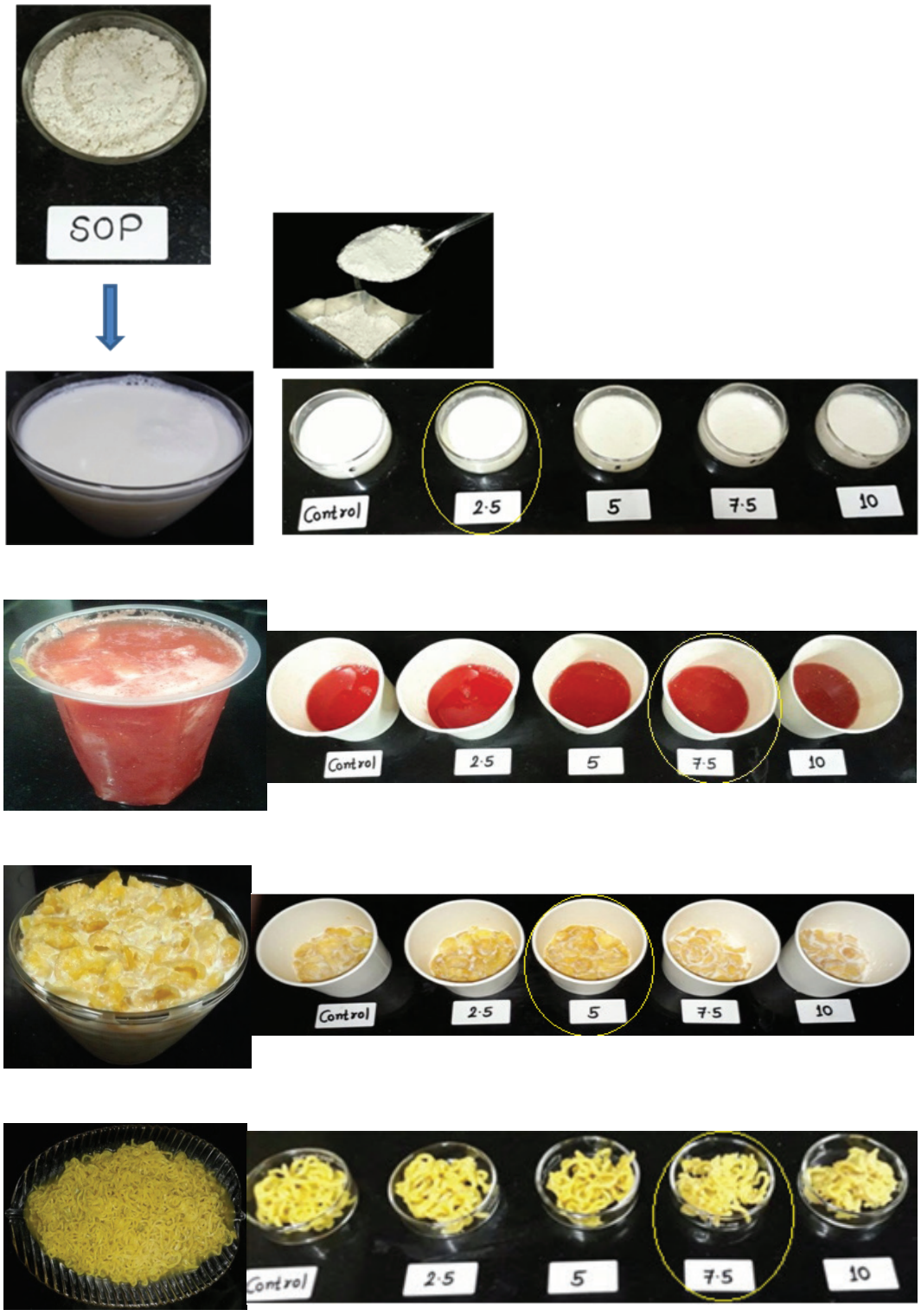


Fig. 8.16 Product acceptability score studies

8.4 Conclusion

The main objective of the present investigation was to compare the efficacy of protein hydrolysate from yellowfin tuna red meat to enhance the structural and oxidative stability of spray dried sardine oil when incorporated as wall material and core material. The results indicated higher encapsulation efficiency for the encapsulates containing protein hydrolysate either as wall or as core material. However, the findings from oxidative stability studies suggested a lower rate of protection when protein hydrolysate was used as wall material, compared to that offered when used as core material along with fish oil. Further the possibilities of incorporating oil encapsulate in selected products (milk, juice, corn flakes, noodles) indicated acceptable sensory characteristics and increased nutritional value in fortified products.

Chapter 9

Utilization of yellowfin tuna protein hydrolysate in health beverage formulation

9.1 Introduction

Food plays a major role in determining the health status of a person. Of the different food components, protein is a major nutrient with a recommended daily intake of 95-120 g and is hydrolysed during the gastrointestinal digestion process into several peptides (Hernandes-Ledesma et al., 2014). Humans require a protein intake sufficient to maintain the body nitrogen balance and allow for desirable rates of deposition during growth. Seafood is an easily available and cheapest food source meeting the protein requirements of approximately 3.1 billion people, globally (FAO, 2016). There is a high potential in marine processing industries to convert and utilize the food and their by-products as valuable functional ingredients. Of the by-products, hydrolysates or bioactive peptides can be suggested as a potential source of natural ingredient and in this context more focus is given by researchers on improving the bioavailability and bioaccessibility of these marine protein hydrolysates for validating as functional ingredients for healthy foods.

Protein hydrolysates have been defined as a mixture of polypeptides, oligopeptides and amino acids derived by hydrolysis of protein sources, to various extent. There has been increasing interest in these preparations over the last two

decades, with novel bioactive peptides continually being discovered, as it has been shown that short-chain peptides from hydrolyzed proteins have a higher nutritive value (Kristinsson and Rasco, 2000; Wisuthiphaet et al., 2015).

Nutritional content is an important factor considered by customers when choosing their foods. Recent food market trend indicates diversification in consumer's food demand with more approach towards health benefit of foods beyond basic nutrition. Knowledge on the association between nutrition and health has resulted in the development of the concept of functional foods, which is a practical and new approach towards improved health status (Girgih et al, 2013). Functional foods defined to be those with specific health benefits, hold a strong market position worldwide and the functional beverage sector accounts for approximately 12.5 % of the world market (Anon, 2011). As studied by Sloan (2003), a wide range of customers (47 %) opine that fortified foods and drinks satisfy their recommended nutritional requirements. Food powders represent a large proportion of the total processed food in the world on account of several reasons viz., low bulk weight, storage, transport and usage conveniences, diverse applications, high stability and the option of high production (Intipunya and Bhandari, 2010). In this regard, fortified powder supplements which can be blended to form drinks are a good option which has enhanced taste as well as improved nutritional value. High energy, proteinaceous drinks help to meet instant energy requirement and compensate electrolytic loss. Hence, new ingredients having superior functional properties are of interest for the development of novel functional beverages. Recently there has been an exceptional demand in the food industry for inexpensive proteins and bioactive peptides for human consumption. Additionally reports indicate the relevance of utilizing protein hydrolysates in the food industry to improve the quality of finished products, especially their storage stability (Rao et al., 2016). Several attempts have been made on utilization of protein hydrolysate in the formulation of various products (Sathivel

et al., 2005b; Sinha et al., 2007; Rao et al., 2016) but, still there is immense scope for its utilization in beverages especially in health based energy drinks on account of its superior functionalities (Singh et al., 2009). Additionally, alternative uses for co-products of the fish processing industry are highly sought as these co-products are excellent sources of nutrients like protein. The utilization of protein hydrolysate from cannery discards like tuna red meat for such health formulations is an ideal approach which is more economical as well as adds on value. Therefore, the current study was performed to formulate a health beverage mix by incorporation of tuna protein hydrolysate (TPH) optimized from yellowfin tuna red meat, a processing discard in tuna canning industry. The base mix was RSM optimized with sensory acceptability as response. Further different levels of TPH were added to the base mix, evaluated for its properties and the best combination was subjected to stability studies under ambient temperature (28^{o}C).

9.2 Materials and methods

9.2.1 Raw material, enzymes and chemicals

Tuna red meat, by-product after canning from Forstar Frozen Foods Pvt. Ltd., a seafood industry at Mumbai, was utilized for the preparation of protein hydrolysate using papain enzyme (Hi Media, India). Ingredients used for health mix preparation included malted barley, malted wheat, milk powder, sugar and vanilla flavor, procured from local suppliers. Malted barley and wheat were prepared from whole barley and wheat, respectively which was soaked in water overnight, washed, drained, spread in a container and covered with a moist cloth for about 16-20 h for facilitating its germination. During this holding period, it was regularly mixed. Sprouted wheat as well as barley was dried to desired moisture content (< 10%) at 60-70^{o}C for 7-8 h and further powdered and roasted. All enzymes and chemicals used for the analysis were of laboratory grade from Merck and Hi media, India.

9.2.2 Hydrolysis - Optimization studies

The hydrolysate used for the formulation of health beverage was derived from tuna red meat using papain enzyme according to an RSM based protocol (discussed in chapter 4) for optimum functional and antioxidative properties (Table 9.1). The optimized hydrolytic conditions were viz., enzyme: substrate (E/S) ratio of 1.08 %, hydrolysis time of 30 min, temperature of 60^{o}C and pH of 6.5. The resultant solution was cooled, coarse filtered and centrifuged at 8000 g at 10^{o}C for 20 min (K-24A, Remi Instruments, Mumbai) and the supernatant was further spray dried (Hemaraj Enterprises, Mumbai) to get protein hydrolysate powder which was used for fortification in health mix.

Table 9.1 Characteristics of optimized tuna protein hydrolysate

Parameters	Values
Degree of hydrolysis	21.93 ± 0.27 %
Foaming capacity	186.7 ± 11.6 %
Foam stability	43.3 ± 5.8 %
Emulsifying activity index	95.74 ± 1.95 m^2/g
Emulsion stability index	30.68 ± 1.07 min
Oil absorption capacity	1.26 ± 0.05 g/g
Bitterness	7.4 ± 0.5
DPPH radical scavenging activity	74.0 ± 0.36 %
FRAP	36.94 ± 0.80 mM Ascorbic Acid/g protein
Metal chelating activity	22.03 ± 2.52 mg EDTA/g protein
ABTS radical scavenging activity	56.27 ± 1.34 %

Values are expressed as Mean ± SD; n = 3; n = 10 (Bitterness)

9.2.3 Formulation of base mix

A base mix was formulated for developing a health beverage using the ingredients viz., malted barley, malted wheat, milk powder, sugar and vanilla flavor. The composition of the base mix: malted barley (20-70 %), malted wheat (10-50 %), milk powder (10-20 %), being process (independent) parameters was optimized based on RSM with a central composite design (12 runs) (Fig. 9.1; Table 9.2). Appearance, flavour, aroma, colour and texture of the prepared product were taken as response (dependent) variables. The overall acceptability was evaluated on the basis of these attributes (Annexure 3). Levels of ingredients viz., sugar and vanilla flavor was kept constant at 10 % and 2.5 %, respectively.

Fig. 9.1 Different formulations of base mix

Table 9.2 Composition of base mix based on RSM and acceptability scores

Run	A:Barley (%)	B:Wheat (%)	C:Milk powder (%)	Sensory acceptability
1	41.25	41.25	17.5	8.0 ± 0.5
2	70	20	10	4.8 ± 0.4
3	40	50	10	7.5 ± 0.5
4	35	50	15	6.5 ± 0.5
5	61.25	26.25	12.5	7.0 ± 0.5
6	61.25	21.25	17.5	8.5 ± 0.5
7	30	50	20	8.8 ± 0.4
8	50	30	20	8.2 ± 0.6
9	70	10	20	8.7 ± 0.5
10	40	50	10	7.3 ± 0.5
11	52.5	32.5	15	8.0 ± 0.7
12	70	15	15	6.5 ± 0.5

9.2.4 Preliminary product acceptability study

Preliminary trials were carried out to understand the effect of protein hydrolysate incorporation on the properties of the health beverage. The selected base health mix was added with optimized tuna protein hydrolysate (TPH) @ 2.5, 5, 7.5 and 10 % levels hereafter referred to as health mix viz., $HM_{2.5}$, HM_5, $HM_{7.5}$ and HM_{10}, respectively (Fig. 9.2). Base mix without addition of TPH was kept as control viz., HM. The samples were subjected to proximate analysis (AOAC, 2012), colour (EZ 45/0, Hunter Associates Lab inc., Reston, Virginia, USA), functional properties viz., foaming (Sathe and Salunkhe, 1981), emulsifying (Pearce and Kinsella, 1978) and oil absorption capacity (Shahidi et al.,1995); antioxidative properties viz., DPPH radical scavenging activity (Shimada et al., 1992) and FRAP (Benzie and Strain, 1996) (described in chapter 3; section 3.2.8 and 3.2.9). Difference and preference tests as adopted by Bilek and Bayram (2015) were slightly modified and carried out to select the most preferred combination of TPH in health beverage mix.

Each sample was served as powder and evaluation was scaled from 1 to 4, 1 being with very good taste to 4 being very bad taste, as influenced by TPH bitterness, by a panel consisting of ten trained members (Annexure 4). Similarly bitterness was sensorily evaluated as per Nilsang et al. (2005) with modifications. Caffeine solution was used as a standard for anchoring the scale of bitterness using a defined 10 line scale anchored from ‘‘no bitterness’’ (as 1) to ‘‘extreme bitterness’’ (10) (Annexure 2). Based on the preliminary study, selected health mix along with base health mix (HM), as control was taken for further characterization and stability studies.

Fig. 9.2 Health mix samples

9.2.5 Characterization of health mix

9.2.5.1 Nutritional profiling

9.2.5.1.1 Fatty acid

Fatty acid composition of health mix was determined in the present investigation. Total lipid was extracted from the sample by the method of Folch et al. (1957) (described in chapter 8; section 8.2.2). Fatty acid composition analysis was performed using gas chromatograph (Varian, Guindy, Chennai; Model no: CP-3800) with a Cpsil 88 FAME column (100 m length x 0.25 mm internal diameter; 0.20 μm film thicknesses and flame ionization detector.

9.2.5.1.2 Amino acid

HPLC (high-performance liquid chromatography) (Shimadzu Prominence, Japan) was employed for amino acid profiling of the health mix (Ishida et al., 1981). (described in chapter 6; section 6.2.2.4.2).

9.2.5.1.3 Mineral

Inductivity Coupled Plasma–Optical Emission Spectrometer (iCAP 6300 Duo, Thermo fisher Scientific, Cambridge, England) with dual configuration (axial and radial) and iTEVA (version 2.8.0.97) operational software was used for elemental analysis (described in chapter 6; section 6.2.2.4.3).

9.2.5.2 Physical properties

9.2.5.2.1 Particle density

About one gram of sample was taken in a 10 ml measuring cylinder with a glass stopper to which petroleum ether (5 ml) was added and well shaken to suspend the powder particles completely. Finally, all the powder particles sticking on the cylinder wall were rinsed down with a further 1 ml of petroleum ether and the total volume of petroleum ether together with suspended powder was noted. The particle density was calculated as follows (Jinapong et al., 2008):

$$\text{Particle density (g/ml)} = \frac{\text{Weight of powder}}{\text{Total volume of petroleum ether with suspended powder - 6}}$$

9.2.5.2.2 Bulk and tapped densities

Bulk and tapped densities were measured as per Chinta et al. (2009). A known quantity of sample powder was loosely packed through a funnel into 10 ml graduated cylinder by slight tapping to collect the powder sticking to the wall of the cylinder and the volume was recorded. Bulk density (ρB) of the powder was calculated by dividing weight of the sample by its volume. Similarly, a known quantity of the sample was poured into the cylinder and tapped until a constant volume was reached to determine the tapped density (ρT).

9.2.5.2.3 Porosity

Porosity of the powder samples was calculated using the equation (Jinapong et al., 2008):

$$\text{Porosity} = \frac{\text{Particle density} - \text{Tapped density} \times 100}{\text{Particle density}}$$

9.2.5.2.4 Flowability and cohesiveness

Hausner ratio (Hausner, 1967) and Carr Index (Carr, 1965) indicative of the flow properties viz., cohesiveness and flowability were determined from the bulk and tapped densities as:

$$\text{Hausner ratio} = \rho T / \rho B$$

$$\text{Carr index} = 100 (1 - \rho B / \rho T)$$

Table 9.3 Classification of powder flowability based on Carr index and Hausner ratio

Flow characteristics	Carr Index (%)	Hausner ratio
Excellent/ Very free flow	1-10	1.00-1.11
Good/free flow	11-15	1.12-1.18
Fair	16 – 20	1.19-1.25
Passable	21-25	1.26-1.34
Poor/Cohesive	26 – 31	1.35-1.45
Very poor/very cohesive	32- 37	1.46-1.59
Extremely poor/ approx. no flow	> 38	> 1.60

9.2.5.2.5 Wettability

Wettability of the powder sample was determined according to Jinapong et al. (2008). About 100 ml of distilled water was taken in a 250 ml beaker. A glass funnel held on a ring stand was set over the beaker with 10 cm spacing between the bottom of the funnel and the water surface. A test tube was placed inside the funnel to block the lower opening of the funnel. About 0.1 g of powder sample was placed around the test tube. The tube was lifted (noted as initial time) to the final time for

the powder to become completely wetted (visually assessed as when all the powder particles penetrated the surface of the water) was recorded to assess the wettability.

9.2.5.2.6 Dispersibility

Dispersibility measurement was performed as reported by Jinapong et al. (2008). About 10 ml of distilled water, was poured into a 50 ml beaker into which about one gram of powder was added. The sample was stirred vigorously with a spoon for 15 s with about 25 complete back and forth movements around the beaker. The reconstituted sample was sieved and the sieved milk (1 ml) was transferred to a preweighed dry petriplate which was oven dried for about four hours at 100°C. The dispersibility of the powder was calculated as:

$$\text{Dispersibility (\%)} = \frac{(10 + a) \times TS\ \%}{a \times \frac{100-b}{100}}$$

where a = amount of powder (g) used, b = moisture content in the powder, and % TS = dry matter in the reconstituted sieved milk.

9.2.6 Antioxidant stability during in vitro gastrointestinal (GI) digestion

Simulated GI digestion using an *in vitro* pepsin–pancreatin hydrolysis was carried out according to the method of Cinq-Mars et al. (2007) and You et al. (2010b), with slight modification. The pH of the powder sample (3 mg, 15 ml) was adjusted to 2.0 with 6 M HCl. Pepsin was then added (E/S 1:35 w/w), and the mixture was incubated with continuous shaking (Shaking bath, Neolab Instruments, Mumbai, India) for 1 h at 37°C. The pH was then adjusted to 5.3 with 0.9 M $NaHCO_3$ solution and further to pH 7.5 with 6 M NaOH. Pancreatin was added (E/S 1:25 w/w), and the mixture was further incubated with continuous shaking for 3 h at 37°C. To terminate the digestion, the solution was submerged in boiling water for 10 min. Then, the GI digest was cooled to room temperature and centrifuged at 5000 g for

15 min. The supernatant was used for analysis. To investigate the changes in DPPH radical-scavenging activity of sample digested during the simulated GI digestion, aliquots of GI digests were removed every one hour for 3 h during the *in vitro* digestion.

9.2.7 Storage stability studies

Health mix selected based on the preliminary product acceptability study together with control viz., base health mix (HM) were packed in airtight plastic bottles, stored under ambient conditions (28^{o}C) and subjected to monthly analysis for indices viz., moisture (AOAC, 2012), pH (ECPH S1042S, Eutech Instruments, Singapore), colour (EZ 45/0, Hunter Associates Lab inc., Reston, Virginia, USA), PV (AOAC, 2012), FFA (AOAC, 2012), TMA-N and TVBN (Conway micro – diffusion assay), sensory (Meilgaard et al., 2006) (Annexure 3) and microbiological parameters (USFDA, 2001) for a period of six months (described in chapter 6; section 6.2.3 and chapter 7; section 7.2.5).

9.2.8 Statistical analysis

The analytical data obtained in triplicate were subjected to analysis of variance (ANOVA). The differences between means were evaluated by duncan's multiple range test and were considered significant at 5 % levels. SPSS statistic programme (SPSS 16.0 for Windows, SPSS Inc., Chicago, IL) was used for interpretation of the results obtained.

9.3 Results and discussion

9.3.1 Formulation of base health mix

Malt-based drinks have acquired reputation over centuries for their nutritional value and have attracted more customers on account of increasing health awareness. Malting is effective with respect to preservation as well as it adds flavour and texture to the product (Murray and Van der Meer, 1997). Various processes are available for the production of malted grains of various qualities (Obuzor and Ajaezi, 2010). In general, the malting practice for converting raw grain into malt involves a three-step process: steeping, germinating and drying and because malt is made from whole grain and minimally processed, it is an all natural ingredient. A health beverage mix was formulated based on RSM based optimization using the ingredients viz., malted barley, malted wheat, milk powder, sugar and vanilla flavor. Sensory studies indicated a combination of malted barley (30 %), malted wheat (50 %), milk powder (20 %) as most acceptable by the panellists (Table 9.2) and hence was selected as the base mix for further studies. Level of ingredients viz., sugar and vanilla flavor was kept constant at 10 % and 2.5 %, respectively.

9.3.2 Preliminary product acceptability study

Incorporation of tuna protein hydrolysate in the base mix @ 2.5 - 10% levels improved the protein content in the sample (Table 9.4). There was a significant increase ($p< 0.05$) in protein content from 9.69 ± 0.14 % to 15.10 ± 0.26 % with incorporation of hydrolysate up to 10 % from control which is attributed to higher protein content in the hydrolysate (86.23 ± 1.54 %). Moisture content of a sample influences its storage stability by influencing the oxidative as well as microbial indices. In the present study, the moisture content of the samples varied from 5.01 ± 0.15 to 5.61 ± 0.16 % (Table 9.4). It was observed that incorporation of protein hydrolysate in the health mix resulted in a proportional decrease in the moisture

content which must be ascribed to the lower moisture content in the protein hydrolysate. Fat content varied significantly ($p < 0.05$) between control and fortified samples and it ranged from 1.23 ± 0.01 to 1.49 ± 0.06 % whereas ash content ranged from 0.93 ± 0.02 to 1.76 ± 0.11 %. These proportional variations must be on account of the addition of hydrolysate in the sample at increasing concentrations.

Table 9.4 Proximate composition of tuna protein hydrolysate and health mix samples

Sample	Proximate composition (%)				
	Moisture	**Protein**	**Fat**	**Ash**	**CHO**
TPH	7.86 ± 0.31	86.23 ± 1.54	0.71 ± 0.11	4.05 ± 0.48	-
HM	5.61^{a} ± 0.16	9.69^{d} ± 0.14	1.23^{c} ± 0.01	0.93^{c} ± 0.02	82.54^{a} ± 0.16
$HM_{2.5}$	5.23^{b} ± 0.11	11.82^{c} ± 0.32	1.36^{b} ± 0.05	0.95^{c} ± 0.02	80.64^{b} ± 0.35
HM_{5}	5.19^{b} ± 0.12	12.84^{b} ± 1.04	1.49^{a} ± 0.06	1.05^{c} ± 0.08	79.43^{c} ± 1.04
$HM_{7.5}$	5.17^{b} ± 0.10	14.36^{a} ± 0.48	1.45^{ab} ± 0.04	1.20^{b} ± 0.06	77.82^{d} ± 0.56
HM_{10}	5.01^{b} ± 0.15	15.10^{a} ± 0.26	1.45^{ab} ± 0.08	1.76^{a} ± 0.11	76.68^{e} ± 0.11

Consumers consider color as one of the most important parameters for evaluating the product quality like freshness, flavor etc (Wu and Sun, 2013). Colour properties viz., lightness, redness and yellowness of the samples varied significantly ($p < 0.05$) with variations in TPH levels in the sample. Lightness ranged from 80.76 ± 0.07 to 81.41 ± 0.03 indicating an increase in lightness with hydrolysate incorporation (Table 9.5; Fig. 9.3a). Similarly yellowness also increased with increase in concentration of protein hydrolysate in samples ranging from 14.77 ± 0.15 to 17.38 ± 0.1 (Fig. 9.3c). Correspondingly, there was a decrease observed in the case of redness from 1.9 ± 0.02 to 1.17 ± 0.03 (Fig. 9.3b). These variations in the colour indices in samples must be on account of the colour characteristics of the incorporated tuna protein hydrolysate which had a creamish white appearance.

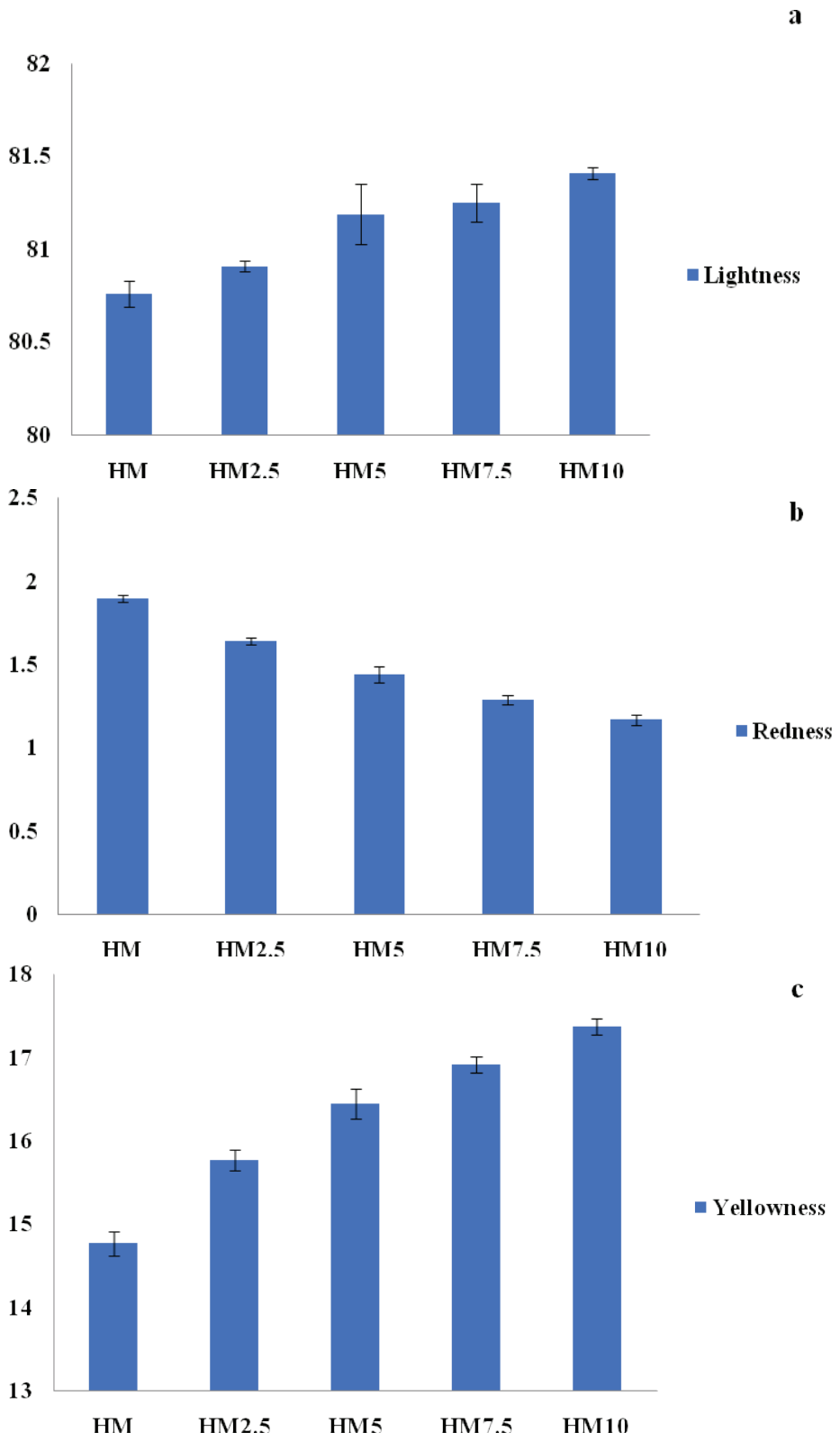


Fig. 9.3 Variations in colour attributes viz., **a**. lightness; **b**. redness; **c**.yellowness of health mix samples incorporated with different levels of TPH

Functional properties viz., foaming capacity improved from 50 ± 0 % (HM) to 83.33 ± 5.77% (HM_{10}) whereas foam stability showed a slight increase by 10 % from 10 ± 0 % in control (Fig. 9.4). Similarly emulsifying property viz., emulsifying activity index (EAI) increased from 48.95 ± 3.65 m^2/g (HM) to 70.81 ± 2.39 m^2/g (HM_{10}) whereas ESI improved from 33.36 ± 2.09 (HM) to 39.67 ± 2.80 min (HM_{10}) (Fig. 9.5). This enhancement in properties must be related to the addition of protein hydrolysate in the samples which are reported to have superior functional properties (Parvathy et al., 2018a,b). Present study also reported superior functional property in the optimized tuna protein hydrolysate which was opted for incorporation in the base mix (Table 9.1). However oil absorption capacity was lower for fortified samples compared to control. A value of 90.22 ± 1.6 % was observed for control while it decreased in hydrolysate incorporated samples, ranging from 86.04 ± 1.15 % to 87.56 ± 0.54 % (Fig. 9.6).

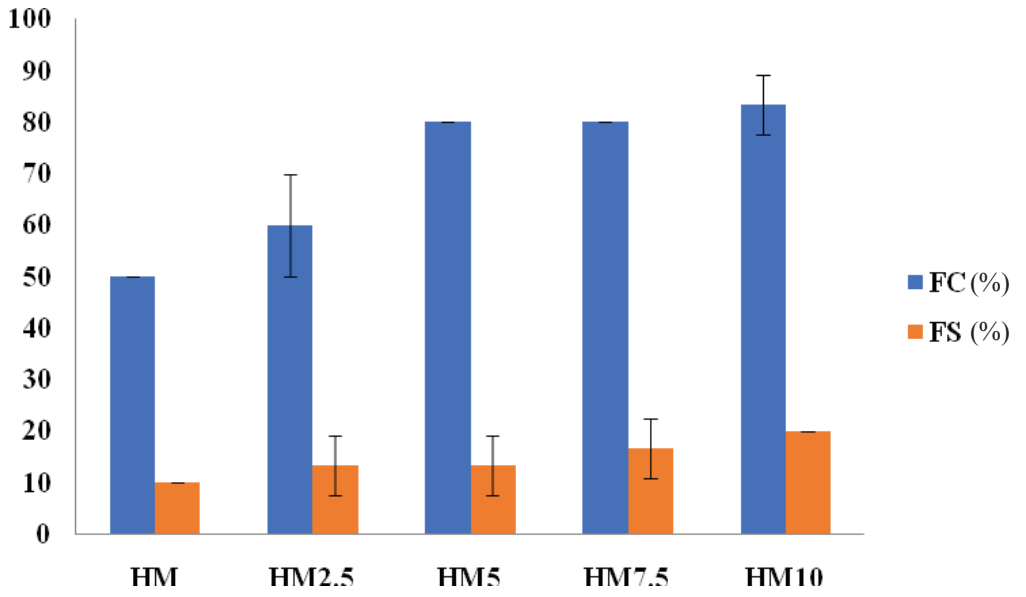


Fig. 9.4 Variations in foaming properties of health mix samples incorporated with different levels of TPH

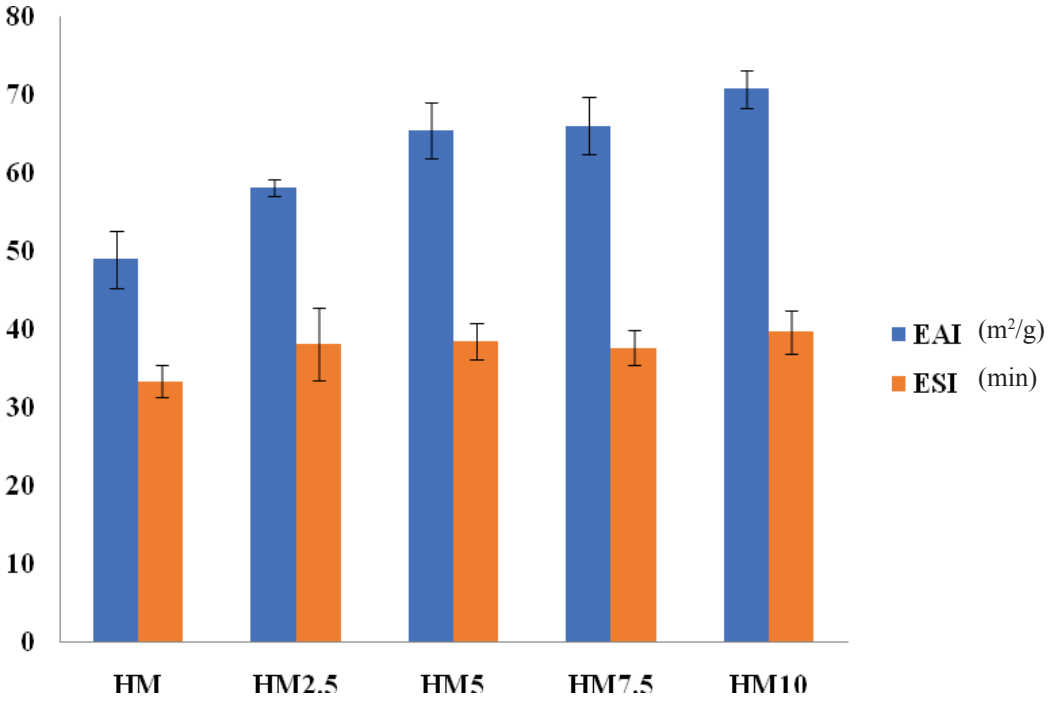


Fig. 9.5 Variations in emulsifying properties of health mix samples incorporated with different levels of TPH

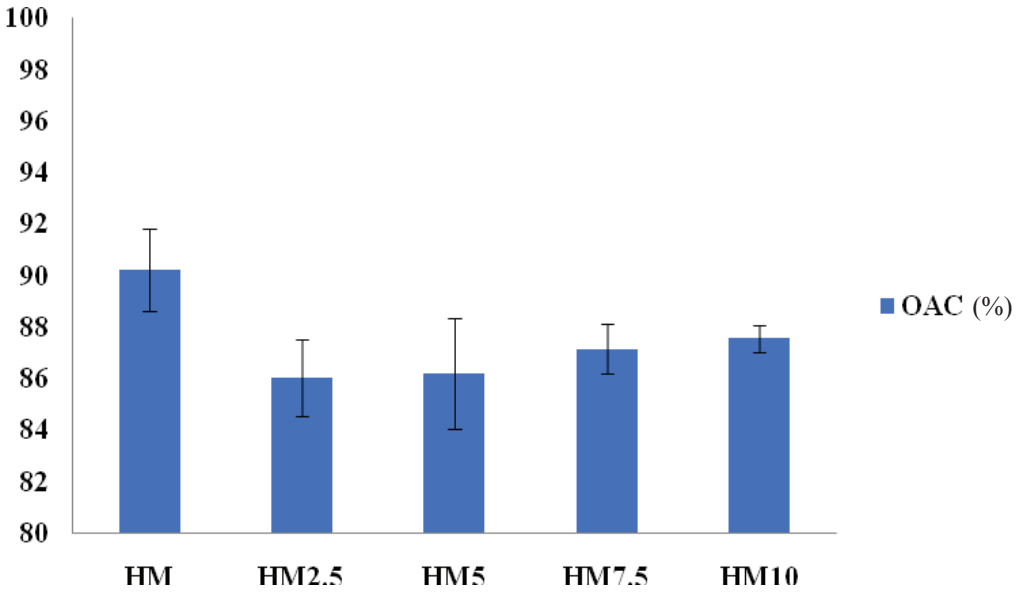


Fig. 9.6 Variations in oil absorption capacity of health mix samples incorporated with different levels of TPH

The antioxidative activities of compounds have been attributed to various mechanisms, such as prevention of chain initiation, binding with transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reducing capacity and radical scavenging ability (Chun-hui et al., 2007). Protein hydrolysates derived from various plant and animal sources are known for its potent antioxidant properties (Qian et al., 2008; Sheriff et al., 2014). The TPH incorporated in the present study also exhibited good antioxidant potential (Table 9.1). In concurrence to this reports, the present study also revealed improved antioxidant properties with increased levels of TPH in the health mix samples. DPPH radical scavenging activity revealed an increase by 4.86 % for HM_{10} from 21.78 ± 0.13 % for HM (Table 9.5; Fig. 9.7). Similarly FRAP also exhibited an increase by 3.45 % from 34.82 ± 0.43 mM AA/g protein in control (Fig. 9.8).

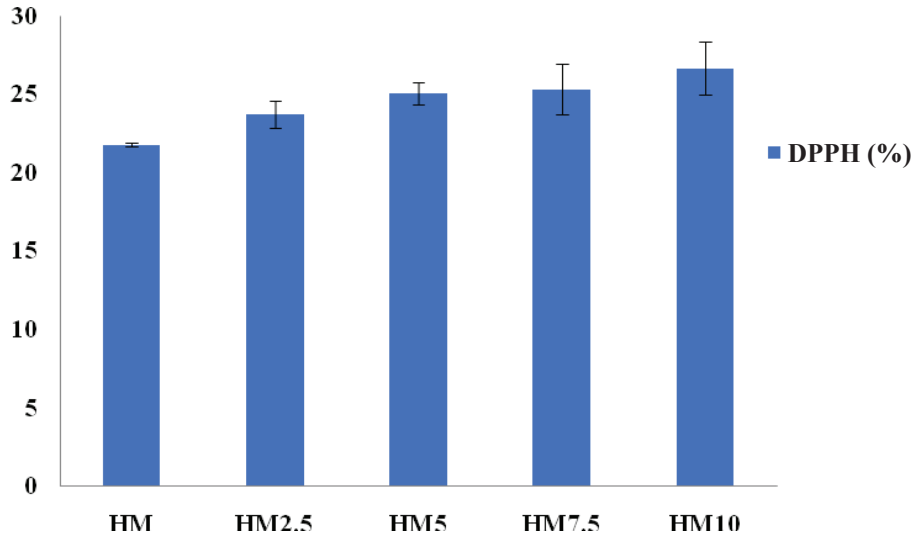


Fig. 9.7 Variations in DPPH radical scavenging activity of health mix samples incorporated with different levels of TPH

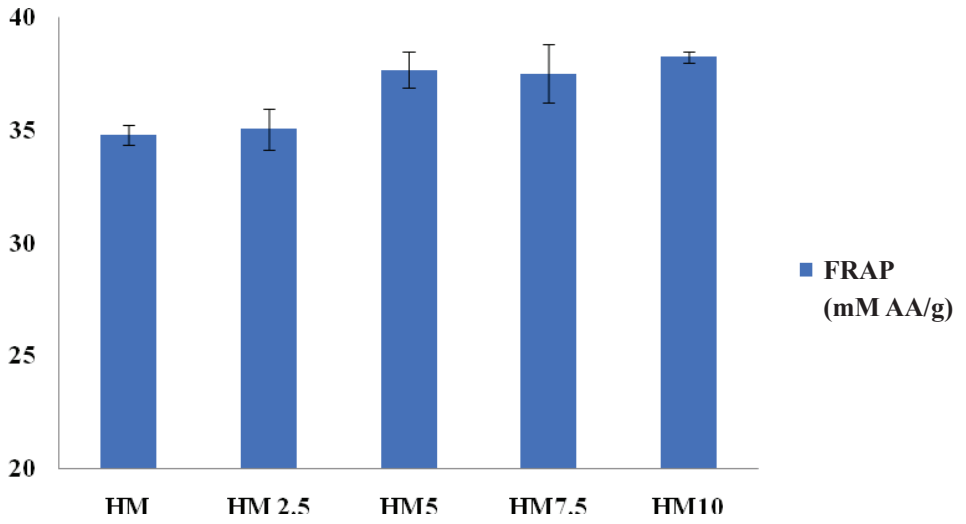


Fig. 9.8 Variations in FRAP of health mix samples incorporated with different levels of TPH

Sensory studies indicated highest acceptability for HM (1.2 ± 0.42) as well as health mix with 2.5 % protein hydrolysate ($HM_{2.5}$) (1.6 ± 0.52) where, as per scoring 1 denoted very good taste and 4 being very bad taste. Acceptability was related to the fish flavor and associated bitterness in the sample which was in turn related to the concentration of protein hydrolysate in the health mix. Bitterness was hardly detectable in control (HM) and $HM_{2.5}$ with a score of 1.1 ± 0.32 and 1.4 ± 0.52, respectively (Table 9.5; Fig. 9.9). However HM_5 experienced slight detection of fish flavor whereas it was prominent in $HM_{7.5}$ and HM_{10} resulting in an abrupt drop in the acceptability to 3.4 ± 0.70 in the latter. This must be on account of the intensification of fish flavor as well as bitterness imparted by protein hydrolysate when incorporated at higher concentrations.

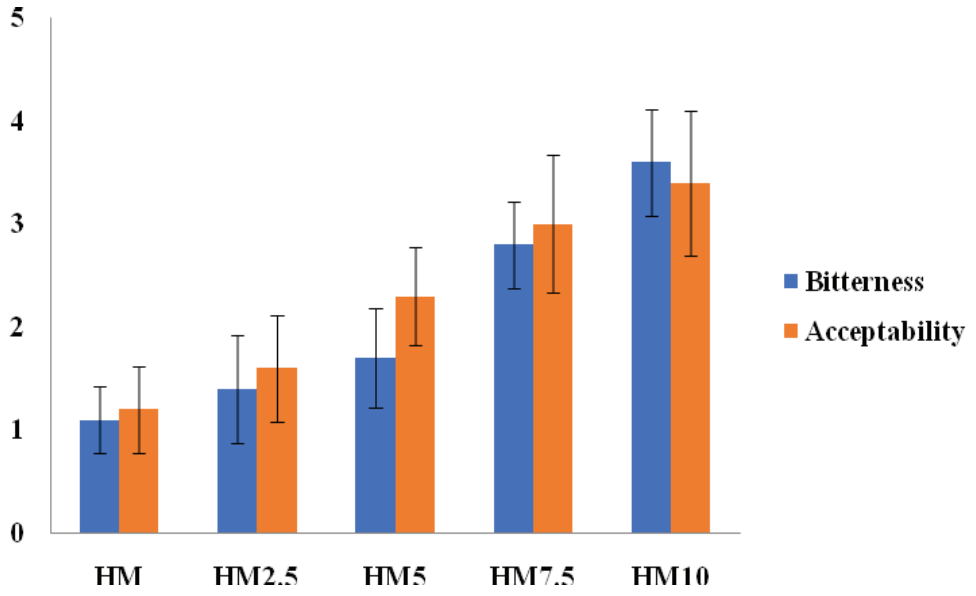


Fig. 9.9 Variations in sensory attributes of health mix samples incorporated with different levels of TPH

The optimized TPH had a bitterness score of 7.4 ± 0.5, indicating high bitterness (Table 9.1). The development of bitterness in hydrolysate is generally associated with the levels of hydrophobic amino acids. There are previous studies reporting the release of bitter tasting peptides during hydrolysis creating acceptability issues during food applications (Yarnpakdee et al., 2015).

Table 9.5 Variations in parameters of health mix samples incorporated with different levels of TPH

Parameters	HM	$HM_{2.5}$	HM_{5}	$HM_{7.5}$	HM_{10}
Lightness	$80.76^{c} \pm 0.07$	$80.91^{c} \pm 0.03$	$81.19^{b} \pm 0.16$	$81.25^{b} \pm 0.1$	$81.41^{a} \pm 0.03$
Redness	$1.90^{a} \pm 0.02$	$1.64^{b} \pm 0.02$	$1.44^{c} \pm 0.05$	$1.29^{d} \pm 0.03$	$1.17^{e} \pm 0.03$
Yellowness	$14.77^{e} \pm 0.15$	$15.77^{d} \pm 0.12$	$16.45^{c} \pm 0.18$	$16.9^{b} \pm 0.1$	$17.38^{a} \pm 0.10$
Foaming Capacity (%)	$50.00^{c} \pm 0.00$	$60.00^{b} \pm 10.00$	$80.00^{a} \pm 0.00$	$80.00^{a} \pm 0$	$83.33^{a} \pm 5.77$
Foam Stability (%)	$10.00^{b} \pm 0.00$	$13.33^{ab} \pm 5.77$	$13.33^{ab} \pm 5.77$	$16.67^{ab} \pm 5.77$	$20.00^{a} \pm 0.00$
Emulsifying Activity Index (m^2/g)	$48.95^{c} \pm 3.65$	$58.16^{b} \pm 1.04$	$65.53^{a} \pm 3.59$	$66.12^{a} \pm 3.67$	$70.81^{a} \pm 2.39$
Emulsion Stability Index (min)	$33.36^{b} \pm 2.09$	$38.13^{ab} \pm 4.62$	$38.51^{ab} \pm 2.38$	$37.65^{ab} \pm 2.26$	$39.67^{a} \pm 2.80$
Oil Absorption Capacity (%)	$90.22^{a} \pm 1.6$	$86.04^{b} \pm 1.51$	$86.19^{b} \pm 2.14$	$87.16^{b} \pm 0.96$	$87.56^{b} \pm 0.54$
DPPH rad. Scav. Act. (%)	$21.78^{c} \pm 0.13$	$23.72^{bc} \pm 0.87$	$25.05^{ab} \pm 0.73$	$25.30^{ab} \pm 1.62$	$26.64^{a} \pm 1.68$
FRAP (mM AA/g)	$34.82^{b} \pm 0.43$	$35.06^{b} \pm 0.92$	$37.69^{a} \pm 0.81$	$37.53^{a} \pm 1.31$	$38.27^{a} \pm 0.24$
Bitterness	$1.10^{d} \pm 0.32$	$1.40^{cd} \pm 0.52$	$1.70^{c} \pm 0.48$	$2.80^{b} \pm 0.42$	$3.60^{a} \pm 0.52$
Acceptability	$1.20^{c} \pm 0.42$	$1.60^{c} \pm 0.52$	$2.30^{b} \pm 0.48$	$3.00^{a} \pm 0.67$	$3.40^{a} \pm 0.70$

Values are expressed as Mean ± SD; n = 3; For bitterness/acceptability: n=10. Different superscript in the same row indicate significant difference ($p < 0.05$)

9.3.3 Characterization of health mix

9.3.3.1 Nutritional profile

The new era of food system has introduced various diets and dietary habits with simultaneous rise in burden on healthcare systems making it decisive to develop novel products, interventions and refined guidelines which will perk up health through diet. Foods that are self sustained to provide adequate nutrients and appropriate calories, is a fundamental requirement for continued health. Nutrient profiling is a synthetic indicator of its overall nutritional quality and aims to classify individual foods based on their nutrient content and their contribution to a healthy diet (Maillot et al., 2008). This gives insights into the composition of the available processed foods in the market facilitating the customers to choose the right product based on their demand (Pivk Kupirovič et al., 2019).

9.3.3.1.1 Fatty acid

Fatty acids play a major role in metabolism both for storage and transport of energy, as vital components of all membranes as well as act as gene regulators. Omega 3 fatty acids such as EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid), well-known for their health benefits (Nettleton, 1995), have emerged as one of the major ingredients in a growing variety of functional foods and beverages. One of the possibilities of enrichment of food products with omega-3 PUFA is the incorporation of marine sources (Kolanowski and Laufenberg, 2006). After enormous daunting trials, recently food industry has succeeded in facilitating this nutrient in getting well into a palatable beverage by optimizing the ingredient formulations. Though fatty acids and lipids contribute only upto the tune of 1-3% of total grain weight, they have a major role in determining the final product. Analysis of fatty acids in the developed health mix samples indicated palmitic and oleic acids to be in good proportion followed by linoleic, stearic and myristic

acids. Similar to the present study reports by Cozzolino et al. (2015), Ozcan et al. (2018), reported palmitic, oleic and linoleic acids to be the major contributors in different malted barley varieties. This concurrence in report must be because the developed beverage had malted grains viz., barley and wheat as major ingredients in the product composition.

Table 9.6 Fatty acid profile of health mix samples

Major Fatty acids	HM	$HM_{2.5}$
Caprylic acid C8:0	1.39	1.68
Capric acid C10:0	1.68	1.81
Lauric acid C12:0	2.50	2.44
Myristic acid C14:0	7.64	9.67
Palmitic acid C16:0	37.12	35.25
Palmitoleic acid C16:1	1.16	1.10
Stearic acid C18:0	9.92	9.72
Oleic acid C18:1	25.79	25.33
Linoleic acid C18:2	11.82	11.92
Arachidic acid C20:0	0.32	0.27
Gondoic acid C20:1	0.13	0.21
Unidentified acids	0.54	0.60

9.3.3.1.2 Amino acid

Proteins, made-up of the fundamental components viz., amino acids have a major role to play in the world of food supplements. These ingredients help novel functional beverages, in maintaining their status as healthy drinks (Gruenwald, 2009). Fortified products should supplement the essential amino acids in a balanced manner to meet the dietary requirements. In the present study, amino acid analysis indicated not much difference between base mix as well as fortified one. However an increase in total essential amino acid content from 40.8 % to 42.18 % was observed on fortification with hydrolysate which was desirable. The essential amino acid profile indicated that leucine was present in the highest amount (Table 9.7). Moreover, the data pertaining to non- essential amino acids indicated glutamic acid to be in highest proportion. The presented health mix samples satisfied the reference EAA value of 40 % (Table 9.7), as recommended by WHO/FAO/UNU (2007) and hence marked to be a suitable dietary nutrient supplement. Similar reports were given by Yasmin et al. (2015) with highest amount of glutamic acid in whey based fructooligosaccharides supplemented low-calorie drink. Presence of branched chain amino acids like isoleucine, leucine and valine promote muscle protein synthesis and helps to increase the bio-availability of high complex carbohydrate intake and are absorbed by muscle cells for anabolic muscle building activity (Jain et al., 2013). The health mix samples had high levels of leucine and moderate levels of isoleucine and valine thus being nutritionally advantageous. Studies by Sinha et al. (2007) on the amino acid profile for whey protein concentrate for beverage formulation also indicated glutamic acid to be the prominent amino acid followed by leucine and aspartic acid.

Table 9.7 Amino acid profile of health mix samples

Amino acid composition	Percentage of total amino acids	
	HM	**$HM_{2.5}$**
Essential Amino acids (EAA)		
Arginine	4.52	4.37
Histidine	3.25	3.67
Isoleucine	3.36	3.62
Leucine	7.95	7.84
Phenyl alanine	4.26	4.36
Threonine	3.72	3.74
Valine	4.41	4.92
Methionine	1.40	1.55
Lysine	4.30	4.93
Tyrosine	3.63	3.18
Total	**40.8**	**42.18**
Non Essential Amino acids (NEAA)		
Alanine	3.77	4.30
Aspartic acid	6.33	6.35
Glycine	3.06	3.45
Glutamic acid	32.19	30.34
Proline	7.59	7.49
Serine	5.51	5.24
Cysteine	0.74	0.66
Total	**59.19**	**57.17**

9.3.3.1.3 Mineral

Micronutrient supplementation from fortified food products has an immense role with regard to the growth and nutrition status of individuals (Solon et al., 2003). The short-term urgency and practical considerations that can counteract micronutrient deficiencies include fortification of commonly consumed foods and supplementation. In the present study, the base health mix samples as well as hydrolysate fortified one was analysed for the minerals. Analysis indicated their richness in minerals like potassium, calcium, phosphorous, sodium and magnesium (Table 9.8). All the heavy metals were either below permissible level or not detected in both the samples. Similar to the present study, Chavan et al. (2015) reported whey based beverage to be rich in minerals like calcium, phosphorous, sodium, magnesium and potassium. The richness in these minerals strengthen the suitability of using the developed health mix for improved nutritional status with involvement in the formation of skeletal structures, constituents of body fluids and tissues; as components of enzyme systems and for proper nerve functioning.

Table 9.8 Mineral profile of health mix samples

Element (ppm)	HM	$HM_{2.5}$
Aluminium	7.38±2.61	7.05±2.54
Boron	1.27±0.46	0.69±0.04
Copper	2.94±1.09	3.65±1.84
Iron	12.05±2.20	14.88±2.40
Zinc	17.25±1.35	18.34±0.67
Magnesium	619.69±4.17	696.10±3.04
Potassium	5650.00±0.97	7102.00±1.07
Calcium	2324.00±1.62	2702.00±1.53
Manganese	7.58±4.34	8.50±3.20
Selenium	0.71±0.00	1.00±0.00
Phosphorous	2220.00±1.13	2635.00±0.763

Barium	2.12±0.75	1.84±0.55
Sodium	907.5±1.99	1198.00±2.18
Nickel	BDL	0.73±0.00
Lead	BDL	BDL
Arsenic	BDL	BDL
Chromium	BDL	BDL
Cadmium	BDL	BDL
Mercury	BDL	BDL
Cobalt	BDL	BDL
Tin	BDL	BDL

9.3.3.2 Physical properties

9.3.3.2.1 Particle density

The particle density or true density, is the density of the particles that make up the powder, in contrast to the bulk density, which measures the average density of a large volume of the powder in a specific medium. The variation in particle size is responsible for the changes in physical properties of the powder. Particle density of HM and $HM_{2.5}$ were 0.716 ± 0.003 and 0.78 ± 0.02, respectively (Fig. 9.10) indicating significant difference ($p < 0.05$) on incorporating hydrolysate in the sample.

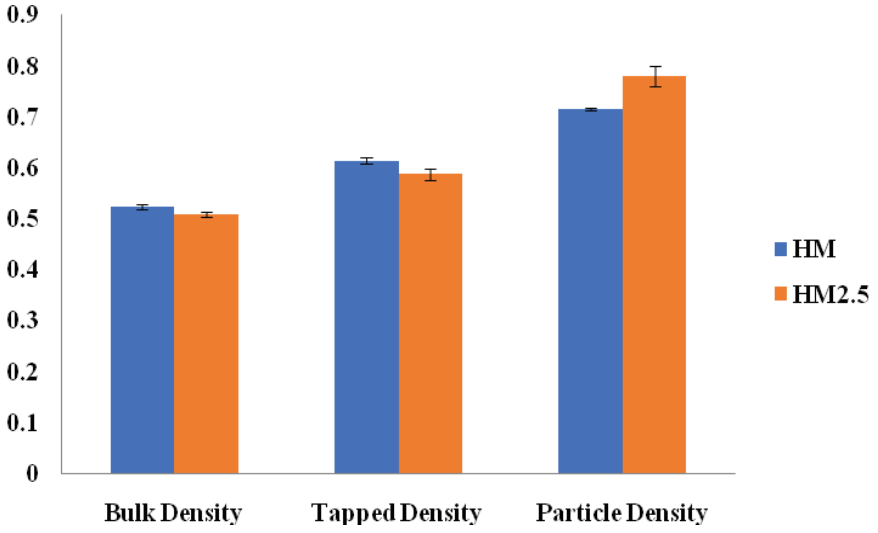


Fig. 9.10 Bulk, tapped and particle densities of health mix samples

9.3.3.2.2 Bulk, tapped densities and porosity

Powder density is an important factor that is required for standardization of process parameters for packaging, distribution, processing, and storage of food. The bulk density, defined as the mass of the solid particles including moisture to the total volume occupied by the particles, surface moisture, and all the pores, closed or open to the surrounding atmosphere, is generally used to characterize the final powder (Johanson, 2005; Kurozawa et al., 2009). It reflects the size, shape and arrangement of particles and voids. The bulk density of a powder is always smaller than its particle density. Bulk density of HM and $HM_{2.5}$ were 0.524 ± 0.005 and 0.509 ± 0.005, respectively (Fig. 9.10). Similarly the tapped densities were 0.614 ± 0.006 and 0.588 ± 0.011, respectively (Fig. 9.10). Porosity of HM and $HM_{2.5}$ were 16.08 ± 3.01 and 25.49 ± 2.38, respectively (Fig. 9.11). The bulk and tapped densities of HM were significantly higher ($p < 0.05$), whereas their inter-granular porosities were significantly lower than those of $HM_{2.5}$. Results suggest $BM_{2.5}$ to be of finer texture due to its comparatively lower bulk density than control. The bulk density varies indirectly with the total pore space of powder and gives a good estimate of the porosity of the product.

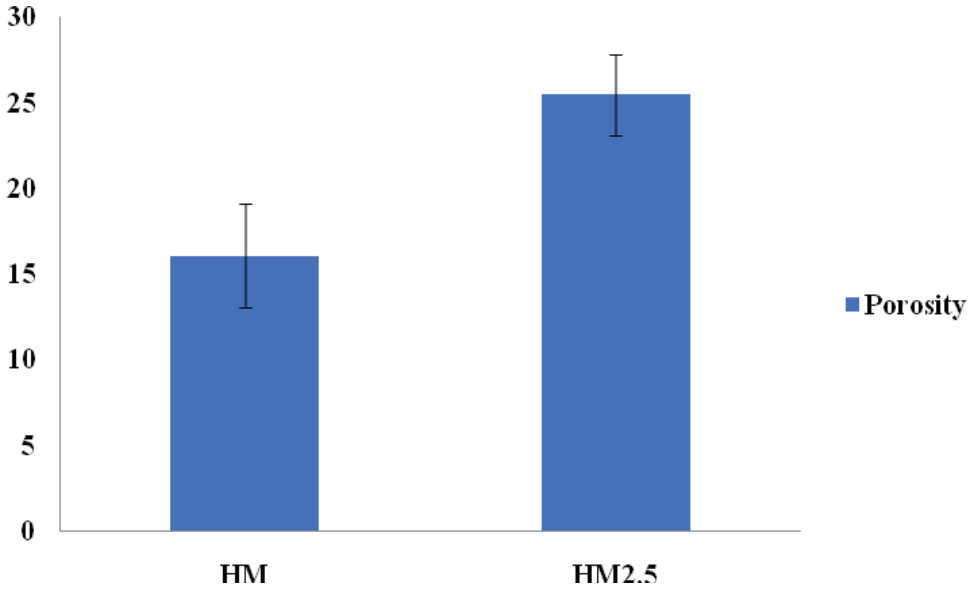


Fig. 9.11 Porosity of health mix samples

9.3.3.2.3 Flowability and cohesiveness

Hausner ratio of HM and $HM_{2.5}$ were 1.17 ± 0.01 and 1.15 ± 0.01, respectively. Similarly the carr index values indicated 14.66 ± 0.27 and 13.42 ± 0.96, respectively (Fig. 9.12). Evaluation of the handling properties of the powders indicated similar flow characteristics without any significant difference between the samples and were classified as free flowing with good flow properties by their Hausner ratio (HR) as classified in table 9.3. This was in accordance with their low Carr index (CI) which indicated their good flowability (Table 9.3).

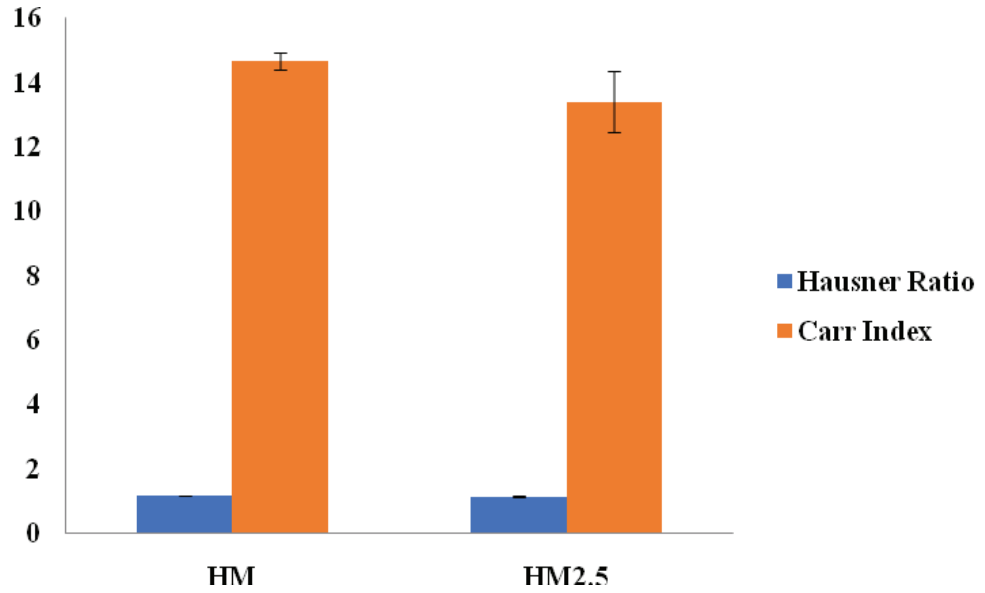


Fig. 9.12 Flowability and cohesiveness of health mix samples

9.3.3.2.4 Wettability and dispersibility

Powder rehydration is important as a critical benchmark of quality for consumption (Selomulya and Fang, 2013). Wettability is an important reconstitution property as it is the preliminary step involved in dispersion or dissolution. The wetting behavior of powders is important to understand especially in pharmaceutical and food industries, where the use of different powder compounds is common. The wetting of powders and pigments involves contact angle phenomena, wherein

contact angles indicate the degree of wetting when a solid and liquid interact. The lower the contact angle, the greater the wetting. Wettability of HM and $HM_{2.5}$ were 4.13 ± 0.03 sec and 2.81 ± 0.13 sec, respectively (Fig. 9.13). The significantly higher wettability or lower wetting time ($p < 0.05$) observed in hydrolysate incorporated sample viz., $HM_{2.5}$ must be on account of the better water absorption capacity possessed by protein hydrolysate, as reported previously (Haldar et al., 2018). As a result an increased rate of water penetration into the fine pores within the powder and consequently shortened wetting time was observed.

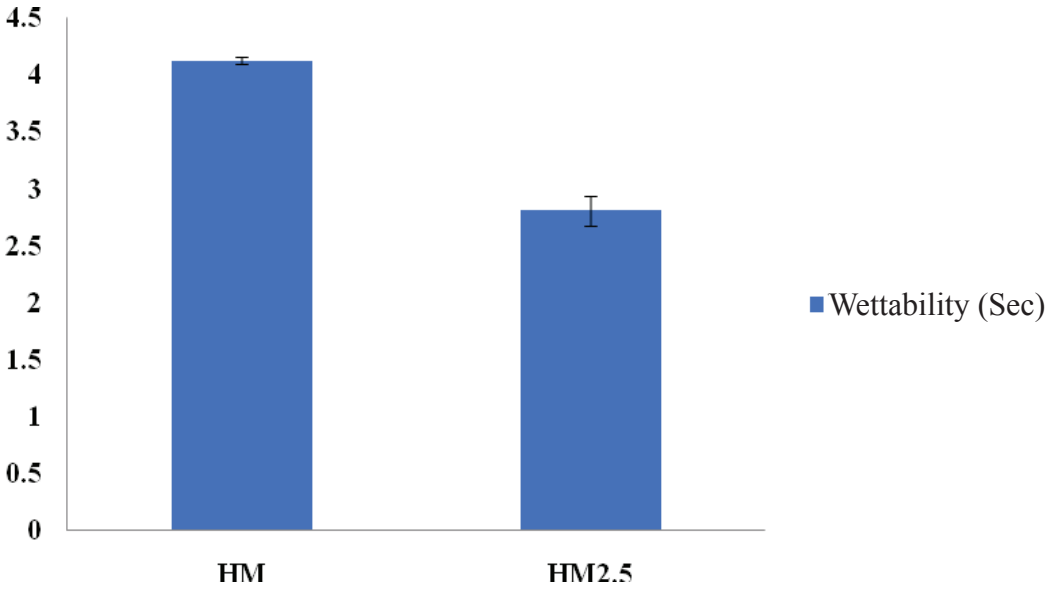


Fig. 9.13 Wettability of health mix samples

Water-protein interaction is one of the key criteria responsible for the functional properties of protein in food system (Haldar et al., 2018). Dispersibility of HM and $HM_{2.5}$ were 60.09 ± 1.58 % and 68.02 ± 1.46 %, respectively (Fig. 9.14). This significantly higher wettability and dispersibility ($p < 0.05$) can be linked to enhanced porosity of fortified sample viz., $HM_{2.5}$ thus being more advantageous for instant incorporation in drinks. Granulation of food powders produces porous granules with higher wettability and dispersibility (Jafari et al., 2015).

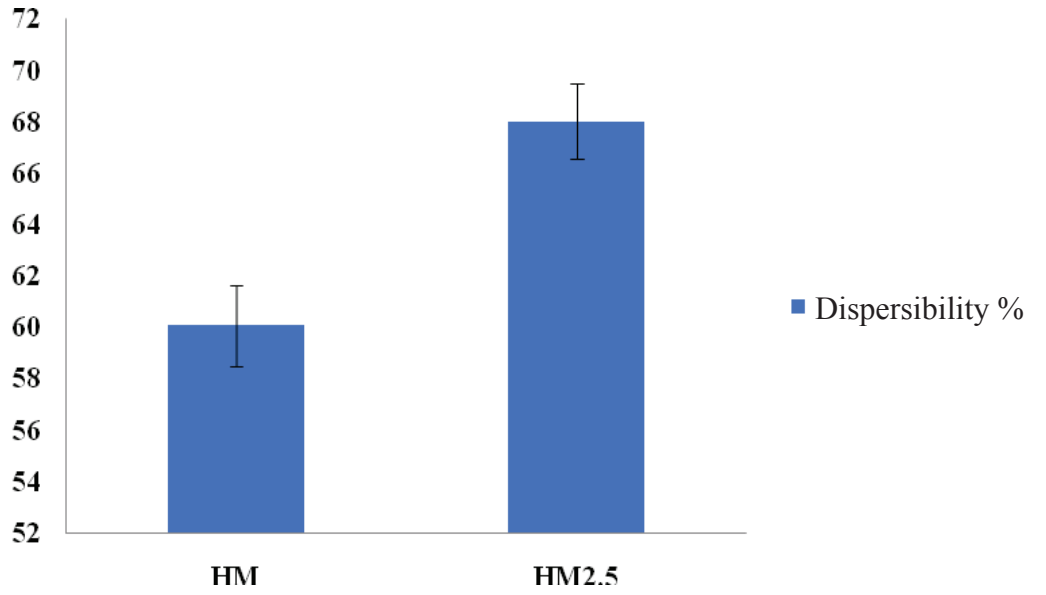


Fig. 9.14 Dispersibility of health mix samples

9.3.4 In vitro digestibility and stability

In vitro digestibility tests offer a rapid and a simple way to mimic *in vivo* conditions. It gives us information about the stability of food and how they could survive the digestion process. In antioxidant assay *in vitro*, the intrinsic properties of the organism and the extraction process itself are major influential factors. In the present *in vitro* digestibility study, results indicated that the samples underwent some degree of degradation in the antioxidant properties. This decrease was less prominent during the initial stages viz., gastric conditions while a more pronounced decrease was observed later in the intestinal conditions (Fig. 9.15) However, evaluation of the efficacy of these samples in animal model and human clinical studies is needed to fully substantiate their role. The gastrointestinal tract is in contact with food digests and therefore, with an important quantity (and variety) of food derived peptides and hence the influence of peptides on different intestinal functions and health are gaining an increasing interest (Moughan et al., 2007; Shimizu and Hachimura, 2011).

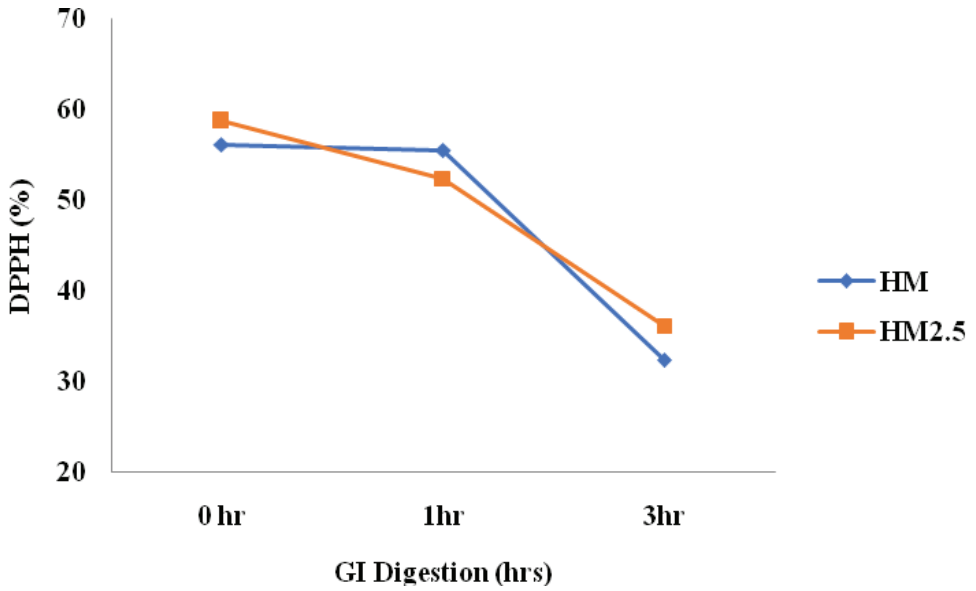


Fig. 9.15 In vitro digestibility and stability of health mix samples

9.3.5 Storage stability studies

9.3.5.1 Moisture

Analysis of moisture content is a critical aspect of material quality as it greatly influences the physical properties and product quality of all food products during processing and subsequent storage. Most food powders have low moisture content which assist in reducing the rate of quality degradation. This facilitates food powders to be stored for a longer time than other forms of food products (Intipunya and Bhandari, 2010). Comparison between the initial moisture content of the samples indicated lower moisture content by $HM_{2.5}$ in comparison to control on account of compositional variation (discussed in section 9.3.2). During storage, moisture content remained nearly constant in both the samples (Fig. 9.16; Table 9.9). However compared to HM, $HM_{2.5}$ indicated a slight tendency of increase in the moisture content which must be on account of the hygroscopic nature of protein hydrolysate incorporated in the sample. Tonon et al. (2008) reported the moisture uptake to be dependent on the permeability of the packaging material used and its

interaction with temperature due to the variation of the relative humidity of the air surrounding the packaging together with the hygroscopicity offered by the product.

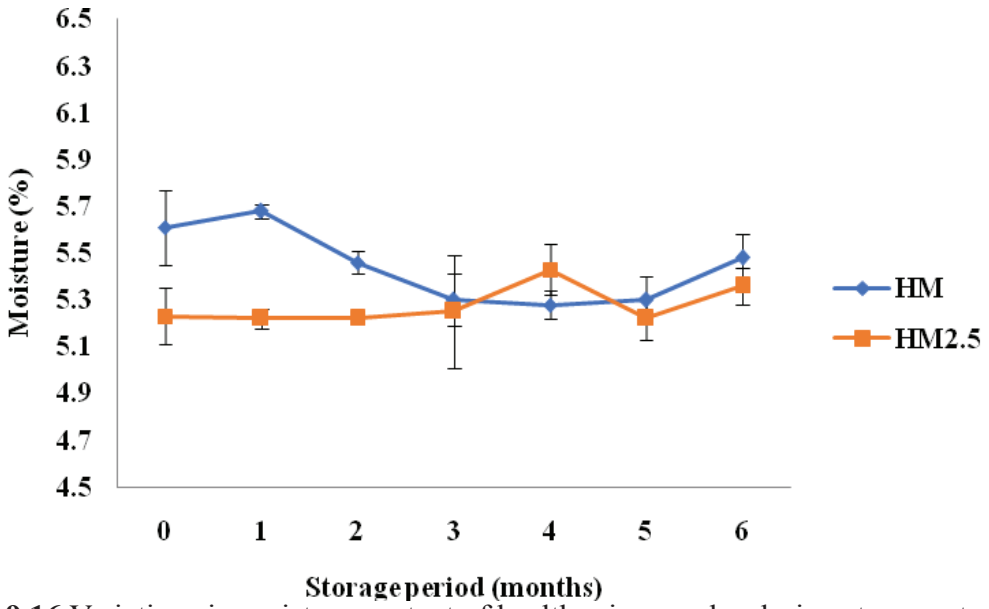


Fig. 9.16 Variations in moisture content of health mix samples during storage at ambient temperature

9.3.5.2 pH

pH is an important physical food characteristic which is related to the level of acidity/alkalinity due to the release or absorption of hydrogen ions. Generally in food, fermentation or microbial activity might cause variations in pH. In the present study, pH of the samples didn't indicate any marked variations during the storage period of six months under ambient conditions which indicated absence of any significant bacterial activities in the sample. It ranged from 6.14 - 6.2 in control (HM) and 6.09 - 6.15 in $HM_{2.5}$ (Fig. 9.17; Table 9.9). Studies conducted by Ahn et al. (2017) indicated the changes in pH of the coffee beverage stored at 4°C for 12 days to be almost consistent during storage.

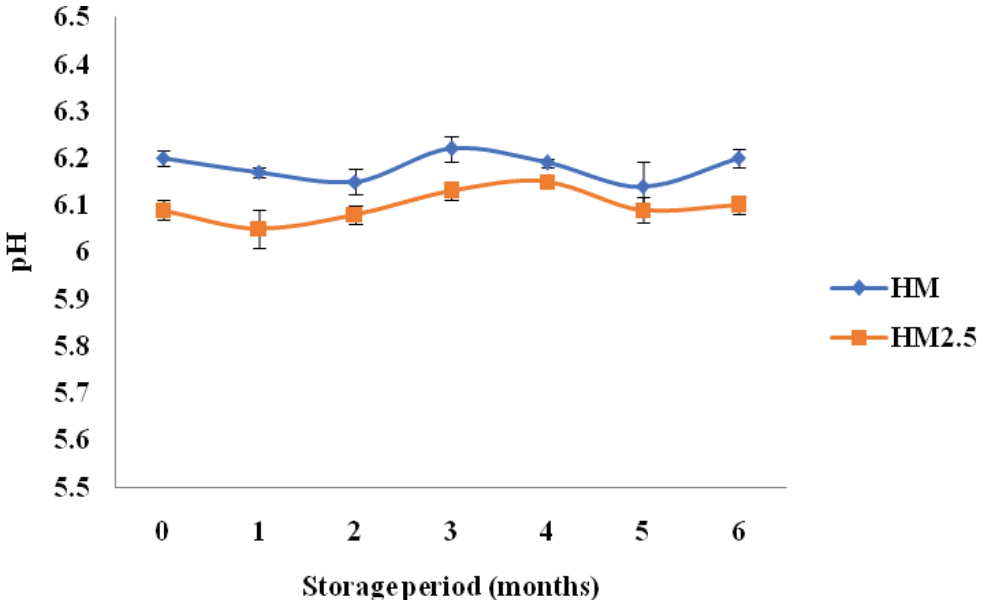


Fig. 9.17 Variations in pH of health mix samples during storage at ambient temperature

9.3.5.3 PV and FFA

Quality degradation in food powders can occur even without variations in the physical appearance depending on their chemical composition and physical states. These degradations mostly involve either chemical or physical deteriorations or both and may be related (Intipunya and Bhandari, 2010). Of the quality problems, lipid oxidation is a major one as it produces compounds that alter textural properties, and adversely affect the color and nutrition of a food product. Eventually, lipid oxidation reduces shelf life and therefore causes food spoilage, an important factor in food security as understood to be the availability to, and accessibility of, high-quality food. The shelf life of a product that is susceptible to lipid oxidation is determined when a consumer can detect lipid oxidation volatiles that impact flavor. Since some lipid oxidation products have very low sensory threshold values, sensory perception of rancidity can sometimes occur prior to being able to chemically detect oxidation products. However, once lipid oxidation products are detected (the end of the lag phase) then it is highly likely that the sensory properties of the product are compromised (Barden, 2014).

Peroxides are the primary products of lipid oxidation and play a central role in auto oxidation of lipids and decompose them into carbonyls and other compounds. PV of the samples indicated an increasing trend ($p < 0.05$) in both cases during storage. Variations in PV were more prominent in $HM_{2.5}$ compared to control (Fig. 9.18; Table 9.9). However, the peroxide values observed in this study were still below the critical limit of peroxide value which is 10-20 meq O_2/kg sample. Reports have indicated fish protein hydrolysates to be prone to oxidation on account of high content of unsaturated fatty acids (Sohn et al., 2005; Yarnpakdee et al., 2012c). In the current study, the fat content was relatively higher ($p < 0.05$) in $HM_{2.5}$ (1.36 ± 0.05 %) in comparison to control (1.23 ± 0.01 %) (Table 9.4) which must have undergone oxidation indicating higher PV in $HM_{2.5}$.

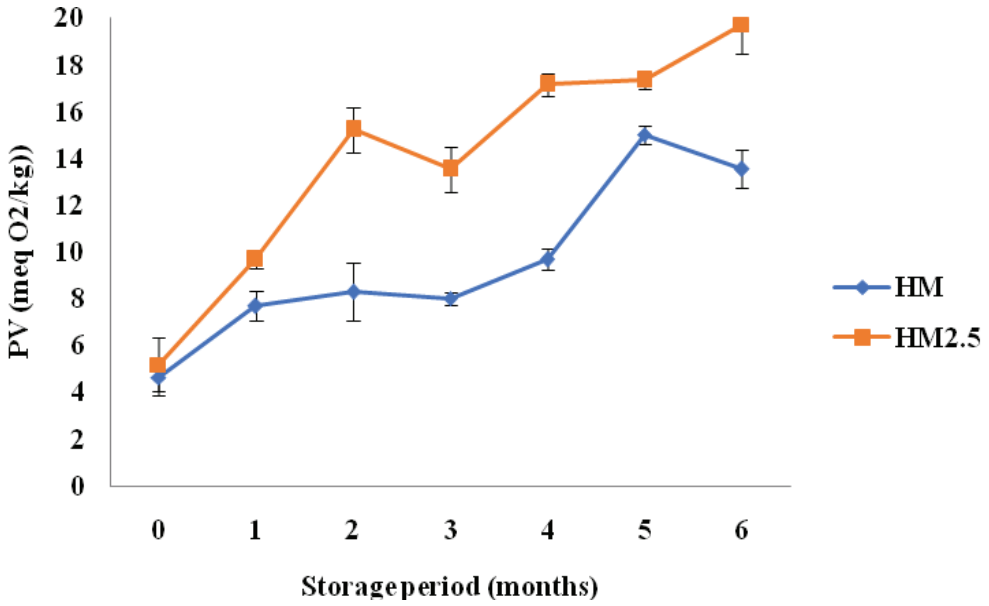


Fig. 9.18 Variations in peroxide value of health mix samples during storage at ambient temperature

Besides oxidation, lipids may undergo hydrolysis resulting in the formation of FFA during storage. Accumulation of FFA is responsible for the textural changes, enhanced oxidation of lipids, and development of off flavors in the food (Sequeira-Munoz et al., 2006). FFA values also indicated an increasing trend with an initial

value of 10.55 ± 0.37 % to 15.25 ± 0.06 % towards six months of storage for HM while it was slightly higher and varied significantly ($p < 0.05$) from an initial value of 9.93 ± 0.28 % to 18.03 ± 1.44 % for $HM_{2.5}$ (Fig. 9.19; Table 9.9).

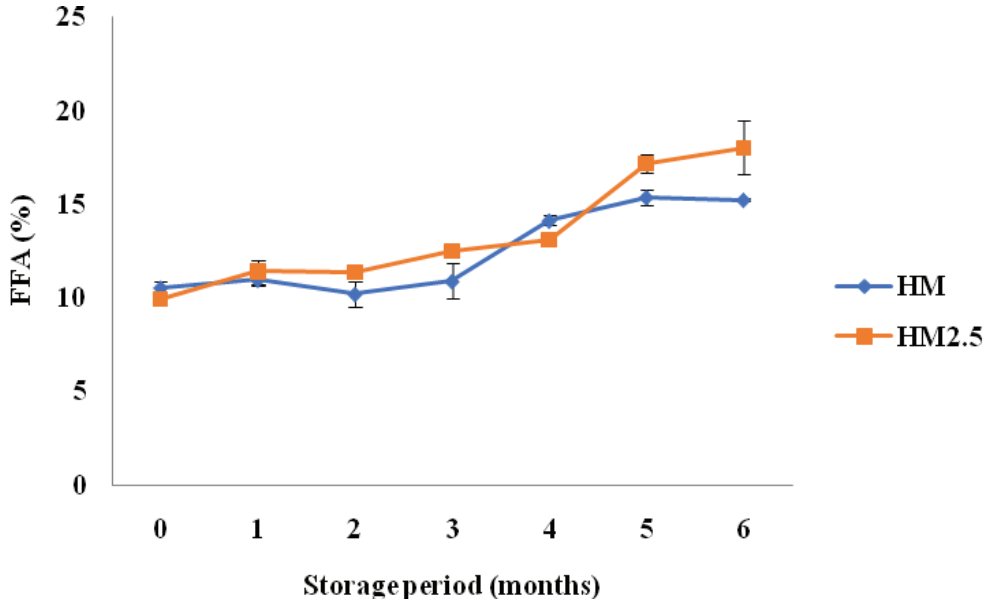


Fig. 9.19 Variations in free fatty acid of health mix samples during storage at ambient temperature

9.3.5.4 TMA-N and TVBN

Total volatile bases in a product is often used as an indicator of its quality and helps to assess the deteriorative changes during storage. TMA-N and TVBN of the samples indicated an increasing trend in both cases during storage. The pattern of increase in TMA-N was similar in both samples during storage reaching 7 mg% towards the end of storage period (Table 9.9; Fig. 9.20).

Variations in TVBN also followed a similar pattern with an initial value of 5.83 ± 2.02 in HM and 2.33 ± 2.02 in $HM_{2.5}$, approaching a value of 17.5 mg% towards the final storage period (Fig. 9.21; Table 9.9). The increase in volatile bases (e.g., ammonia and trimethylamine) during storage can be related to the activity of spoilage bacteria and the present study indicated not much significant variations in the indices indicating lower chemical variations on account of bacterial activity.

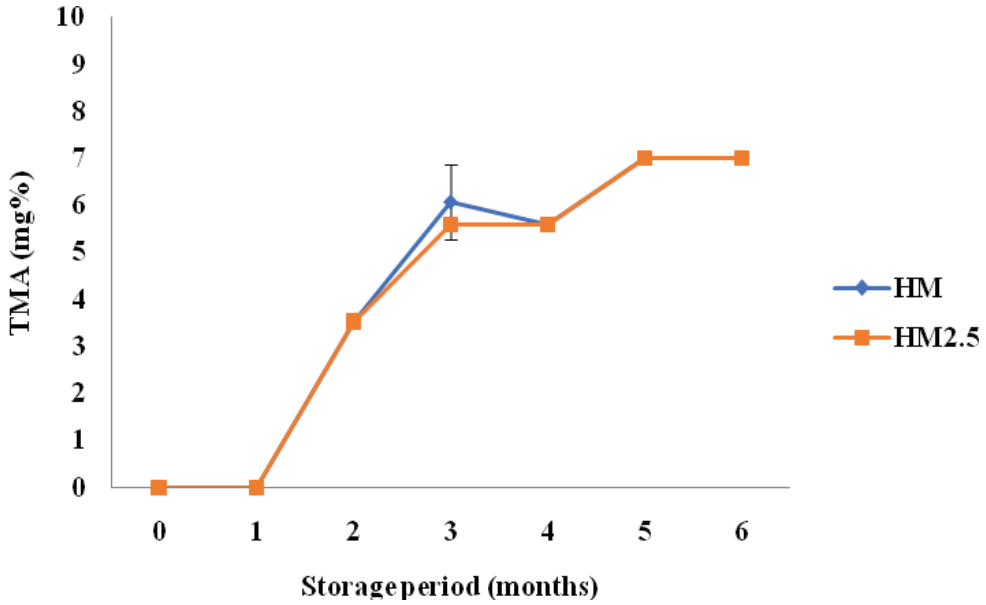


Fig. 9.20 Variations in TMA-N of health mix samples during storage at ambient temperature

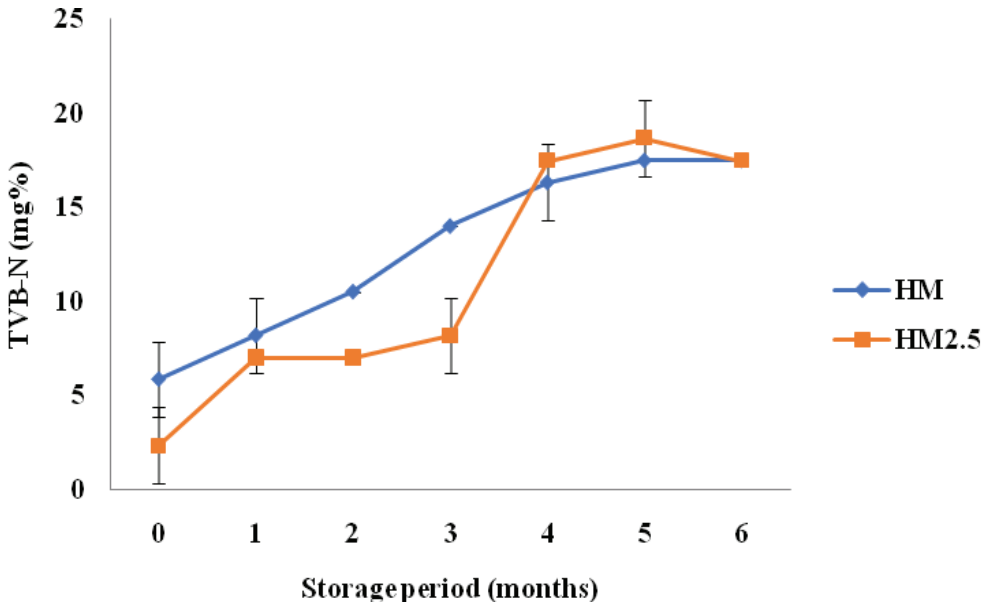


Fig. 9.21 Variations in TVBN of health mix samples during storage at ambient temperature

9.3.5.5 Sensory analysis

Effectiveness in recognising the complexities marks sensory analysis as the ultimate quality evaluation technique for determining the shelf stability of a product. Sensory indices indicated a slight reduction in the overall acceptability during the storage (Fig. 9.22; Table 9.9). The initial acceptability score of HM was 8.7 ± 0.50 which got reduced to 8.3 ± 0.50. Similarly the acceptability of 8.4 ± 0.50 was decreased to 7.8 ± 0.40 upon storage for six months under ambient conditions in $HM_{2.5}$ which must be on account of slight intensification of hydrolysate flavor in the sample. However during the storage period, the degree of acceptability was favourable for both the samples.

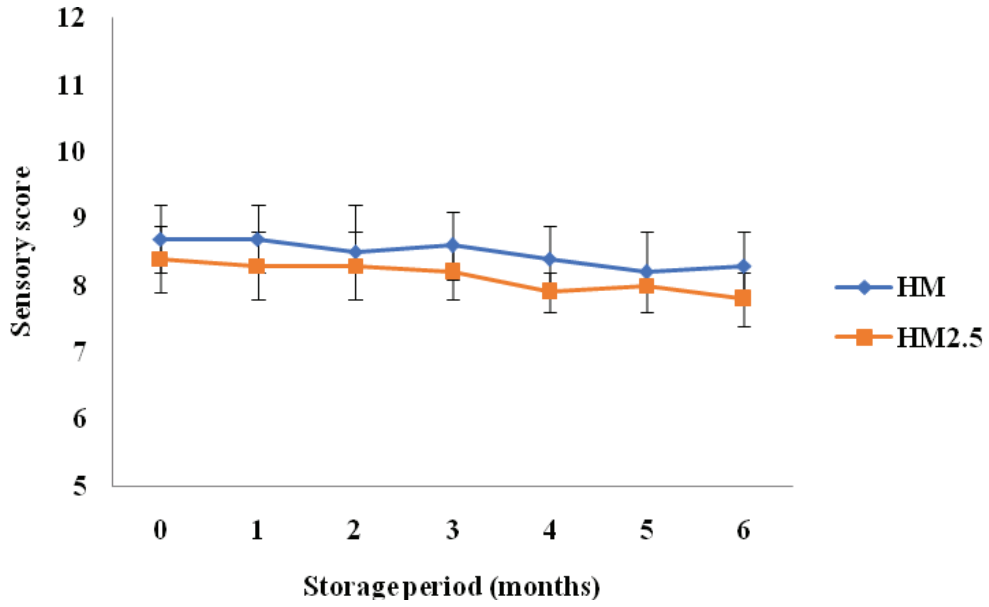


Fig. 9.22 Variations in sensory attributes of health mix samples during storage at ambient temperature

9.3.5.6 Microbiological analysis

Storage stability of food products is a measure of how long they retain optimal quality after production, of which microbial quality is a major aspect. The variations in TPC indicated an increasing trend during storage ($p < 0.05$) for both samples, being more prominent in HM in comparison to $HM_{2.5}$. It varied from an initial value of 4.98 to 5.41 log cfu/g in control and 4.91 to 5.21 log cfu/g in $BM_{2.5}$ (Fig. 9.23; Table 9.9). The findings further strengthen the possible antimicrobial property of TPH which is in concurrence with previous observations reported for hydrolysates from various protein sources (Da Rocha et al., 2018). Nevertheless, both sample lots remained within the microbial acceptable limit of 7 log cycle (ICMSF, 1998) during storage.

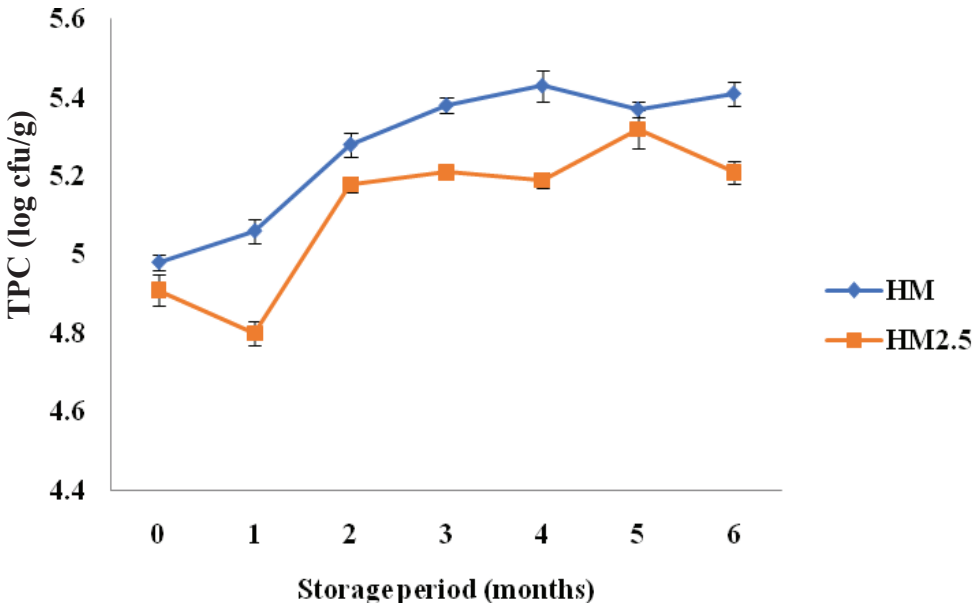


Fig. 9.23 Variations in microbiological attributes of health mix samples during storage at ambient temperature

Table 9.9 Variations in different attributes of health mix samples during storage at ambient temperature (28°C)

Parameters	Storage period (months)	HM	$HM_{2.5}$
Moisture (%)	0	$5.61^{abA} \pm 0.16$	$5.23^{aB} \pm 0.12$
	1	$5.68^{aA} \pm 0.03$	$5.22^{aB} \pm 0.04$
	2	$5.46^{bcA} \pm 0.05$	$5.22^{aB} \pm 0.01$
	3	$5.30^{cA} \pm 0.11$	$5.25^{aA} \pm 0.24$
	4	$5.28^{cA} \pm 0.06$	$5.43^{aA} \pm 0.11$
	5	$5.30^{cA} \pm 0.10$	$5.22^{aA} \pm 0.09$
	6	$5.48^{bA} \pm 0.10$	$5.36^{aA} \pm 0.08$
pH	0	$6.20^{abA} \pm 0.02$	$6.09^{bcdB} \pm 0.02$
	1	$6.17^{bcdA} \pm 0.01$	$6.05^{dB} \pm 0.04$
	2	$6.15^{cdA} \pm 0.03$	$6.08^{cdB} \pm 0.02$
	3	$6.22^{aA} \pm 0.03$	$6.13^{bB} \pm 0.02$
	4	$6.19^{abcA} \pm 0.01$	$6.15^{aA} \pm 0.01$
	5	$6.14^{dA} \pm 0.05$	$6.09^{bcdB} \pm 0.03$
	6	$6.20^{abcA} \pm 0.02$	$6.1^{bcB} \pm 0.02$
PV (Meq O_2/kg)	0	$4.65^{eB} \pm 0.80$	$5.2^{fA} \pm 1.17$
	1	$7.70^{dB} \pm 0.63$	$9.69^{eA} \pm 0.39$
	2	$8.29^{cdB} \pm 1.24$	$15.23^{cA} \pm 0.94$
	3	$8.01^{dB} \pm 0.28$	$13.55^{dA} \pm 0.96$
	4	$9.69^{cB} \pm 0.46$	$17.16^{bA} \pm 0.51$
	5	$15.03^{aB} \pm 0.40$	$17.35^{bA} \pm 0.38$
	6	$13.55^{bB} \pm 0.81$	$19.72^{aA} \pm 1.26$
FFA (%)	0	$10.55^{cA} \pm 0.37$	$9.93^{dA} \pm 0.28$
	1	$10.98^{cA} \pm 0.31$	$11.41^{cA} \pm 0.64$
	2	$10.20^{cB} \pm 0.71$	$11.38^{cA} \pm 0.26$
	3	$10.90^{cB} \pm 0.95$	$12.49^{bA} \pm 0.36$
	4	$14.14^{bA} \pm 0.27$	$13.1^{bB} \pm 0.19$
	5	$15.39^{aB} \pm 0.41$	$17.21^{aA} \pm 0.48$
	6	$15.25^{aB} \pm 0.06$	$18.03^{aA} \pm 1.44$

TMA-N (mg%)	0	$0.00^{eA} \pm 0.00$	$0.00^{bA} \pm 0.00$
	1	$0.00^{eA} \pm 0.00$	$0.00^{bA} \pm 0.00$
	2	$3.50^{dA} \pm 0.00$	$3.50^{bA} \pm 0.00$
	3	$6.07^{bA} \pm 0.81$	$5.60^{bB} \pm 0.00$
	4	$5.60^{cA} \pm 0.00$	$5.60^{aA} \pm 0.00$
	5	$7.00^{aA} \pm 0.00$	$7.00^{aA} \pm 0.00$
	6	$7.00^{aA} \pm 0.00$	$7.00^{aA} \pm 0.00$
TVBN (mg%)	0	$5.83^{dA} \pm 2.02$	$2.33^{cB} \pm 2.02$
	1	$8.17^{dA} \pm 2.02$	$7.00^{bA} \pm 0.00$
	2	$10.50^{cA} \pm 0.00$	$7.00^{bB} \pm 0.00$
	3	$14.00^{bA} \pm 0.00$	$8.17^{bB} \pm 2.02$
	4	$16.33^{aA} \pm 2.02$	$17.50^{aA} \pm 0.00$
	5	$17.50^{aA} \pm 0.00$	$18.67^{aA} \pm 2.02$
	6	$17.50^{aA} \pm 0.00$	$17.50^{aA} \pm 0.00$
Sensory	0	$8.70^{aA} \pm 0.50$	$8.40^{aA} \pm 0.50$
	1	$8.70^{aA} \pm 0.50$	$8.30^{abA} \pm 0.50$
	2	$8.50^{aA} \pm 0.70$	$8.30^{abA} \pm 0.50$
	3	$8.60^{aA} \pm 0.50$	$8.20^{abA} \pm 0.40$
	4	$8.40^{aA} \pm 0.50$	$7.90^{abA} \pm 0.30$
	5	$8.20^{aA} \pm 0.60$	$8.00^{abA} \pm 0.00$
	6	$8.30^{aA} \pm 0.50$	$7.80^{bA} \pm 0.40$
TPC (log cfu/g)	0	$4.98^{eB} \pm 0.02$	$4.91^{cA} \pm 0.04$
	1	$5.06^{dA} \pm 0.03$	$4.80^{dB} \pm 0.03$
	2	$5.28^{cA} \pm 0.03$	$5.18^{bB} \pm 0.02$
	3	$5.38^{bA} \pm 0.02$	$5.21^{bB} \pm 0.01$
	4	$5.43^{aA} \pm 0.04$	$5.19^{bB} \pm 0.02$
	5	$5.37^{bA} \pm 0.02$	$5.32^{aB} \pm 0.05$
	6	$5.41^{abA} \pm 0.03$	$5.21^{bB} \pm 0.03$

9.4 Conclusion

Utilization of 2.5% protein hydrolysate from yellowfin tuna red meat for formulation of a health beverage mix containing malted barley and wheat was attempted. Incorporation of tuna protein hydrolysate improved the nutritional status of the health mix formulation. Further on account of its bioactive properties, the health mix functionalities also improved upon TPH addition. Shelf stability analysis of health mix samples under ambient temperature (28^{o}C) indicated good stability throughout the study period of six months. Present study explored the potentiality of utilizing protein hydrolysates in beverage formulation for improved quality and better storage stability.

Chapter 10
Summary

- A study was carried out with the aim of standardization of enzymatic hydrolytic conditions to obtain protein hydrolysate from yellowfin tuna (*Thunnus albacares*) red meat with specific functional and bioactive properties for their potential applications, characterization, storage stability analysis and their performance evaluation in the incorporated food formulations.
- Initially a comparative evaluation of the peptides from white and red meat of yellowfin tuna (*Thunnus albacares*) was carried out to explore the extent to which the properties vary in red meat derived hydrolysate in comparison to its white meat.
- Protein hydrolysate was prepared employing hydrolytic conditions viz., 1% (w/w) papain for one hour at optimized temperature of 60^0C and pH of 6.5, from white and red meat of tuna to derive tuna white meat protein hydrolysate (TWPH) and tuna red meat protein hydrolysate (TRPH), respectively.
- Assessment of the peptide properties indicated better antioxidative activity for TWPH. However, except oil absorption capacity (OAC), functional properties viz., protein solubility, foaming capacity and emulsifying properties were higher for TRPH indicating the application potential of tuna red meat hydrolysate in food and pharmaceutical sector.

- Process optimisation studies for the selective extraction of functional and antioxidant hydrolysates from cooked tuna red meat (*Thunnus albacares*) using RSM with a central composite design, with emphasis on protein recovery was carried out.
- For the study, the major hydrolytic variables viz., enzyme-substrate (E/S) ratio (0.25-1.5 %) and hydrolysis time (30-240 min) were considered at a pre-optimized temperature of 60^{o}C and pH of 6.5.
- The optimum hydrolytic conditions for superior functional properties were achieved at an E/S ratio of 0.34 % for hydrolysis duration of 30 minutes while the optimum conditions to exhibit the maximum antioxidative properties were: 0.98 % E/S and 240 minutes of hydrolysis time.
- Enzyme-substrate ratio was more influential in explaining the response variations than hydrolysis time. A few properties of hydrolysate having the same degree of hydrolysis varied significantly and hence, could not be entirely explained based on the degree of hydrolysis.
- The optimized hydrolysates were assessed for its nutritional profile, molecular weight, surface morphology, thermal characteristics, physico-chemical properties as well as functional/antioxidant properties.
- Storage stability studies of the spray dried hydrolysates at ambient (28^{o}C) and chilled storage temperature (4^{o}C) for up to six months indicated an uptake of moisture, increase in oxidative indices as well as changes in functionality during storage which was more prominent under ambient temperature.
- Efforts were made in the investigation to develop and upscale the laboratory outcomes to facilitate industrial production of tuna red meat protein hydrolysate with specific properties for their potential applications in food system.

- The economic feasibility study indicated profitability of producing TPH on an industrial production. The net profit ratio was calculated to be 23.72 % with a rate of return of 29.35 %. The breakeven point was assessed to be 50.68 % and an investment payback time of 1.6 years was observed.
- Similar to hydrolysis optimization carried out for cooked tuna red meat protein, studies were also conducted for separate extraction of functional and antioxidant hydrolysates from raw yellowfin tuna red meat under similar conditions.
- Comparative studies done on cooked and raw tuna red meat indicated protein recovery during hydrolysis to be higher from raw tuna red meat than from cooked meat. Hydrolysate from cooked tuna red meat exhibited superior functional properties except OAC, whereas except ABTS radical scavenging activity, hydrolysates from raw tuna red meat exhibited dominance with regard to antioxidative activities.
- Application potentials of derived hydrolysates was explored by attempts in microencapsulation of fish oil. Studies were carried out to compare the efficacy of yellowfin tuna red meat hydrolysate (optimized for antioxidative properties) in protecting the core sardine oil, when used as wall and core polymer during encapsulation. Observations suggested the advocation of protein hydrolysate as core material along with sardine oil for obtaining shelf stable spray dried oil encapsulates.
- Fortification and stabilization of mayonnaise by incorporating protein hydrolysate as a partial replacer of egg yolk in the product was done. Tuna hydrolysates optimized for superior functional properties was used in mayonnaise formulation.
- The storage stability parameters of the mayonnaise samples under chilled conditions (4^{o}C) indicated better oxidative and physicochemical stability

for fortified samples compared to control.

- Utilization of protein hydrolysate from yellowfin tuna red meat for formulation of a health beverage mix was carried out. Tuna protein hydrolysate (TPH), optimized for functional and antioxidative properties using papain under a hydrolytic condition viz., E/S of 1.08 %, 30 minutes hydrolysis time, temperature and pH of 60^{o}C and 6.5, respectively was used.
- Incorporation of TPH in the health mix improved nutritional, functional as well as antioxidative properties of the sample.
- Sensory studies indicated highest acceptability for health mix added with TPH @ 2.5% ($HM_{2.5}$) and further storage studies of $HM_{2.5}$ samples under ambient temperature (28^{o}C) indicated good stability throughout the study period of six months.

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ANNEXURE I

SENSORY EVALUATION SCORE CARD

Assessor:……..
Date:……

(Please score the sample characteristics by placing the relevant score)

An honest expression of your personal feeling will help us in deriving meaningful conclusions.

Sample:…….

GENERAL CHARACTERISTICS

Attributes	**Sample A**	**Sample B**	**Sample C**	**Sample D**
Appearance				
Colour				
Odour				
Flavour/taste				
Overall acceptability				

Please score the sample characteristics according to the following scale

Quality Grade Description	**Score**
Like extremely	09
Like very much	08
Like moderately	07
Like slightly	06
Neither likes nor dislikes	05
Dislike slightly	04
Dislike moderately	03
Dislike very much	02
Dislike extremely	01

ANNEXURE II

SENSORY EVALUATION SCORE CARD

Assessor:……..
Date:.....……

(Please score the sample characteristics by placing the relevant score for **the attribute - bitterness**)

An honest expression of your personal feeling will help us in deriving meaningful conclusions.

Sample: …….....…….

SPECIFIC CHARACTERISTICS

Attributes	**Sample A**	**Sample B**	**Sample C**	**Sample D**
Bitterness				

Please score the sample characteristics according to the following scale

Bitterness		
Quality Grade Description	**Score**	**Caffeine Standard (ppm)**
Extreme bitterness	10	900
Intense bitterness	09	800
Strong bitterness	08	700
High bitterness	07	600
Moderate bitterness	06	500
Mild bitterness	05	400
Slight bitterness	04	300
Very slight but detectable bitterness	03	200
Neither bitter nor bitterless	02	100
No bitterness	01	50

ANNEXURE III

SENSORY EVALUATION SCORE CARD

Assessor:…….....……..
Date:…….....…

(Please score the sample characteristics by placing the relevant score)

An honest expression of your personal feeling will help us in deriving meaningful conclusions.

Sample:…….....…….

GENERAL CHARACTERISTICS

Attributes	**Sample A**	**Sample B**	**Sample C**	**Sample D**
Appearance				
Colour				
Odour				
Flavour				
Texture				
Overall acceptability				

Please score the sample characteristics according to the following scale

Quality Grade Description	**Score**
Like extremely	09
Like very much	08
Like moderately	07
Like slightly	06
Neither likes nor dislikes	05
Dislike slightly	04
Dislike moderately	03
Dislike very much	02
Dislike extremely	01

ANNEXURE IV

SENSORY EVALUATION SCORE CARD

Assessor:…….....……..
Date:…….....…

(Please score the sample characteristics by placing the relevant score)

An honest expression of your personal feeling will help us in deriving meaningful conclusions.

Sample:…….....…….

CHARACTERISTICS

Attributes	Sample A	Sample B	Sample C	Sample D
Taste				

Please score the sample characteristics according to the following scale

Quality Grade Description	Score
Very good	01
Good	02
Bad	03
Very bad	04

Publications and Award

Research articles

- **Parvathy, U.,** Binsi P.K., Madhurima Anant Jadhav, Visnuvinayagam, S., P. Muhamed Ashraf, George Ninan and Zynudheen A.A. (2019). Protein hydrolysate from yellowfin tuna red meat as fortifying and stabilizing agent in mayonnaise. *Journal of Food Science and Technology*, https://doi.org/10.1007/s13197-019-04069-x
- **Parvathy, U.,** Binsi, P.K., Jeyakumari, A., George Ninan, Zynudheen, A.A. and Ravishankar, C.N. (2019). Tuna red meat hydrolysate as core and wall polymer for fish oil encapsulation: a comparative analysis. *Journal of Food Science and Technology*, 1-13.
- **Parvathy, U**., Binsi, P.K., Joshy, C.G., Jeyakumari, A., Zynudheen, A.A., George Ninan and Ravishankar, C.N. (2019). Selective Extraction of Surface-active and Antioxidant Hydrolysates from Yellowfin Tuna Red Meat Protein using Papain by Response Surface Methodology. *The Indian Journal of Nutrition and Dietetics*, [S.l.]: 10-25.
- **Parvathy, U.,** Binsi, P.K., Zynudheen, A.A., George Ninan and Murthy, L.N. (2018). Peptides from white and red meat of yellowfin tuna (*Thunnus albacares*): A comparative evaluation. *Indian Journal of Fisheries*, 65(3): 74-83.
- **Parvathy, U**., Binsi, P.K., Joshy, C.G., Jeyakumari, A., Zynudheen, A.A., George Ninan and Ravishankar, C.N. (2018). Functional Hydrolysates from Yellow Fin Tuna Red Meat Using RSM Based Optimization. *International Journal of Current Microbiology and Applied Sciences,* 7(11): 1462-1474.

Popular articles

- **Parvathy, U**., Binsi P.K., George Ninan and Zynudheen A.A. (2019). Marine peptides: Application potentials in food and nutraceutical sector. *Beverage and Food World,* 46(6): 37-38.

- **Parvathy, U**., Binsi, P.K., Zynudheen, A.A. and George Ninan (2018). Utilization of yellowfin tuna protein hydrolysate in health beverage formulation. *FishTech Reporter*, 4 (1), January-June: 19-20.
- **Parvathy, U**., Binsi, P.K., Jeyakumari, A., George Ninan, Zynudheen, A.A. and Ravishankar, C.N. (2018). Fish Protein Hydrolysates: A potential additive in foods. *Aquastar*, August: 31-35.
- Jeyakumari, A., **Parvathy, U**., Murthy, L.N. and Visnuvinayagam, S. (2017). Fish protein hydrolysate: Properties and Application. *Beverage and Food World,* 44(2): 40-42.

Abstracts

- **Parvathy, U.,** Binsi, P.K., Madhurima Anant Jadhav, George Ninan and Zynudheen, A.,A. (2018). Tuna protein hydrolysate as fortifying and stabilizing agent in mayonnaise. Book of Abstracts: Swadeshi Science Congress, Thiruvananthapuram, 7-9th November, 2018, p 85.
- **Parvathy, U.,** Binsi, P.K., Joshy, C.G., Zynudheen, A.A., George Ninan and Ravishankar, C.N. (2017). Enzymatic hydrolysis for the selective extraction of surface active and antioxidant hydrolysates from yellowfin tuna red meat: Optimization using RSM. Book of Abstracts: 11th IFAF, 21-24th November, Cochin, 2017, p 342.
- **Parvathy, U.,** Binsi, P.K., Jeyakumari, A., George Ninan, Zynudheen, A.,A. and Ravishankar, C. N. (2017). Protein hydrolysate from yellowfin tuna (*Thunnus albacares*) red meat for oxidative and structural stabilization of microencapsulated fish oil. Book of Abstracts: 11th IFAF, 21-24th November, Cochin, 2017, p 519-520.

Brochures

- **Parvathy, U**. (2019). TunaPro. In: ICAR-CIFT Aquaceuticals
- **Parvathy, U**. (2019). NutriMayo. In: ICAR-CIFT Aquaceuticals
- **Parvathy, U**. (2019). OmegaPro Nutrimix. In: ICAR-CIFT Aquaceuticals
- **Parvathy, U**. (2019). HealthPro+. In: ICAR-CIFT Aquaceuticals
- **Parvathy, U**., Jeyakumari, Murthy, L.N. and Visnuvinayagam, S. (2017). Fish protein hydrolysate (In: English, Hindi and Marathi)

Award

- Received the **'Young Scientist Award'** for the paper entitled 'Protein hydrolysate from yellowfin tuna (*Thunnus albacares*) red meat for oxidative and structural stabilization of microencapsulated fish oil by **Parvathy U.,** Binsi P.K., Jeyakumari A., George Ninan, Zynudheen A.A. and C. N. Ravishankar in the 11th Indian fisheries and aquaculture forum on fostering innovations in fisheries and aquaculture during 21-24th November, 2017 organised by Asian Fisheries Society, Indian branch.