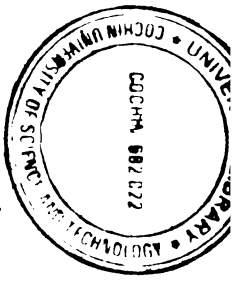
OPTIMIZATION OF PROCESS PARAMETERS FOR THE EXTRACTION OF GELATIN FROM THE SKIN OF FRESHWATER FISH AND THE EVALUATION OF PHYSICAL AND CHEMICAL CHARACTERISTICS



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BY

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DECLARATION

I, George Ninan do hereby declare that the thesis entitled "OPTIMIZATION OF PROCESS PARAMETERS FOR THE EXTRACTION OF GELATIN FROM THE SKIN OF FRESHWATER FISH AND THE EVALUATION OF PHYSICAL AND CHEMICAL CHARACTERISTICS " is a genuine record of bonafide research carried out by me under the supervision of Dr. Jose Joseph, Principal Scientist , Fish Processing Division, Central Institute Of Fisheries Technology, Cochin and has not previously formed the basis of award of any degree, diploma, associateship, fellowship or any other similar titles of this or any other university or Institution.

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CERTIFICATE

This is to certify that this thesis entitled "Optimization of Process Parameters for the Extraction of Gelatin from the Skin of Freshwater Fish and the Evaluation of Physical and Chemical Characteristics" embodies the original work done by George Ninan, under my guidance and supervision. I further certify that no part of this thesis has previously been formed the basis of award of any degree, diploma, associateship, fellowship or any other similar titles of this or in any other university or Institution. He has also passed the Ph.D qualifying examination of the Cochin University of Science And Technology, Cochin held in October 2006.

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CONTENTS

No	Titles	Page No.
1.0	**INTRODUCTION**	1
2.0	**REVIEW OF LITERATURE**	8
2.1.	**Fishery Industry Waste**	9
2.1.1	*Uses of Fish Waste*	12
2.1.1.1	*Animal Feed*	12
2.1.1.2	*Biodiesel / Biogas*	14
2.1.1.3	*Natural Pigments*	14
2.1.1.4	*Food Industry/Cosmetics*	15
2.2	**Fish Collagen**	16
2.3	**Gelatin**	18
2.3.1	Molecular Structure	19
2.3.1.1	*Structural Unit*	20
2.3.2	**Manufacture of Gelatin.**	23
2.3.2.1.	*Acid Process*	24
2.3.2.2.	*Alkali Process*	24
2.3.3	**Properties of Gelatin**	25
2.3.3.1	*Solubility in Water.*	26
2.3.3.2	*Gelling Properties*	26
2.3.3.3	*Emulsifying and Foaming Properties*	27
2.3.3.4	*Protective Colloid / Crystal Habit Modifying Properties.*	29
2.3.3.5	*Film Forming Properties.*	29
2.3.3.6	*Micro Encapsulation-Mixed Film Forming Properties.*	31
2.3.3.7	*Milk - Food Stabilising Properties.*	31
2.3.3.8	*Fruit Juice Clarifying Properties.*	31
2.3.3.9	*Texturising Properties.*	32
2.3.3.10	*Nutritional Properties.*	33
2.3.3.11	*Stability.*	33
2.3.3.12	*Corrosive Properties.*	33
2.3.4	**Applications of Gelatin**	34
2.3.4.1	*Food Applications*	34
2.3.4.2	*Pharmaceutical Applications*	35
2.3.4.3	*Photographic applications*	36
2.3.5	**Gelatin from Fish**	37

2.3.5.1	*Sources of Fish Gelatin*	38
2.3.5.2	*Marine Fish*	41
2.3.5.3	*Fresh Water Fish*	43
2.3.5.4	*Gelatin from the Bone, Cartilage and Scales of Fish*	44
2.3.5.5	**Physical Properties of Fish Skin Gelatin**	45
2.3.5.5.1	*Extractability and Yield*	45
2.3.5.5.2	*Viscosity*	48
2.3.5.5.3	*Clarity*	49
2.3.5.5.4	*Melting and Setting Temperatures*	49
2.3.5.5.5	*Odour*	50
2.3.5.5.6	*Colour*	51
2.3.5.6	**Functional Properties of Fish Skin Gelatin**	51
2.3.5.6.1	*Gel strength, Gelling and Melting Points*	51
2.3.5.6.2	*Foam Formation Ability and Foam Stability*	53
2.3.5.6.3	*Water Holding and Fat Binding Capacities*	53
2.3.5.6.4	*Texture Profile*	54
2.3.5.7	**Chemical Properties of Fish Skin Gelatin**	56
2.3.5.7.1	*Proximate Composition & pH*	56
2.3.5.7.2	*Amino Acid Composition*	57
2.3.5.7.3	*Molecular Weight Distribution*	59
2.3.5.7.4	*Fourier Transform Infrared Spectroscopic (FTIR) Spectra of Gelatin*	60
2.3.5.8	**Mammalian Gelatin Vs Fish Gelatin**	61
2.3.5.9	**Optimization of Process Parameters for the Production of Gelatin from Fish Skin using Response Surface Methodology**	64
2.3.5.10	**Gelatin / Collagen based Edible Films**	65
2.3.5.11	**Plasticizers in Edible Films**	67
2.3.5.12	**Fish Skin Gelatin based Films**	68
2.3.5.13	**Fish Gel based Products**	70
2.3.5.14	**Microbiological Quality of Fish Gelatin**	72
2.3.5.15	**Safety of Gelatin as a Food Ingredient**	74
3.0	**MATERIALS AND METHODS**	76
3.1	**Materials**	77
3.1.1.	*Chemicals*	77
3.1.2.	*Equipment and Glassware*	78
3.1.3	*Fish Skin*	80
3.2	**Methods**	81

3.2.1	*Preparation of the Fish Skin for Gelatin Extraction*	81
3.2.2.	*Process of Gelatin Extraction*	81
3.2.2.1	*Pretreatment of fish skins prior to main extraction*	81
3.2.2.2	*Gelatin extraction*	82
3.2.3	**Experimental Design for Process Optimization**	84
3.2.3.1	*Screening Experimental Design*	84
3.2.3.2	*Optimization of Experimental Design*	86
3.2.4	**Gelatin Water Dessert Preparation**	88
3.2.5	**Preparation of Gelatin Films**	89
3.2.6	**Analytical Methods**	90
3.2.6.1	*Determination of yield*	90
3.2.6.2	*Determination of pH*	90
3.2.6.3	*Determination of Colour*	90
3.2.6.4	*Viscosity*	90
3.2.6.5	*Clarity*	91
3.2.6.6	*Foam Formation Capacity and Foam Stability*	91
3.2.6.7	*Water holding and Fat binding capacities*	91
3.2.6.8	*Determination of Melting point*	92
3.2.6.9	*Determination of Setting point and Setting time*	92
3.2.6.10	*Gel strength determination*	93
3.2.6.11	*Determination of Odour*	94
3.2.6.12	*Texture Profile Analysis*	94
3.2.6.13	*Determination of Moisture*	95
3.2.6.14	*Determination of Crude Protein*	95
3.2.6.15	*Determination of Crude Fat*	95
3.2.6.16	*Determination of Ash Content*	96
3.2.6.17	*Estimation of Arsenic, Lead, Copper, Zinc, Cadmium and Chromium using Atomic Absorption Spectrophotometer*	96
3.2.6.18	*SDS-Polyacrylamide Gel Electrophoresis (SDS – PAGE)*	96
3.2.6.19	*Estimation of Amino Acids*	99
3.2.6.20	*Fourier Transform Infrared Spectroscopy (FTIR)*	101
3.2.6.21	**Methods for the Testing of Physical Properties of Gelatin Films.**	101
3.2.6.21.1.	*Determination of Colour of Gelatin films*	101
3.2.6.21.2.	*Determination of Thickness*	101
3.2.6.21.3.	*Determination of Tensile Strength and Elongation at Break*	102

3.2.6.21.4.	*Determination of Water Vapour Transmission Rate*	103
3.2.6.21.5	*Determination of Gas Transmission Rate*	103
3.2.7.	**Microbiological Analyses**	105
3.2.7.1	*Total Aerobic Count at 30°C*	105
3.2.7.2	*Coliforms (30°C)*	106
3.2.7.3	*Coliforms (44.5°C)*	107
3.2.7.4	*Sulphite-Reducing Anaerobic Spores*	108
3.2.7.5	*Clostridium perfringens Spores*	109
3.2.7.6	*Staphylococcus aureus*	111
3.2.7.7	*Salmonella*	113
3.3.	**Statistical Analysis**	115
4.0	**RESULTS AND DISCUSSION**	116
4.1.	**Optimization of Process Parameters for the Extraction of Gelatin from Fish Skin**	117
4.1.1	*Screening Factors for Gelatin Extraction and the Effect of Factors on the Responses during Screening Experiments*	117
4.1.2	*Response Surface Model building and Optimization of Extraction Conditions*	121
4.1.3.	*Numerical Optimization and Assignment of Optimization Parameters for obtaining Solutions with Desirability Values*	129
4.1.4	*Verification of Predicted Values*	137
4.2	**Quality characteristics of Gelatin Extracted from the Skin of freshwater fishes**	138
4.2.1	*Skin Yield from Freshwater Fish*	138
4.2.2	*Physical Properties of Gelatins*	139
4.2.2.1	*Yield*	139
4.2.2.2	*Viscosity*	140
4.2.2.3	*Clarity*	140
4.2.2.4	*Melting Temperature*	141
4.2.2.5	*Setting Temperature & Setting Time*	142
4.2.2.6	*Odour*	143
4.2.2.7	*Colour*	144
4.2.3	*Functional properties of Gelatins*	145
4.2.3.1	*Gel strength*	145
4.2.3.2	*Foam Formation Ability and Foam Stability*	147
4.2.3.3	*Water Holding and Fat Binding Capacities*	147
4.2.3.4	*Textural Properties*	148

4.2.4	*Chemical Properties of Gelatins*	151
4.2.4.1	*Proximate Composition*	151
4.2.4.2	*pH*	154
4.2.4.3	*Amino acid composition*	154
4.2.4.4.	*Molecular weight distribution*	157
4.2.4.5	*FTIR Spectra of Carp Skin Gelatins*	161
4.2.5	*Heavy Metal Content in the Carp Skin Gelatin*	167
4.2.6	*Microbiological Quality of Carp Skin Gelatin*	168
4.3	**Physical, Chemical and Functional Properties of Carp Skin Gelatin compared to Mammalian Gelatins**	169
4.3.1	**Carp Skin Vs Mammalian Gelatin - Physical Properties**	169
4.3.1.1.	*Viscosity*	169
4.3.1.2.	*Melting point*	170
4.3.1.3.	*Clarity*	171
4.3.1.4	*Setting Point and Setting Time*	171
4.3.1.5.	*Sensory Evaluation*	172
4.3.2	**Carp Skin Vs Mammalian Gelatin – Amino Acid Composition**	174
4.3.3	**Carp Skin Vs Mammalian Gelatin – Functional Properties**	176
4.3.3.1	*Gel Strength*	176
4.3.3.2.	*Foaming Ability and Foam Stability*	177
4.3.3.3	*Water holding capacity and Fat binding capacity*	179
4.4	**Comparison of Physical, Mechanical and Barrier Properties of Edible Films Prepared from Rohu, Common carp and Grass carp gelatin with Mammalian Skin Gelatin Films.**	181
4.4.1	*Physical and Mechanical Properties of Gelatin Films*	181
4.4.2	*Barrier Properties of Gelatin Films*	184
4.5	**Comparison of Gel Strength, Melting Point, Odour and Texture Profile of Gelatin water Desserts**	187
4.5.1	*Gel Strength, Melting Point and Odour of Gelatin Water Desserts*	187
4.5.2	*Texture Profile Analysis of Gelatin Water Desserts*	189
5.0	**SUMMARY AND CONCLUSIONS**	192
6.0	**REFERENCES**	201

LIST OF TABLES

TABLE NO.	TITLE	PAGE NO.
2.1	Waste generation in industrial fish processing in India	10
2.2	Inputs and outputs of various fish processes	11
2. 3	References cited in literature on the extraction and characterisation of fish gelatin	40
3.1	Size description of the selected fish species	80
3.2	Process variables adopted for the pretreatment of fish skins	82
3.3	Process variables adopted for the extraction of gelatin	82
3.4	Independent Variables and their Levels in the 6 Factor, 2 Level Fractional Factorial (2 6-3 /Iii) Screening Design	85
3.5	Fractional Factorial Screening Design in Coded Units	85
3.6	Independent variables and their levels in the 4 factor, 5 level Central Composite Rotatable Design for optimization of the extraction conditions of gelatin from fish skin	86
3.7	Central Composite Design for Optimizing the Extraction Condition of Fish Skin Gelatin	87
3.8	Composition and pH of Gelatin Desserts	89
3.9	Ingredients of Selective liquid culture medium	106
3.10	Ingredients for Selective liquid culture medium-LGA	107
3.11	Ingredients for Beef extract-yeast extract-sulphite-iron agar	108
3.12	Ingredients for Enrichment broth	110
3.13	ingredients for LS medium	110
3.14	Ingredients for Selective liquid enrichment broth	111
4.1.1(a)	Experimental results for Rohu gelatin using Fractional Factorial screening design ($2^{6-3/iii}$) in coded units	118
4.1.1(b)	Experimental results for Common carp gelatin using Fractional factorial screening design ($2^{6-3/iii}$) in coded units	118
4.1.1 (c)	Experimental results for Grass carp gelatin using Fractional factorial screening design ($2^{6-3/iii}$) in coded units	119
4.1.1(d)	Screening Experiment – Range of Responses for independent factors (Rohu Gelatin)	119
4.1.1(e)	Screening Experiment – Range of Responses for independent factors (Common carp Gelatin)	120
4.1.1 (f)	Screening Experiment - Range of Responses for independent factors (Grass carp Gelatin)	120
4.1.2(a)	Central Composite Design for Optimizing the Extraction Conditions of Rohu Skin Gelatin in coded units together with experimental data and their predicted value	122

4.1.2(b)	Central Composite Design for Optimizing the Extraction Conditions of Common carp Skin Gelatin in coded units together with experimental data and their predicted value	124
4.1.2(c)	Central Composite Design for Optimizing the Extraction Conditions of Grass carp Skin Gelatin in coded units together with experimental data and their predicted value	126
4.1.2(d)	Rohu Gelatin- Analysis of Variance (ANOVA) for Response Surface Quadratic Model	127
4.1.2(e)	Common carp Gelatin- Analysis of Variance (ANOVA) for Response Surface Quadratic Model	128
4.1.2 (f)	Grass carp Gelatin- Analysis of Variance (ANOVA) for Response Surface Quadratic Model	129
4.1.3(a)	Rohu Skin Gelatin- Optimization Parameters in the response optimizer	131
4.1.3(b)	Common carp Skin Gelatin- Optimization Parameters in the response optimizer	132
4.1.3(c)	Grass carp Skin Gelatin- Optimization Parameters in the response optimizer	132
4.1.3(d)	Rohu Skin Gelatin- Optimization solutions obtained using the response optimizer	133
4.1.3(e)	Common carp Skin Gelatin- Optimization solutions obtained using the response optimizer	133
4.1.3(f)	Grass carp Skin Gelatin- Optimization solutions obtained using the response optimizer	133
4.1.4	Experimental and predicted results of verification under optimized conditions	137
4.2.1	Yield percentage during different stages of separation of skin from the carcass of Rohu, Common carp and Grass carp	138
4.2.2	Physical properties of Gelatins extracted from the skin of Rohu, Common carp and Grass carp	143
4.2.3	Colour of Gelatin from the skin of Rohu, Common carp and Grass carp	145
4.2.4	Functional Properties of Gelatin from the skin of Rohu, Common carp and Grass carp	148
4.2.5	Texture Profile Analysis of Gelatin from the Skin of Rohu, Common carp and Grass carp	151
4.2.6	Proximate Composition of Fresh Raw Skin of Carps	152
4.2.7	Proximate Composition and pH of Carp Skin Gelatin	153
4.2.8	Amino Acid Composition of Gelatin from Carp Skin	157
4.2.9	FTIR Spectra Peak Position and Assignments for Carp Skin	163

	Gelatin	
4.2.10	Heavy Metal Content of Carp Skin Gelatin	167
4.2.11	Bacteriological Quality of Fish Skin Gelatin	168
4.3.1	Amino Acid Profile of Mammalian and Carp Skin Gelatin	175
4. 4 .1	Physical and mechanical properties of gelatin based Edible Films	183
4. 4 .2	Colour of gelatin based Edible Films	184
4. 4.3	Barrier Properties of gelatin based Edible Films	186
4.5.1	Gel Strength, Melting Point and Odour of Gelatin Water Desserts	189
4.5.2	Instrumental Textural Parameters of Gelatin Water Desserts	191

LIST OF FIGURES

FIGURE	TITLE	PAGE NO.
1	Typical structure of gelatin	20
3.1	Flow chart for pretreatment and extraction of gelatin from fish skin	83
4.1.3 (a)	Three-dimension response surface plots of Rohu skin gelatin extraction –Gel strength	134
4.1.3 (b)	Three-dimension response surface plots of Rohu skin gelatin extraction – Yield	134
4.1.3(c)	Three-dimension response surface plots of Common carp skin gelatin extraction –Gel strength	135
4.1.3 (d)	Three-dimension response surface plots of Common carp skin gelatin extraction–Yield	135
4.1.3 (e)	Three-dimension response surface plots of Grass carp skin gelatin extraction– Gel strength	136
4.1.3 (f)	Three-dimension response surface plots of Grass carp skin gelatin extraction — Yield	136
4.2.1	Electrophoretic Profile of the Carp Skin Gelatins	160
4.2.2(a)	FTIR Spectra of Rohu skin Gelatin	164
4.2.2 (b)	FTIR Spectra of Common carp skin Gelatin	165
4.2.2 (c)	FTIR Spectra of Grass carp skin Gelatin	166
4.3.1(a)	Viscosity of Mammalian and Carp Skin Gelatin	169
4.3.1(b)	Melting Point of Mammalian and Carp Skin Gelatin	170
4.3.1(c)	Clarity of Mammalian and Carp Skin Gelatin	171
4.3.1(d)	Setting Point of Mammalian and Carp Skin Gelatin	172
4.3.1(e)	Setting time of Mammalian and Carp Skin Gelatin	172
4.3.1(f)	Odour Score of Mammalian and Carp Skin Gelatin	173
4.3.2(a)	Bloom Value of Mammalian and Carp Skin Gelatin	177
4.3.2(b)	Foaming Ability of Mammalian and Carp Skin Gelatin	178
4.3.2(c)	Foam Stability of Mammalian and Carp Skin Gelatin	178
4.3.2(d)	Water Holding Capacity of Mammalian and Carp Skin Gelatin	179
4.3.2(e)	Fat binding Capacity of Mammalian and Carp Skin Gelatin	180

LIST OF ABBREVIATIONS

Anon	-	Anonymous
ANOVA	-	Analysis of Variance
APS	-	Ammonium per sulphate
B	-	Bloom
BF_3	-	Boron trifluoride
BG	-	Bovine Skin Gelatin
BSA	-	Bovine serum albumin
BSE	-	Bovine Spongiform Encephalopathy
BP	-	Boiling point
^0C	-	Degree celsius
cm	-	Centimeter
cP	-	Centipoise
CFU	-	Colony forming Units
CG	-	Common carp Skin Gelatin
$CuSO_4$	-	Copper sulphate
EDTA	-	Ethelene diamine tetra acetic acid
Fig	-	Figure
FTIR	-	Fourier Transform Infrared Spectroscopy
g	-	Grams
GTR	-	Gas Transmission Rate
GME	-	Gelatin Manufacturers of Europe
GG	-	Grass carp Skin Gelatin
h	-	Hours
HCl	-	Hydrochloric acid
H_2SO_4	-	Sulphuric acid
HPLC	-	High performance liquid chromatography
KBr	-	Potassium bromide
KH_2PO_4	-	Potassium dihydrogen phosphate
kDa	-	Kilo Dalton
Kg	-	Kilogram
Kgf	-	Kilogram force
L	-	Litre
M	-	Molar

mg	-	Milligram
µ moles	-	Micromoles
µg	-	Microgram
µ l	-	Microlitre
min	-	Minutes
ml	-	Millilitre
mM	-	Millimolar
mm	-	Millimeter
Na_2HPO_4, 12 H_20	-	Sodium monohydrogen phosphate
NaOH	-	Sodium hydroxide
OD	-	Optical density
OTR	-	Oxygen Transmission Rate
OPA	-	Ortho phthalaldehyde
TN	-	Total nitrogen
PG	-	Porcine Skin Gelatin
RG	-	Rohu Skin Gelatin
r p m	-	Revolution per minute
RSM	-	Response Surface Methodology
SD	-	Standard deviation
SDS-PAGE	-	Sodium dodecyl sulphate –polyacrylamide gel electrophoresis
TEMED	-	Tetramethylethylenediamine
TPA	-	Texture Profile Analysis
v/v	-	Volume / Volume
w/v	-	Weight / Volume
WVTR	-	Water Vapour Transmission Rate

1.0 Introduction...

Global fish and shrimp production has been in a steadily increasing trend over the last decade and this trend is expected to continue. Of the estimated 131 million tonnes of fish produced in the year 2000 in the World, nearly 74% (97 million tonnes) was used for direct human consumption. The remainder (about 26%) was utilized for various non-food products, mostly for reduction to meal and oil. As a highly perishable commodity, fish has a significant requirement for processing. In 2000, more than 60% of total world fisheries production underwent some form of processing (FAO, 2002). An important waste reduction strategy for the industry is the recovery of marketable byproducts from fish wastes. Hydrolyzed fish wastes can be used for fish or pig meal as well as fertilizer components (www.earthprint.com). The three most common methods for utilization of aquatic waste (either from aquaculture or wild stock) are the manufacture of fishmeal/oil, the production of silage and the use of waste in the manufacture of organic fertilizer (www.fao.org). The utilization of byproducts is an important cleaner production opportunity for the industry, as it can potentially generate additional revenue as well as reduce disposal costs for these materials. The transportation of fish residues and offal without the use of water is an important factor for the effective collection and utilization of these byproducts

Of the total world fish production of 132.9 million tons, more than 75 % is utilized for human consumption and the rest is used for other purposes. Out of the 32.25 million tons used for other purposes, 78% is used for reduction and remaining for miscellaneous purposes (FAO, 2002).The fish landing in India is around 6 million tons in 2005 of which the marine sector is contributing about 2.9 million tons, against the estimated potential of 3.9 million tons (Anon. 2006).

The fish processing industry in India by and large depends on the shrimp which constitutes about 20% of the total landings. The trawling operations for prawn results in the landings of many low value varieties of fish, most of which are thrown back to the sea. The bycatch from Indian seas is mostly composed of jew fish, perches, sole, barracuda, lizard fish, anchovies, lactarius, crab, bulls eye, threadfin breams etc. Industrial fish processing for human consumption yields only 40% edible flesh and the remaining 60% is thrown away as waste (Raa & Gildberg, 1982). Annual discard from the world

fisheries were estimated to be approximately 20 million tonnes (25%) per year. This includes "waste" or byproducts also. Only 36000 tons of the byproducts were used for human consumption, which amounts to about 15.5 % of the total (Rubin, 2001).Presently, the world export trade of fish waste is 6,75,970 tons in different forms worth US $205.4 million. The import figures are 1,23,3602 tons (value 328.1 million dollars). In India the export figure of the fish waste for the year 2002 is 2016 tons worth 11.03 million US dollars (FAO 2002).

Processing of fish leads to enormous amounts of waste. It is estimated that fish processing waste after filleting accounts for approximately 75% of the total fish weight and 30% of the waste is in the form of bones and skins (Gomez-Guillen *et al.,* 2002). About 30% of the total fish weight remains as waste in the form of skins and bones during preparation of fish fillets (Shahidi, 1994). This waste is an excellent raw material for the preparation of high value products including protein foods. The utilization of fish wastes helps to eliminate harmful environmental aspects and improve quality in fish processing.

With a view to utilize the by catch and processing waste, efforts have been made to develop methods for converting them into products for human consumption, animal nutrients and products of commercial importance. Among the most prominent current uses for fish waste are fishmeal production, extraction of collagen and antioxidants, isolation of cosmetics, biogas/biodiesel, production of chitin and chitosan, food packaging (gelatin, chitosan) and enzyme isolation (proteases).

The fish skins and bones can be processed into gelatin, thus solving the problem of fish waste disposal in addition to creating value-added products. Gelatin is a substantially pure protein food ingredient, obtained by the thermal denaturation of collagen, which is the structural mainstay and most common protein in the animal kingdom. It is one of the most versatile gelling agents in food applications due to its special texture and the 'melt-in-mouth' perception. In addition to foodstuffs, gelatin has found a variety of applications in the pharmaceutical and photographic industry. Generally, gelatin is produced from skin and bone collagen by acid or alkali treatment to give type A and type B gelatins, respectively (Veis, 1964; Ward & Courts,

1977). The World production of gelatin is currently pegged at 315,000MT of which 45.8% is produced from pigskin, 52.6% from bovine hides and bones and 1.6% from other sources (GME, 2007).

Gelatin forms thermally reversible gels with water, and the gel melting temperature (<35°C) is below body temperature, which gives gelatin products with unique organoleptic and flavour releasing properties. The disadvantage of gelatin is that it is derived from animal hide and bones hence there are problems with regard to kosher and Halal status and vegetarians also have objections to its use. Competitive gelling agents like starch, alginate, pectin, agar, carrageenan etc. are all carbohydrates from vegetable sources, but their gels lack the melt in the mouth and elastic properties of gelatin gels.

There are two main types of gelatin. Type A, with isoionic point of 7 to 9, is derived from collagen with exclusively acid pretreatment. Type B, with isoionic point of 4.8 to 5.2, is the result of an alkaline pretreatment of the collagen. However, gelatin is sold with a wide range of special properties like gel strength, to suit particular applications.

Gelatin is a gelling protein, which has widely been applied in the food and pharmaceutical industries. Most commercial gelatins are derived from mammalian sources, mainly pigskin and cowhide but for many socio-cultural reasons alternative sources are increasingly demanded. Among such reasons are religious proscription of Judaism and Islam, and diseases like Bovine Spongiform Encephalopathy (BSE). Byproducts of poultry and fish are rarely used as a resource of gelatin.

The amount of gelatin used in the worldwide food industry is increasing annually (Montero & Gomez-Guillen, 2000). However, frequent occurrences of BSE and foot/mouth diseases limited the utility of mammalian gelatins in processing of functional food, cosmetic and pharmaceutical products. Therefore, the significance of study of gelatin from fish by-products, such as skin and bone, has increased for the replacement of mammalian resources (Gudmundsson, 2002).

A few fish gelatins are available commercially, but fish gelatin is not commonly utilized because it is inferior to mammalian gelatin in rheological properties, which affect product quality (Choi & Regenstein, 2000). The fish gelatins available commercially are not well characterized. One of the most important differences between mammalian and fish gelatins are that the latter have normally low gelling and melting temperatures and also lower gel strength (Norland, 1990)

The use of byproducts from fish for gelatin production as an alternative to mammalian gelatin raises some practical problems. First, fish collagen is highly susceptible to deterioration when compared to mammalian collagen which is more stable. Second, the raw material for gelatin production from fish viz. skin can undergo rapid enzymatic and microbial damage when kept along with the rest of byproducts including gut contents causing wide variations in the quality of gelatins produced.

In order to be suitable for application in food and pharmaceutical industries, fish gelatin must possess the following characteristics. First, there should be a large quantity of fish processing waste to make the collection of sufficient quantity of skin and bones economical to run the production continuously. Secondly, gelatin from fish byproducts must have rheological properties (gel strength, gelling and melting points, etc.) at the level of mammalian gelatin. However, it is not easy for fish byproducts to satisfy the above two categories because of their typical physical properties.

Fish byproducts from freshwater are seldom used as a source of raw materials for gelatin extraction. They are mainly used for animal feed supplements due to their small size (Gildberg *et. al.*, 2002). However, some studies have ascertained that freshwater fish have vast amounts of waste after removal of useful edible parts and high gelatin yield is expected from them (Jamilah & Harvinder, 2002; Grossman & Bergman, 1992: Muyonga *et.al.*, 2004a). Additionally, most findings suggest that gelatin from tropical fish species has an advantage over those extracted from cold water species, the

former having better rheological properties nearly similar to mammalian gelatins (Veis, 1964 ; Cho *et. al.,* 2005; Gilsenan & Ross-Murphy, 2000a).

India ranks second to China in the global aquaculture production. The aquaculture production in the country was estimated to be 2.47 million tonnes in 2004 which is more than 50% of the total fish production. Freshwater fin fish contributed almost 97% of the total freshwater aquaculture. Among the freshwater species Indian major carps (Catla - *Catla catla,* Rohu – *Labeo rohita* and Mrigal *Cirrhinus mrigala)* predominated with 85% of the total landings. Generally the freshwater fish is consumed fresh in the country. However with the change in consumer preferences and constant demand for value added and convenience products, there is scope for the development of organized fish processing units in the inland sector. With the establishment of such units, there will be generation of significant quantity of fishery waste, which, if not properly utilized can be a serious environmental hazard. In major carps the skin accounts for almost 6% of the live weight. Fish skin forms a major portion of the fishery waste, particularly in the case of production of mince based and fillet based value added products.

The present study aims to evaluate the suitability of the skin of the freshwater fish as a raw material for the production of gelatin. The objectives of this study are:

- To study the suitability of the fish skin from selected species of Indian major carps and exotic carps for the production of gelatin

- To optimize the process condition for the extraction of gelatin from the skin of selected species of Indian major carps and exotic carps

- To study the physico-chemical properties of the gelatin prepared from the skin of selected species of Indian major carps and exotic carps

- To compare the physicochemical properties the gelatin prepared from the skin of selected species of Indian major carps and exotic carps with that of gelatin from mammalian sources

- To prepare and study the physical and mechanical properties of edible films fabricated with fish skin gelatin from the above species

- To formulate gel based edible products from fish skin gelatin and study their physical and sensory properties.

2.0 Review of Literature...

2.1 Fishery Industry Waste

Fishery products are prepared from a wide variety of species in the aquatic environment and usually only the most desirable and easy extractable portion of the carcass is used as human food. This leaves a large portion of the highly nutritious tissue, most of which will end up as 'waste' and some will be utilized as byproduct. In addition many other species are often unintentionally caught as 'trash fish' while fishing for target species which is also a rich source of protein and often underutilized. Fishery products for direct human consumption can be broadly categorized into fresh whole products, cured products, chilled and frozen products, surimi and fish mince based products and canned items. Major fishery byproducts are fish meal and oil, fish protein extracts, fish silage, fish protein hydrolysates, chitin & chitosan from crustacean shell etc. Fish processing generates solid wastes that can be as high as 50 - 80% of the original raw material (Wasswa, *et. al.,* 2007). About 30% of these wastes consist of skin and bone with high collagen content.

Global production of fish and shrimp has been increasing steadily over the last decade and this trend is expected to continue. Of the estimated 131 million tonnes of fish produced in 2000 in the world, nearly 74% (97 million tonnes) was used for direct human consumption. The remainder (about 26%) was utilized for various non-food products, mostly for reduction to meal and oil. As a highly perishable commodity, fish has a significant requirement for processing. In 2000, more than 60% of total world fisheries production underwent some form of processing (FAO, 2002).In India, the industrial fish processing generate 3, 02,750 tonnes of waste (Anon. 2005). Among the maritime states, maximum waste generation was observed in Gujarat (30.51%) followed by Maharashtra (23%) and Kerala (17.5%). The waste generation during the processing of major marine products from India is given below in Table 2.1.

Table 2.1 Waste generation in industrial fish processing in India*

Products	Waste generated (%)
Shrimp products (PD, PUD, HL, etc.)	50
Fish Fillets	70
Fish steaks	30
Whole and gutted fish	10
Cuttlefish rings	50
Cuttlefish whole	30
Cuttlefish fillets	50
Squid whole cleaned	20
Squid tubes	50
Squid rings	55

*Anon.2005

Fish used as human food accounts for 78% of the total fish catch in developed and developing countries, leaving about 21% for non-food uses (Vannuccini, 2004). Processing leads to the generation of a large biomass of fish to the tune of approximately 7.3 million tons/year which is generally discarded (Kelleher, 2005). Inputs and outputs of the various activities involved in fish processing show that the highest energy requirements occur in the following processes in descending order: drying of press cake, sterilization of cans, canning and cooking (Table 2.2). In the case of wastewater generated, the processes responsible for the greatest amount are skinning of nobbed fish and canning (17 and 15m^3, respectively per 1000 Kg of input as fresh or whole fish).

An important waste reduction strategy for the industry is the recovery of marketable byproducts from fish wastes. Hydrolysed fish wastes can be used for fish or pig meal as well as fertilizer components (www.earthprint.com). The three most common methods for utilisation of aquatic waste (either from aquaculture or wild stock) are the manufacture of fishmeal/oil, the production of silage and the use of waste in the manufacture of organic fertilizer (www.fao.org). The utilization of byproducts can potentially generate additional revenue as well as reduce disposal costs for these materials. The transportation of fish residues and offal without the use of water is an important factor for the effective collection and utilisation of these byproducts (www.earthprint.com).

Table 2.2 Inputs and outputs of various fish processes*

Process	Inputs			Outputs
	Fresh or frozen fish (kg)	*Energy (kW h)*	*Wastewater (m^3)*	*Solid waste generated (kg)*
White fish filleting	1000	Ice: 10–12 Freezing: 50–70 Filleting: 5	5–11	Skin: 40–50 Heads: 210–250 Bones: 240–340
Oily fish filleting	1000	Ice: 10–12 Freezing: 50– Filleting: 2–5	5–8	400–450
Canning	1000	150–190	15	Heads/entrails: 250 Bones: 100–150
Fish meal and fish oil production	1000	Fuel: 49 L Electricity: 32	-	-
Frozen fish thawing	1000	–	5	–
De-icing and washing	1000	0.8–1.2	1	0–20
Scaling of white fish	1000	0.1–0.3	10–15	Scales: 20–40
De-heading of white fish	1000	0.3–0.8	1	Heads and debris: 270–320
Filleting of de-headed white fish	1000	1.8	1–3	Frames and off cuts: 200–300
Filleting of un-gutted oily fish	1000	0.7–2.2	1–2	Entrails, tails, heads and frames: 400
Skinning white fish	1000	0.4–0.9	0.2–0.6	Skin: 40
Skinning oily fish	1000	0.2–0.4	0.2–0.9	Skin: 40
Trimming and cutting white fish	1000	0.3–3. 0	0.1	Bones and cut-off: 240–340
Packaging of fillets	1000	5.0–7.5	–	–
Freezing and storage	1000	10.0–14.0	–	–
Unloading fish for canning	1000	3.0	2.0– 5.0	–
Grading of fish	1000	0. 15	0.2	0.30
Nobbing and packing in cans	1000	0.4–1.5	0.2–0.9	Heads and entrails: 150

Skinning of nobbed fish	1000	–	17.0	Skin: 55
Precooking of fish to be canned	1000	0.3–1.1	0.07–0.27	Inedible parts: 150
Draining of cans containing precooked fish	1000	0.3	0.1–0.2	–
Sauce filling	1000	–	–	Spillage of sauce and oil: varies
Can sealing	1000	5.0–6.0	–	–
Washing of cans	1000	7.0	0.04	–
Sterilization of cans	1000	230	3.0–7.0	–
Handling and storage of fish	1000	10.0–12.0	-	–
Unloading of fish	1000	3.0	2.0–5.0	-
Cooking of fish	1000	90.0	–	–
Pressing the cooked fish	1000	–	-	Press cake: 100 dry matter
Drying of press cake	1000	340.0	–	–
Fish oil polishing	1000	Hot water	0.05–0.1	–
Stick water evaporation	1000	475.0	–	Concentrated stick water: 250 Dry matter: 50

* www.agrifood-forum.net

2.1.1 Uses of Fish Waste

Fish industry wastes are important as sources for environmental contamination. Research has been carried out in order to develop methods to convert these wastes into useful products (Perea, *et. al.,* 1993; Kristinsson & Rasco, 2000; Larsen *et. al.*, 2000; Guerard *et. al.*, 2001; Coello *et. al.*, 2002; Laufenberg, *et. al.,* 2003). Probably, more than 50% of the remaining material from the total fish capture is not used as food and constitutes almost 32 million tonnes of waste (Kristinsson & Rasco, 2000).

2.1.1.1 Animal Feed

Nowadays, the use of food wastes as animal feed is an alternative of high interest, because it stands for environmental and public benefit besides reducing the cost of production (Samuels, *et. al.,* 1991; Westendorf, *et. al.,* 1998; Myer, *et. al.,* 1999; Westendorf, 2000). Offal from the fishing industry could be used as a feed ingredient, as it represents a valuable source of high-quality protein and energy (New, 1996; Gabrielsen & Austreng, 1998).

Fish waste (mainly heads, bones, skin, viscera and sometimes whole fish) is heated at 65, 80, 105 and 150 ^{0}C for 12 h in order to reduce the moisture content to 10–12%, which is the recommended moisture content in animal feed (NRC, 1998). Fish waste is a good source of protein [58% dry matter], minerals and fat (19% dry matter) including mono polyunsaturated fatty acids are abundant in fish waste. Toxic substances (such as As, Pb, Hg and Cd) were detected in fish waste at rather low concentrations. Fish waste can be used as alternative feedstuffs in swine diets to meet partially the protein requirements and serve as a substitute for common sources of protein i.e., soybean meal and commercial fishmeal (Esteban, *et. al.,* 2006).

Fish silage is a liquid product resulting from the liquefaction of a whole fish or a part (Tatterson & Windsor, 1974). Liquefaction is an autolytic process carried out by enzymes already present in the fish and accelerated by an acid that induces the proper conditions for the enzymes to breakdown the tissues and limits the growth of spoilage bacteria (Gildberg, 1993). Ensilage of fish waste, although practised in some countries several years ago, is not widely used nowadays because of the high water content, which may render transportation expensive. Moreover, fish waste silage is characterized by a disagreeable odour and this may considerably limit its use in high proportion of feed formulations. Fish silage can be used as a nitrogen source and possibly as a probiotic ingredient for poultry feeding (Hammoumi, *et. al.,* 1998).

Chitin is a structural component in crustacean exoskeletons, which contain 15–20% chitin by dry weight. The production of chitin and chitosan from seafood industry waste has proved environmentally attractive and economically feasible, especially when it includes the recovery of carotenoids.

Considerable amounts of chitin are present in the wastes and are marketed as a fish food additive (Arvanitoyannis, 1999; Kumar, 2000). Chitosan can find many applications on its own or as blends either as dietic product or as edible films for food preservation purposes (Arvanitoyannis, *et. al.*, 1997a, 1998).

2.1.1.2 Biodiesel / Biogas

Biodiesel fuel, acquired from the oils and fats of vegetables and animals, is a substitute for, or an additive to, diesel fuel derived from petroleum (Alcantara, *et. al.,* 2000). However, during the early 1980s, engine tests showed that the combustion of vegetable oils caused durability problems related to incomplete combustion such as nozzle choking, engine deposits, ring sticking and crankcase lubricant contamination (Dunn & Bagby, 2000). Furthermore, the higher viscosity of vegetable oils compared with diesel fuel caused excessive carbon deposition and thickening of lubricating oil, and was largely responsible for the problems encountered in using vegetable oils as a diesel fuel especially in relative cold areas and during cold seasons (Clark, *et. al.*, 1983).

Kato *et. al.* (2004) evaluated the ozone-treated fish waste oil as a transportation diesel fuel. The oil was found to have suitable properties for use in diesel engines, such as almost identical higher heating value (10700 kcal kg^{-1}) and density (at 15 ^0C, 0.87 g cm^{-3}), lower flash and pour points (37 and 16 ^0C, respectively), no production of sulphur oxides, lowered or no soot and lower poly aromatic and carbon dioxide emissions when compared with commercial diesel fuel. These properties suggested that the obtained oil had better properties than methyl-esterified vegetable oil waste and was suitable for diesel engines especially at low-temperature areas. The net energy production from the biogas was 43–47 MW h $year^{-1}$ and could cover 2–4% of the energy demand in flow-through hatcheries.

2.1.1.3 Natural Pigments

Shrimp waste is one of the most important sources of natural carotenoids (Shahidi *et. al.*, 1998). Shrimp waste, such as head and body carapace, was used for carotenoids extraction with various organic solvents and solvent mixtures (Sachindra *et. al.,* 2001). The recovered carotenoids can

be effectively used in aquaculture feed formulations, and the residue available after extraction may be used for the preparation of chitin/chitosan (Sachindra *et. al.*, 2006).

2.1.1.4 Food Industry/Cosmetics

The recovery of chemical components from seafood waste materials, which can be used in other segments of the food industry, is a promising area of research and development for the utilization of seafood wastes. Studies have shown that a number of useful compounds can be isolated from seafood waste including enzymes, gelatin and proteins that have antimicrobial and anti tumor capabilities. Chitosan, produced from shrimp and crab shell, has wide range of applications from the cosmetic to pharmaceutical industries (http://ift.confex.com).The shrimp waste consisted of 71.4% head and 28.6% shell (Meyers, 1986) which contains useful components such as protein, lipid and astaxanthin pigment in addition to chitin, thus making the commercial shrimp waste an attractive material for extraction of the above-mentioned components.(Mandeville *et. al.,* 1992). Whole shrimp heads from Northern pink shrimp (*Pandalus eous*), Endeavour shrimp (*Metapenaeus endeavouri*) and Black tiger shrimp (*Penaeus monodon*) were used for Shrimp Head Protein Hydrolysates (SHPH) isolation. SHPH can be used as a natural food additive to suppress the denaturation of myofibrillar proteins and maintain moisture in intermediate moisture foods (Ruttanapornvareesakul *et. al.,* 2005).

Proteases are the most important group of industrial enzymes used in the world and find several applications in the food industry (Garcia-Carreno *et. al.,* 1994). Proteases are mainly derived from plant, animal and microbial sources, whereas their counterparts derived from marine and other aquatic sources had not been extensively used (Haard & Simpson, 1994). Shrimp proteases can be used at industrial scale in food industry as they proved to be effective for beef meat tenderization, inactive after mild heat treatment, and active at low temperatures, thus resulting in energy savings through operation at room temperature.

Fish Protein Hydrolysate (FPH) prepared from the scrap of marine species was found to be effective as a cryoprotectant for the suppression of

denaturation of muscle protein of lizard fish meat during frozen storage, because it suppressed the decrease of unfrozen water, maintained a high gel-forming ability and Ca-ATPase activity (Khan *et. al.* 2003). Hydrolysates can be utilized as suppressive additives against myofibrillar protein denaturation and as a reagent to maintain moisture in food. FPH can also be utilised as an alternative substrate for culture of microorganisms viz., *Halobacterium salinarum, Escherichia coli, B. subtilis and Staphylococcus epidermidis* (Martone, *et.al.*, 2005).

The extraction of milk-clotting enzymes from fish stomach mucosa for cheese manufacture would provide an inexpensive alternative to rennet substitutes for domestic use or to export to cheese-producing nations, and would become a new food-related industry. However, further studies are required for testing tuna protease as rennet substitute at industrial scale (Tavares, *et.al.*, 1997).

Fish skin, bone and fin (from Skipjack tuna (*K pelamis)*, Japanese sea bass (*L japonicus)*, Ayu (*P altivelis)*, Yellow sea bream (*D. tumifrons),* Chub mackerel (*S. japonicus)*, Bullhead shark (*H. japonicus)* and Horse mackerel (*T. japonicus*) were examined for potential isolation of collagen. It was found that collagen recovery ranged from 36% to 54%, with the highest value recorded at Ayu (*P. altivelis)* bone, and the lowest at Japanese sea bass (*L. japonicus)* fin. Collagen from fish waste can be utilised in industrial level only for supplementing the skin of land vertebrates, and as alternatives to mammalian collagen in foods, cosmetics and biomedical materials (Nagai & Suzuki, 2000). The collagen-rich products can be used as functional material in the food industry, where jellification stands for a major application (Montero & Borderias ,1990).

2.2 Fish Collagen

Collagen is the major structural protein found in the skin and bones of all animals. Collagen is the most abundant protein of animal origin, comprising approximately 30% of total animal protein. Being a major constituent of the connective tissues, collagen plays an important part in creating mechanical strength, integrity and rheological properties of the muscles and fillets (Sikorski & Bordreias ,1994) Collagen molecules composed of three α-chains

intertwined in the so-called collagen triple-helix, adopt a 3D structure that provides an ideal geometry for inter-chain hydrogen bonding (Te Nijenhuis, 1997). Each chain in the helix rotates counter clockwise. The triple-helix is approximately 300 nm in length, and the chain has a molecular weight of approximately 10^5 kDa (Papon, *et.al.,* 2007). The collagen molecule is stabilized by interchain hydrogen bonds and by interactions of the radially extending amino acids residues with water molecules. The rod like molecules is aggregated into fibrils (Bailey & Etherlington, 1980). There are at least 19 variants of collagen, named type I–XIX (Bailey *et.al.*, 1998). Types I, II, III and V are the fibrous collagens. Type I collagen is found in all connective tissue, including bones and skins. It is a heteropolymer of two α-1 chains and one α-2 chain. It consists of one-third glycine, contains no tryptophan or cysteine and is very low in tyrosine and histidine. The triple helices are stabilized by inter-chain hydrogen bonds. There are twenty different amino acids in each α chain, and for each animal type of gelatin, these amino acids are in a specific repeated pattern. Glycine, which represents a third of the amino acids content, is in repeated sequence with two other amino acids. This might be represented as glycine-x-y. It is not unusual for x to be proline and y to be a hydroxyproline residue.

In fish, the greatest concentrations of collagen exist in the skeleton, fins and skin. Several studies have focussed on the characterization of different fish collagens (Kimura & Ohno, 1987; Montero, Alvarez *et.al.*, 1995; Montero *et.al.*, 1999; Nagai & Suzuki, 2000; Piez, 1965; Rigby, 1968; Sato *et.al.*, 1989; Sivakumar *et. al.,* 2000). Most fish collagens have been found to consist of two a-chain variants, which are normally designated as α -1 and α-2 (Gomez-Guillen *et. al.*, 2002; Nagai *et.al.*, 2001). These α chain variants, though having approximately the same molecular weight (95,000 Da), can be separated by SDS PAGE due to their different affinity for SDS. α 2 has a higher affinity for SDS and consequently exhibits a higher mobility than α 1 (Kubo & Takagi, 1984). Piez (1965) isolated three variants of α chains (α-1, α-2 and α-3) from cod skin collagen and found that these variants differed in their amino acid composition. Alpha 3 has also been isolated from Rainbow trout (Saito, *et. al.,* 2001), Horse mackerel and Eel (Kimura, *et.al.*, 1988; Yoshida, *et.al.*, 2001)

In addition to differences in molecular species, fish collagens have been shown to vary widely in their amino acid composition. In particular, the levels of imino acids (proline and hydroxyproline) vary significantly among fish species (Balian & Bowes, 1977; Gudmundsson & Hafsteinsson, 1997; Poppe, 1992). The amount of imino acids, especially hydroxyproline, depends on the environmental temperature in which the fish lives and it affects the thermal stability of the collagens (Balian & Bowes, 1977; Kimura *et.al.*, 1988; Rigby, 1968). Collagens derived from fish species living in cold environments have lower contents of hydroxyproline and they exhibit lower thermal stability than those from fish living in warm environments. This is because hydroxyproline is involved in inter-chain hydrogen bonding, which stabilizes the triple helical structure of collagen (Darby & Creighton, 1993). Cold water fish species are also reported to contain higher levels of hydroxyl amino acids, serine and threonine (Balian & Bowes, 1977). Grossman and Bergman (1992) showed that gelatin from tilapia, a warm water fish species, contains higher levels of imino acids than cold water fish collagens.

Collagen extracted from fish skin, a polymer that is a by-product of food manufacture, has various industrial applications in cosmetology and medicine. The engineering of type I collagen for medical exploitation, in the form of membranes, sponges or gels, originates mainly from calf skin dermis (Miyata &Taira1992; Chevallay, *et.al.*, 1998). Concern about prion contamination has stimulated the search for other, non-bovine, sources of this protein. Dermis fish collagen presents an interesting new source of collagen as it is a by-product of food fabrics and already has cosmetic uses. Differences in the amino acid composition of calf and flat fish collagens have been described; in particular a lower content in proline and Hydroxyproline content inducing a lower denaturation temperature in fish samples (Mathews, 1975).

2.3 Gelatin

In ancient times, gelatin was used as a biological adhesive and in the course of time it progressed to industrial manufacture and diverse applications. The use of gelatin for health purposes has been documented as early as the middle ages. This is a very important biopolymer that has found widespread use in the food and photographic industries over the years.

Traditionally it occurs as a transparent dessert jelly, but it is widely used in confectionery, jellied meats and chilled dairy products. Gelatin is a protein derived from collagen, the major constituent of animal connective tissue. The source and type of collagen will influence the properties of the resulting gelatins. The amino acid content and sequence varies from one source to another but always consists of large amounts of proline, hydroxyproline and glycine. Since most of the commercial gelatins are obtained from either pigskin or cowhide, there has been considerable interest in using alternative substitutes. This has especially been the case since the recent BSE crisis.

Gelatin is a soluble polypeptide derived from insoluble collagen. Procedures to derive this soluble polypeptide involve the breakdown of cross-linkages between polypeptide chains of collagen along with some amount of breakage of polypeptide chain bonds. When tissues that contain collagen are subjected to mild degradative processes i.e., treatment with alkali or acid followed or accompanied by heating in the presence of water, the systematic fibrous structure of collagen is broken down irreversibly and gelatin is formed (Ward & Courts 1977). It is the only food material that gels and melts reversibly below the normal human body temperature (37°C). Gelatin's unique and outstanding functional properties, along with its reasonable cost, make it one of the most widely used food and pharmaceutical ingredients.

2.3.1 Molecular Structure

Gelatin is a heterogeneous mixture of single or multi-stranded polypeptides, each with extended left-handed proline helix conformations and containing between 300 - 4000 amino acids. The triple helix of type I collagen extracted from skin and bones, as a source for gelatin, is composed of two α1(I) and one α2(I) chains, each with molecular mass ~95 kD, width ~1.5 nm and length ~0.3 μm. Gelatin consists of mixtures of these strands together with their oligomers and breakdown (and other) polypeptides. Solutions undergo coil-helix transition followed by aggregation of the helices by the formation of collagen-like right-handed triple-helical proline/hydroxyproline rich junction zones. Higher levels of these pyrrolidines result in stronger gels. There is some dispute over whether each of the three chains in the helical

structure has a 10/1 helix (the three strands forming a 10/3 helix) with a 85.8 Å axial repeat or a 7/1 helix (the three strands forming a 7/2 helix) with a 60 Å axial repeat, with tripeptides forming each unit. Although the former view seems prevalent at the present time, recent evidence indicates the latter to be correct (Okuyama *et.al.*, 2006a; 2006b). Each of the three strands in the triple helix require about 21 residues to complete one turn; typically there would be between one and two turns per junction zone (Oakenfull &.Scott, 2003). Gelatin films containing greater triple-helix content swell less in water and are consequentially much stronger [Bigi *et al*, 2004]. Chemical cross-links can be introduced, to alter the gel properties, using transglutaminase to link lysine to glutamine residues (Babin & Dickinson, 2001) or by use of glutaraldehyde to link lysine to lysine.

2.3.1.1 Structural Unit

Gelatin contains glycine (almost 1 in 3 residues, arranged every third residue), proline and 4-hydroxyproline residues. A typical structure is -Ala-Gly-Pro-Arg-Gly-Glu-4Hyp-Gly-Pro- (Figure 1)

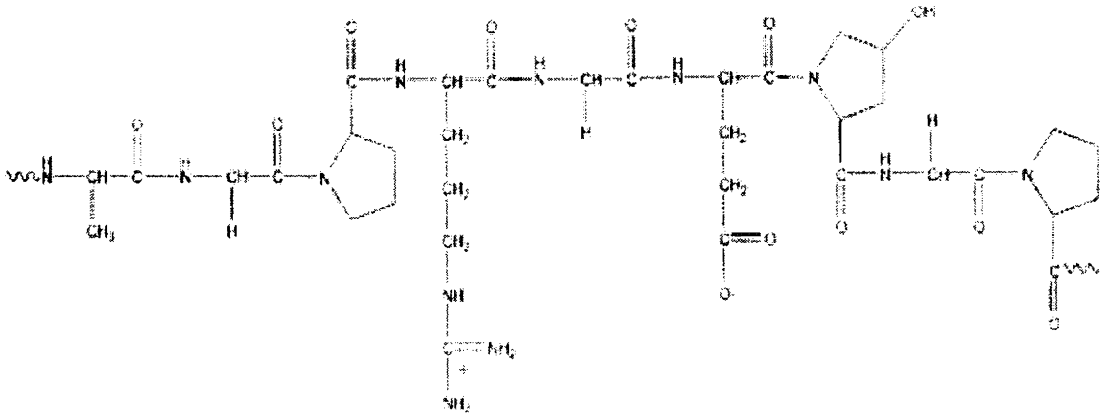

Fig. 1 Typical structure of gelatin

Gelatin is an amphoteric protein with isoionic point between 5 and 9 depending on raw material and method of manufacture. Like its parent protein, collagen (3), it is unique in that it contains 14% hydroxyproline, 16 % proline and 26 % glycine. The only other animal product containing hydroxyproline is elastin and then at a very much lower concentration, so hydroxyproline is used to determine the collagen or gelatin content of foods. In brief, the protein is

made up of peptide triplets, glycine - X - Y, where X and Y can be any one of the amino acids but proline has a preference for the X position and hydroxyproline the Y position. Approximately 1050 amino acids produce an alpha-chain with the left-handed proline helix conformation. Collagen exists in many different forms but gelatin is only derived from sources rich in Type I collagen which contains no cystine; however, hide or skin contains some Type III collagen which can be the source of cystine. Although Type I collagen contains no cystine, the alpha procollagen chains excreted by the cell do contain cystine at the C terminal end of the protein which is thought to be the site of assembly of 3 alpha-chains. The three chains then spontaneously coil together, in zipper fashion, to form a right-handed helix. After spontaneous helix formation, cross links between chains are formed in the region of the N terminal telopeptides (globular tail portion of the chains) and then the telopeptides (containing the cystine and tyrosine of pro-collagen) are shed leaving the rod-like ca. 3150 amino acid containing triple helix. These collagen rods assemble together with a quarter-stagger to form the collagen fibre and the fibres are stabilised by further cross-links.

Gelatin is primarily used as a gelling agent (Ledward, 1986) forming transparent elastic thermoreversible gels on cooling below about 35°C, which dissolve at low temperature to give 'melt in the mouth' products with useful flavor-release. In addition, the amphiphilic nature of the molecules endows them with useful emulsification (for example, whipped cream) and foam-stabilizing properties (for example, mallow foam). On dehydration, irreversible conformational changes take place (Mogilner *et.al.*, 2002) that may be used in the formation of surface films. Such films are strongest when they contain greater triple-helix content. Gelatin is also used as a fining agent to clarify wine and fruit juice.

Gelatin is the product of denaturation or disintegration of collagen. Initially the alpha-chains of collagen are held together with several different but easily reducible cross-links. As the collagen matures, so the cross-links become stabilised (Baily & Light,1989). Then as time progresses the eta-amino groups of lysine become linked to arginine by glucose molecules

(Maillard reaction) to form the pentosidine type cross-links which are extremely stable (Cole &. Roberts,1997). Hence when the alkaline processing is used on young animal skin the alkali breaks one of the initial (pyridinoline) cross-links and as a result, on heating, the collagen releases, mainly, denatured alpha-chains into solution (Cole &. Roberts,1997). Once the pentosidine cross-links of the mature animal have formed in the collagen, the main process of denaturation has to be thermal hydrolysis of peptide bonds resulting in protein fragments of various molecular weights i.e. polydisperse protein fragments. With the "acid process", the collagen denaturation is limited to the thermal hydrolysis of peptide bonds with a small amount of alpha-chain material from acid soluble collagen in evidence (Cole &. Roberts,1996). Nutritionally, gelatin is not a complete protein food because the essential amino acid tryptophan is missing and methionine is present only at a low level.

Type A gelatin (dry and ash free) contains 18.5 % nitrogen, but due to the loss of amide groups, Type B gelatin contains only about 18 % nitrogen (Eastoe & Leach, 1958.). Gelatin is abnormally stable and a special catalyst has to be used to obtain the correct Kjeldahl nitrogen content. The amino acid analysis of gelatin is variable, particularly for the minor constituents, depending on raw material and process used, but proximate values by weight are: glycine 21 %, proline 12 %, hydroxyproline 12 %, glutamic acid 10 %, alanine 9 %, arginine 8%, aspartic acid 6 %, lysine 4 %, serine 4 %, leucine 3 %, valine 2 %, phenylalanine 2 %, threonine 2 %, isoleucine 1 %,hydroxylysine 1 %, methionine and histidine <1% with tyrosine < 0.5 %. The peptide bond has considerable aromatic character, hence gelatin shows an absorption maximum at ca. 230 nm (Stevens, 1992).

The functional properties of gelatin are related to their chemical characteristics. The gel strength, viscosity, setting behaviour and melting point of gelatin depend on their molecular weight distribution and the amino acid composition (Johnston-Banks, 1990). It is generally recognized that the imino acids like proline and hydroxyproline are important in the renaturation of gelatin subunits during gelling (Johnston-Banks, 1990). As a result, gelatin with high levels of imino acids tends to have higher gel strength and melting

point. The molecular weight distribution is also important in determining the gelling behaviour of gelatin. According to Johnston-Banks (1990), the sum of intact α and β fractions together with their peptides is proportional to the gel strength while the viscosity, setting rate and melting point increase with increase in the amount of the high molecular weight fraction.

While the amino acid composition is mainly dependent on the source species (Eastoe & Leach, 1977), the molecular weight distribution of gelatin depends to a large extent on the extraction process (Muller & Heidemann, 1993). During conversion of collagen to gelatin, the inter- and intra-molecular bonds linking collagen chains as well as some peptide bonds are broken. The more severe the extraction process, the greater the extent of hydrolysis of peptide bonds and therefore the higher the proportion of peptides with lesser molecular weight. The age of the source animal may influence the ease with which gelatin can be extracted and the extent of peptide hydrolysis during the extraction (Cole & McGill, 1988; Reich *et.al.*, 1962). Older animal collagen is more cross linked and a more severe process is required to denature it to form gelatin (Reich et al., 1962). There are differences in the extent and type of cross linking found in bones and skins (Sims & Bailey, 1992). This may also affect the ease with which collagen may be solubilised and transformed to gelatin and may result in differences between the properties of gelatins extracted from the two tissues.

2.3.2 *Manufacture of Gelatin*

There are a large number of unit processes in the manufacture of gelatin. The raw materials from which it is derived are demineralised bone (ossein), pigskin, cow hide and fish skin. In China, donkey hide is also used quite extensively. In theory there is no reason for excluding any collagen source from the manufacture of gelatin, but the ones mentioned above are the currently commercially available raw materials. However, in countries where pork is sold with its skin intact, there is no pigskin available for gelatin manufacture.

2.3.2.1. Acid Process (Reich et. al., 1962)

The acid process is carried out with pigskin, fish skin and sometimes with bone as raw material. It is basically one in which the collagen is acidified to about pH 4 and then heated stepwise from 50°C to boiling to denature and solubilize the collagen. Thereafter the denatured collagen or gelatin solution has to be defatted, filtered to high clarity, concentrated by vacuum evaporation or membrane ultra-filtration treatment, to a reasonably high concentration for gelation and then drying by passing dry air over the gel. The final process is one of grinding and blending to customer requirements and packaging. The resulting gelatin has an isoionic point of 7 to 9 based on the severity and duration of the acid processing of the collagen which causes limited hydrolysis of the asparagine and glutamine amino acid side chains. The gelatin obtained by this method is referred as Type A gelatin which contains 18.5% nitrogen.

2.3.2.2. Alkali Process (Cole, 1966)

The alkali process is used for gelatin extraction from bovine hide and other collagen sources where the animals are relatively old at slaughter. The process is one in which collagen is submitted to a caustic soda or lengthy liming process prior to extraction. The alkali hydrolyses the asparagine and glutamine side chains to aspartic and glutamic acid (Veis, 1964) with the result that the gelatin has a traditional isoionic point of 4.8 to 5.2. However, with shortened (7 days or less) alkali treatment, isoionic points as high as 6 are produced. After the alkali processing, the collagen is washed free of alkali and treated with acid to the desired extraction pH (which has a marked effect on the gel strength to viscosity ratio of the final product). The collagen is then denatured and converted to gelatin by heating, as with the acid process. Because of the alkali treatment, it is often necessary to demineralise the gelatin solution to remove excessive amounts of salts using ion-exchange or ultrafiltration. Thereafter the process is the same as for the acid process - vacuum evaporation, filtration, gelation, drying, grinding and blending. The gelatin obtained by this method is referred as Type B gelatin which contains 18% nitrogen.

In the past, little emphasis has been placed on the animal age of the raw material, particularly in the case of gelatins from bovines, however it is now known that this factor plays a significant role in the molecular structure of the derived gelatin. The role of liming in the alkali process used to be considered one of progressive alkali hydrolysis of the collagen, which made it possible to denature the collagen at lower temperatures and thus maximise the yield of top quality gelatin. Recently, however, it has been shown that the role of liming is limited to the hydrolysis of one collagen cross-link which fluoresces at 290/380 nm and that liming has less and less effect on "extractability" as the animal gets older. The result is that alkali treatment times have been greatly reduced. One of the less well recognised effects of alkali treatment is the "opening up" of the hide collagen, as it is termed in leather manufacture, or the destruction of the proteoglycans associated with the collagen fibrils and this probably results in a more pure gelatin via the alkali process as is indicated by electrophoresis of the gelatin proteins (Cole & Roberts 1996).

2.3.3 *Properties of Gelatin*

Gelatin, the product of partial hydrolysis of collagen, finds a variety of applications in the food, photographic and pharmaceutical industries. Viscosity, gel strength, gelling and melting temperatures etc. govern the applications of gelatin. The properties of gelatin gels depend on the source and pretreatment of the raw material and parameters of the process. They are also affected by concentration of the gelatin, pH, the presence of interacting compounds, gel maturation time and temperature.

Gelatin displays multiple functional roles in food processing and formulations. The functional properties of gelatin can be divided into two groups (Schrieber & Gareis, 2007). The first has properties that are associated with gelling, for example, gel strength, gelling time, setting and melting temperatures, viscosity, thickening, texturizing, and water binding. The second group relates to the surface behavior of the gelatin, for example, emulsion formation and stabilization, protective colloid function, foam formation and stabilization (such as in marshmallow), film formation, and adhesion/cohesion (Schrieber &

Gareis, 2007). None of the hydrocolloids currently on the market is capable of covering all of the above-mentioned properties in all applications (Schrieber & Gareis, 2007).

2.3.3.1 Solubility in Water.

Gelatin is only partially soluble in cold water, however dry gelatin swells or hydrates when stirred in water. Such mixtures should generally not exceed 34 % gelatin. On warming about 40°C gelatin that has been allowed to hydrate for 30 minutes melts to give a uniform solution. Alternatively, dry gelatin can be dissolved by stirring in hot water, but stirring must be continued until dissolution is complete. This method is normally only used for dilute solutions of gelatin. (Cole, 2000)

If gelatin solutions are spray dried or drum dried from the sol state, the resulting gelatin is "cold water soluble" and such gelatins gel quickly when stirred into cold water. These gels are generally not clear, so the use of this form of gelatin is limited to milk puddings and other products where solution clarity is not required. The compatibility of gelatin in aqueous solution with polyhydric alcohols like glycerol, propylene glycol, sorbitol etc. is virtually unlimited and they are used to modify the hardness of gelatin films.

In products with limited moisture availability, as in confectionery, and where there is another polymer, as in glucose syrup, competing for the available water, then gelatin can be precipitated resulting in loss of gelation and cloudiness. In these cases the gelatin solubility is very dependent on the charge on the protein molecule or the pH of the product. Hence, the farther the product pH from the isoionic pH, the better will be the solubility and performance of the gelatin.

2.3.3.2 Gelling Properties.

The most common use of gelatin is its thermally reversible gelling properties with water as, for example, in the production of table jellies. An aqueous solution of a few percent gelatin forms thermally reversible gels with water, and the gel-melting temperature ($<35^0$C) is below body temperature,

which gives gelatin products unique organoleptic properties and flavor release (Glicksmann, 1969).The thermo reversibility of this process gives the gelatin gel its unique "melt-in-mouth" quality. Other gelling agents such as starch, alginate, pectin, agar, and carrageenan are all polysaccharides from plant sources, but their gels lack the melt-in-the-mouth, elastic properties of gelatin gels. Gelatin is notable for its gelling properties and clean flavor profile. The gelatin gel has been described as having a sparkling and clear appearance with clean melt-in-the- mouth texture that has yet to be duplicated by any polysaccharide (Baziwane & He, 2003).

In confectionery, gelatin is used as the gelling binder in gummy products, wine gums etc. In the manufacture of these products gelatin is combined with sugar and glucose syrups. Incompatibility between gelatin and glucose syrup can occur (Marrs,1982) and is a function of the concentration of glucose polymers containing more than 2 glucose units, contained in the syrup. Competition between gelatin and glucose polymers for water in low water content products can result in, at worst, precipitation of the gelatin and at best a marked loss in gelling properties or hardness of the product. It is also known that different gelatins with similar properties in water can have very different properties in confectionery formulations. Some raw fruits like pineapple contain proteolytic enzymes like bromelin which hydrolyse gelatin and destroy its gelling ability. In such cases it is essential that the fruit is cooked to destroy the protease before the fruit is added to gelatin solutions.

In general, the lower the mean molecular weight of a gelatin, the lower will be the gel strength and viscosity of its solution. However it has been shown that the collagen alpha-chain (MW 100 kDa and gel strength = 364 g Bloom) is the main contributor of gel strength and that higher molecular weight components (beta-chain with MW 200 kDa, gama-chain with MW 300 kDa and "microgel" with MW > 300 kDa) make a relatively low contribution to gel strength. (Cole,2000)

2.3.3.3 Emulsifying and Foaming Properties.

Gelatin, and to some extent also collagen, is used as a foaming, emulsifying, and wetting agent in food, pharmaceutical, medical, and technical applications due to its surface-active properties. Previous studies have shown that gelatin is surface-active and that it is capable of acting as an emulsifier in oil-in-water emulsions (Lobo, 2002). The hydrophobic areas on the peptide chain are responsible for giving gelatin its emulsifying and foaming properties (Cole, 2000; Galazka, *et.al.*, 1999). However, gelatin is generally a weaker emulsifier than other surface-active substances such as globular proteins and gum arabic. Therefore, when used on its own, gelatin often produces relatively large droplet sizes during homogenization (Chesworth, *et.al.*, 1985; Dickinson & Lopez, 2001; Lobo, 2002), and it has to be either hydrophobically modified by the attachment of nonpolar side-groups (Toledano & Magdassi, 1998), or used in conjunction with anionic surfactants to improve its effectiveness as an emulsifier (Muller & Hermel, 1994; Olijve, *et.al.*, 2001; Surh, *et.al.*, 2005).

The versatility of the emulsifying and foaming properties of gelatin is particularly valued in products like emulsified powders (Klaui, *et.al.*,1970). In such products, its surface active and film-forming characteristics can be successfully exploited during the emulsification process. Its stabilization and gelation characteristics are useful during the subsequent drying and encapsulation stages. In marshmallows, the gel-forming properties of gelatins are used to stabilize the foam upon cooling. In most applications, gelatin is chosen not only for its surface-active properties, but rather because of its unique combination of surface active, chemical, rheological, and gelling properties. For example, in gelatin-foamed foods and ice creams, the unique gel melting behavior in the range of 10–30 ^{0}C results in the melting of gelatin gels in the mouth (De Wolf, 2003).

Dickinson and Lopez (2001) have compared the emulsion stabilizing properties of a set of commercial casein and whey protein ingredients, under neutral pH conditions, with the properties of commercial fish gelatin as an emulsifying agent in oil soluble vitamin encapsulation. They noted that when gelatin is used as an emulsifying agent, the protein/oil ratio should be

optimized in order to avoid the presence of large droplets that could lead to coalescence, especially at high ionic strength. Conversely, where milk protein is intended as a replacement for gelatin in existing emulsion products, attention should be given to the effect of flocculation of whey protein-coated droplets on storage.

2.3.3.4 Protective Colloid/Crystal Habit Modifying Properties.

If a jelly is frozen, the product will suffer from syneresis and on thawing the clear jelly will disintegrate with much exuded water. However, if water containing 0.5 % gelatin is frozen, the water will freeze as millions of small discrete crystals, instead of forming a single solid block of ice. This effect is most desirable in "ice lollies" and is also used in ice cream manufacture to obtain a smooth product with small ice crystals and also to ensure that any lactose precipitates as fine crystals avoiding the development of graininess with time. (Cole,2000)

2.3.3.5 Film Forming Properties.

Edible films are thin films prepared based on a biopolymer. Edible films and coating materials are potentially used to extend the shelf-life and improve the quality of almost any food system by serving as mass transfer barriers to moisture, oxygen, carbon dioxide, lipid, flavor and aroma between food components and the surrounding atmosphere (McHugh, 2000). The main biopolymers used in the edible films preparations are polysaccharides (Nisperos– Carreido, 1994) and proteins (Torres, 1994). Among the proteins, collagens(Gennadios, *et.al.*, 1994) and fish myofibrillar proteins(Cuq *et.al.*, 1995: Cuq *et.al.*, 1997a,b,c) were studied as film bases for edible film preparations.

Among all proteins, gelatin has been attracted the attention for the development of edible films due to its abundance and biodegradability (Bigi *et.al.*, 2002). The use of gelatin in the preparation of edible films or coatings was very well studied and produced many patents, particularly in the field of pharmaceuticals (Gennadios *et.al.*, 1994: Torres, 1994). Collagen and gelatin films have been used for sausage casing (Johnston-Banks, 1990), production

of hard and soft capsules, wound dressing and adsorbent pad in the pharmaceutical industry (Digenis *et.al.*, 1994).Gelatin edible films, with high puncture strength, low puncture deformation and high water vapor permeability, prepared from bovine and porcine skin have been reported (Sobral, *et.al.*, 2001). Edible wrappings based on blends of gelatin with other constituents have been marketed (Torres, 1994). Carvalho *et.al.*, (1997a) prepared edible films from bovine hide gelatin and determined its mechanical properties. The drying properties of films of gelatin plasticized with sorbitol, has been studied in detail by Carvalho *et.al.*, (1997b) and Menegalli *et.al.*, (1999). Sobral (1999) studied the influence of thickness on the mechanical properties, water vapour permeability and the colour of the films made from bovine hide and pigskin gelatins, plasticized with sorbitol. Arvanitoyannis, *et.al.*, (1997 &1998) elaborated on the thermal and functional properties of film made from the blend of gelatin and edible starch incorporating plasticizers.

There has been a review on gelatin films (Arvanitoyannis, 2002), and a considerable body of recently published work on the use of gelatin to obtain edible films is available in the literature (Menegalli, *et.al.*, 1999; Sobral, *et.al.*, 2001; Simon-Lukasik & Ludescher, 2004; Bertan, *et.al.*, 2005). However, the bulk of this information concerns commercial mammalian gelatins. Although researchers are now increasingly turning their attention to fish gelatin films (Muyonga, *et.al.*, 2004a; Jongjareonrak, *et.al.*, 2006a,b; Gómez-Guillén, *et.al.*, 2007; Carvalho, *et.al.*, 2008), the list of literature references dealing with these latter films is considerably shorter. The present literature seems to bear out that there are some differences in the physical properties of films obtained from mammalian and fish gelatins, the former being stronger and more permeable to water vapour and the latter more elastic (Sobral, *et.al.*, 2001; Thomazine, *et.al.*, 2005; Avena-Bustillos, *et.al.*, 2006; Gómez-Guillén, *et.al.*, 2007) although it remains somewhat unclear. Comparability of the data is limited because of the wide range of different experimental conditions employed for film producing, i.e., plasticizer type and concentration, dehydration temperature, film thickness and conditioning, etc. Gomez *et.al.*, (2009) made a comparative study on the physico-chemical and film forming properties of tuna skin gelatin and bovine hide gelatin. It was observed that bovine hide gelatin film has high water vapour permeability whereas

deformability was considerably higher (10 times higher) in the tuna-skin gelatin film. In contrast, breaking force and water solubility were basically unaffected by gelatin origin. Analysis of the thermal properties revealed both films to be wholly amorphous with similar glass transition temperature values

2.3.3.6 Micro Encapsulation - Mixed Film Forming Properties.

One of the major applications of fish gelatin is in the micro encapsulation of vitamins and other pharmaceutical additives such as azoxanthine. Fish gelatin may also be used in the micro encapsulation of colorants. Soper (1999) described a method for micro encapsulation of food flavors such as vegetable oil, lemon oil, garlic flavor, apple flavor, or black pepper with warm-water fish gelatin (150–300 Bloom). Besides being precipitated by polymers competing for water, gelatin is amphoteric. Hence, at a pH where the basic side chains do not carry a charge, acid groups for example from gum arabic can react with the basic groups of gelatin to form an insoluble gelatin-arabate complex which can be precipitated around emulsified oil droplets, forming micro-ecapsulated oil. The microcapsules are hardened with formaldehyde or glutaraldehyde before harvesting and drying. In this application the pH of the gelatin is critical. This process has been used in the food industry for encapsulating flavours.

2.3.3.7 Milk - Food Stabilising Properties.

Gelatin is used as a stabilizer particularly in yoghurt, where the addition of 0.3 - 0.5 % prevent syneresis thus allowing the production of stirred fruit containing products. In this instance the gelatin reacts with the casein in the milk to reduce its tendency to separate water from the curd. Gelatin can also be used in cheese manufacture to improve yield and in the stabilisation of thickened cream. (Cole, 2000)

2.3.3.8 Fruit Juice Clarifying Properties.

In "fining" applications, gelatin reacts with polyphenols (tannins) and proteins in fruit juices forming a precipitate which settles leaving a supernatant which is stable to further cloud formation with storage time. In wine, usage

levels are about 1 to 3 g/hL and excess usage, which would lead to protein instability, needs to be avoided. Traditionally, low bloom strength gelatins are used but it has been shown that high bloom strengths are equally effective (Bestbier, 1983). However, from the practical point of view, the use of low bloom Strength gelatin is cheaper and makes it easier to mix the gelatin into the bulk of the cold juice before gelation can occur. In this regard, it has become common practice to treat cold grapes, during the initial crushing process, with gelatin that has been hydrolysed to the extent that it can no longer gel.

2.3.3.9 Texturising Properties.

The texture of commercial gelatin desserts is usually evaluated by measuring the Gel strength, which in gelatin industry refers to non fracture rigidity (Wainewright, 1977). For the same measurement conditions i.e., sample shape and size, maturation temperature and time, and instrumental parameters, the gel strength of a gelatin dessert would depend mainly on the properties of the gelatin and its concentration. Although gel strength is one of the important commercial criteria for gelatin desserts, this parameter may not represent all the textural properties encountered during human consumption of the product, which includes processes using the fingers (sometimes), and the teeth and tongue under both nondestructive and destructive conditions. Previous studies using small strain rheological tests (Gilsenan & Ross-Murphy, 2000; Gudmundsson, 2002; Joly-Duhamel, *et.al.*, 2002;Zhou, *et.al.*, 2006)provided useful information on the properties of gelatin gels, but these small strain rheological measurements generally did not correlate well with sensory evaluation (Bourne, 2002; Foegeding, *et.al.*, 2003). On the other hand, the large deformation rheological tests were found to correlate better with the sensory studies on gelled systems (Munoz, *et.al.*, 1986a, 1986b; Barrangou, *et.al.*, 2006), and have been applied to measure gelatin gel textures (Bot, *et.al.*, 1996; Surowka, 1997). In addition, texture profile analysis (TPA), a method introduced by Szczesniak and co-workers (Szczesniak, *et.al.*, 1963; Friedman, *et.al.*, 1963) to measure the mechanical textural characteristics of foods, was also found to correlate well with specific sensory

characteristics. This method was further developed by Bourne (1968, 1978, 1995, 2002), who adapted the Instron Universal Testing Machine to food studies and clarified the mechanical textural parameters that could be calculated from a TPA curve.

2.3.3.10 Nutritional Properties.

Gelatin is not a complete protein source because it is deficient in tryptophan and low in methionine content. However, the digestibility is excellent and it is often used in feeding invalids and the high level of lysine (4 %) is noteworthy. Studies have shown that the consumption of 7 to 10 g/day can significantly improve nail growth rate and strength (Schwimmer & Mulinos,1957) and it also promotes hair growth (U. S Patent 4,749,684., 1988) Gelatin has also been shown to benefit arthritis sufferers in a large proportion of cases (Adam, 1991, 1995).

2.3.3.11 Stability.

Dry gelatin has an almost infinite shelf life as long as the moisture content is such that to ensure the product is stored below the glass transition temperature. The stability of gelatin in solution depends on temperature and pH. Generally, to minimize loss of gel strength and viscosity with time, the pH of the solution should be in the range 5 to 7 and the temperature should be kept as low as possible, consistent with the avoidance of gelation and the suitability of the solution viscosity to the particular application. Often the cause of degradation or hydrolysis of gelatin in solution is microbial proliferation, so gelatin solutions should not be stored for longer than is absolutely necessary, and after addition of the acid to confectionery formulations, the solution should be used and cooled/gelled with minimal delays. (Cole, 2000)

2.3.3.12 Corrosive Properties.

Gelatin attacks 304 stainless steel and containers will be perforated after a few months of continuous usage. With gelatin, it is essential to use 316 stainless steel for storage and extraction purposes (Cole, 2000).

2.3.4 Applications of Gelatin

A simplified characterization of the applications of gelatin would be into the following four uses:

1. Edible gelatin - Free of heavy metals and aesthetically suitable for eating.
2. Industrial gelatin - Where the chemical and physical properties are uniquely suitable for an industrial application. A good example would be gelatin used for the micro encapsulation of dye precursors for carbonless paper.
3. Photographic gelatin - The requirements being extremely critical. Photographic film requires a long shelf-life, and the gelatin has a major impact on the silver halide chemistry that requires the ability to take a picture and be able to develop it later with standard developing conditions.
4. Glue - Essentially for adhesive or gluing applications.

2.3.4.1 Food Applications

Gelatin has a considerable number of food applications and uses (Cole, 2000; Hudson, 1994; Keenan, 1994; Poppe, 1997). Gelatin has been used in foods as a beverage clarifier, a fining agent for white wine, as a beer clarifier, and to clarify fruit and vegetable juice (especially for clarified apple juice and pear juice). Gelatin is used in desserts at 8-10% of the dry weight, in yogurt at 0.3-0.5% as a thickener, in ham coatings at 2-3%, and in confectionery and capsules (vitamin supplements) at 1.5-2.5% (Igoe, 1983). Further uses include: fruit toppings for pastry, instant gravy, instant sauces and soups, edible films for confectionery products (McCormick, 1987), and as a stabilizer in ice cream, cream cheese, and cottage cheese as well as in food foams and fruit salads. Overall functional uses include as a stabilizer, thickener, and texturizer. Schrieber & Gareis (2007) have discussed in detailed various food

and non-food applications of gelatin. In spreads its roles are low-fat products stabilizer, water-binding agent (in part), fat substitution and excellent mouth feeling. Fish gelatin and pectin have been used to make a low-fat spread (Cheng, *et.al.*, 2007). It was found that a decrease in the fish gelatin to pectin ratio resulted in an increase in bulk density, firmness, compressibility, adhesiveness, elasticity, and meltability.

The largest single use of Gelatin in food products is in water gel desserts. Gelatin desserts consist of the mixtures of gelatin powder, sweetener, water, and appropriate flavors and colors, along with a pH balancing of ingredients. The melt-in-the-mouth property is one of the most important characteristics of gelatin-based gel desserts (Zhou and Regenstein, 2007). Some other biopolymers, such as agar and carrageenan, can also form thermally reversible gels with water, but the melting temperature of these gels is higher than the human body temperature. Commercial water gelatin desserts are generally made of gelatins from pork or non-religiously slaughtered beef, which are unacceptable to Jews and Muslims. Some consumers are also concerned about BSE in mammalian gelatins. Fish gelatins can satisfy the requirements of these consumer groups. In addition, gelatin desserts made from various gelatins may provide variety in textural and gel melting properties, offering new product development opportunities. The flavored fish gelatin dessert product had less undesirable off flavour and off odour and a more desirable release of flavour and aroma than the product made from an equal bloom, higher melting point pork gelatin(Choi & Regenstein, 2000).

2.3.4.2 Pharmaceutical Applications

These are the dosage form of choice for medicines and food supplements. They are also the ideal packaging for powdered and granulated products. High Bloom gelatin (between 200 and 260 Bloom) is used for the production of hard gelatin capsules. Fish gelatin has also been used in the preparation of pharmaceutical products. Park, *et.al.*, (2007) patented a process describing the preparation of a film-forming composition for hard capsules composed of fish gelatin. Using transglutaminase for crosslinking

circumvented the problems caused by the low gelling temperature property of fish gelatin. Another patent (Hansen, *et.al.*, 2002) described the use of fish gelatin (Bloom value higher than 100) as an ingredient in drug tablets.

Oil-soluble vitamins can be processed into easily dosable, freely flowing powders with the help of gelatin. An emulsion consisting of gelatin, sugar, and the active substance is spray-dried and cross-linked to a degree dependent on the release profile required. Low bloom pigskin and hide split gelatins are used for this application.

Very special types of gelatin – those that conform to the most stringent requirements and which are subsequently modified by the pharmaceutical industry – can be used as a blood substitute or plasma expanders. High bloom bone gelatin (220–270 Bloom) is used for this purpose. In emergency medicine, natural pure gelatin can also be used as a "biological adhesive" in hitherto risky microsurgery.

Sponges made from pharmaceutical gelatin are completely resorbed by the body. They are used routinely in dental medicine and in surgery and – if required –can be impregnated with antibiotics. High bloom hide split and pigskin gelatin are used for sponges.

Gelatin is a natural binding agent used in sugar- coated tablets, wound dressings, suppositories or granulation. Depending on the application medium Bloom to high Bloom hide split, pigskin or bone gelatin is used. The amino acid composition of gelatin corresponds to a high degree to that of joint cartilage; it can thus help in the prevention of arthritis. Relief is provided to already existing conditions and already after just a few weeks of administration, increased mobility of the joints and relief from pain can be observed. Gelatin stops bone loss in patients with osteoporosis, relieves pain and increases mobility. Gelatin hydrolysate is found to be effective in the treatment of osteoporosis with a dosage 10g per day as ready to use liquid product (Eggersglüss, 1999)

2.3.4.3 Photographic applications

In the manufacture of photographic film, only gelatin allows the light-sensitive substances to be coated onto the carrier materials in numerous thin

layers. Photographic gelatin due to its thermoreversible gelling properties, binds light-sensitive silver halides. It is coated onto a special carrier material where its swelling power facilitates picture processing. Gelatin also stabilizes the coupler and dye emulsions that are used in color photography. *Imagel,* an innovative gelatin product has specific surface properties, which are eminently suited to the production of ink jet printing paper and foil resulting in professional and realistic prints of brilliant photographic quality. The low gelling temperature of gelatin from cold-water fish makes it useful as the base for light-sensitive coatings. Cold-water fish gelatin is also a good medium for precipitating silver halide emulsions since this process can be carried out at a lower temperature with fish gelatin than with warm-blooded animal gelatin (Norland, 1990).

2.3.5 *Gelatin from Fish*

Gelatin from marine sources (warm and cold-water fish skins, bones, and fins) is a possible alternative to bovine gelatin (Kim & Mendis, 2006; Rustad, 2003; Wasswa, *et.al,* 2007). One major advantage of marine gelatin sources is that they are not associated with the risk of Bovine Spongiform Encephalopathy. Fish gelatin is acceptable for Islam, and can be used with minimal restrictions in Judaism and Hinduism. Furthermore, fish skin, which is a major byproduct of the fish-processing industry, causing waste and pollution, could provide a valuable source of gelatin (Badii & Howell, 2006). Fish skin contains a large amount of collagen: Nagai & Suzuki (2000) reported that the collagen contents in the fish skin waste of Japanese sea-bass, chub mackerel, and bullhead shark were 51.4%, 49.8%, and 50.1% (dry basis), respectively.

Production of fish gelatin is actually not new as it has been produced since 1960 by acid extraction, although most of it has been used for industrial applications (Norland, 1990). Detailed extraction procedures and characterization of the properties of fish gelatin were described by Grossman & Bergman (1992) in a United States patent. Since then, multiple research groups have further investigated the various aspects of fish gelatin. Gelatin has been extracted from skins and bones of various cold-water (e.g., cod, hake, Alaska pollock, and salmon) and warm-water (e.g., tuna, catfish, tilapia, Nile perch, shark, and megrim) fish. Table 2.3 lists the various reports

currently available in the literature on the extraction and characterization of fish gelatin.

Fish skin gelatin is available commercially and can be produced for kosher use provided that the appropriate conditions are met (such as the use of fish having scales). Fish gelatin with normal gel strength has normal hydroxyproline content and is made from warm water fish and not necessarily from fresh water, although this is normally the case. Fish gelatin with low or no gel strength (Ward, 1958) has a low hydroxyproline content (Eastoe & Leach, 1958.) and is produced from cold water species which are sourced typically from the sea. Fish glue is a crude gelatin product only suitable for technical application. At present, the fish gelatin production is minor, yielding about 1% of the annual world gelatin production of 250,000 tons.

The melting and gelling temperature of gelatin has been found to correlate with the proportion of the imino acids proline and hydroxyproline (both with a 5-membered pyrrolidine ring) in the original collagen. This is typically, 24% for mammals and 16–18% for most fish species. Cold water fish, for example cod, have a very low hydroxyproline content and coupled with this a very low gelling and melting temperature.

2.3.5.1 Sources of Fish Gelatin

The fish skins and bones can be processed into gelatin, thus contributing to solving the problem of waste disposal with the advantage of value addition. In order to be applied to food and pharmaceutical industries, fish gelatin must possess the following characteristics. First, a large quantity of by-product and its economical collection are essential to be continuously produced in industry. Second, gelatin from fish byproducts must have rheological properties (gel strength, gelling and melting points, etc.) at the level of mammalian gelatin. However, it is not easy for fish byproducts to satisfy the above two categories because of their typical physical properties. The main draw back of the fish gelatins are the gels based on them tend to be less stable and have inferior rheological properties than mammalian gelatins. It may be noted that fish gelatin has its own unique properties like better release of a product's aroma and flavor with less inherent off-flavor and off-odor than a commercial pork gelatin, which offer new opportunities to product developers (Choi &

Regenstein 2000). The amount of gelatin obtained commercially from fish and other species increased consistently from 2003 to 2005. Over this period, the percent of gelatin from fish and other marine species increased from 0.7% to 1.3% of total world production (GME, 2007).

In the past decade, fish gelatin extraction has been reported from many species viz., sole(Devictor, *et.al.*, 1995),cod (Gudmundsson & Hafsteinsson, 1997), hake(Montero, *et.al.*, 1999), blue shark (Yoshimura,*et.al.*,2000b), megrim (Montero & Gomez-Guillen, 2000;Gomez-Guillen &Montero, 2001), black tilapia & red tilapia (Jamilah & Harvinder, 2002), yellowfin tuna (Lefebvre, *et.al.*, 2002; Cho, *et.al.*, 2005), Alaska pollock(Zhou &Regenstein, 2004, 2005), horse mackerel(Badii & Howell, 2006), skate (Cho, *et.al.*, , 2006) Catfish) (Yang, *et.al.*, 2007; Liu, *et.al.*, 2008), Nile Perch (Muyonga, *et.al.*, 2004a), Grass carp , (Kasankala, *et.al.*, 2007) Bigeye snapper and Brown eye red snapper (Jongjareonrak, *et.al.*, 2006). The main drawbacks of the fish gelatins are the gels based on them tend to be less stable and have inferior rheological properties than mammalian gelatins.

Table 2. 3 References cited in literature on the extraction and characterisation of fish gelatin

Source	Reference
Baltic cod (*Gadus morhua*), salmon (*Salmo salar*), herring (*Clupea harengus*)	Kolodziejska *et.al.*, (2008)
Flounder (*Platichthys flesus*)	Fernandez-Diaz *et.al.*, (2003)
Alaska pollock (*Theragra chalcogramma*)	Zhou & Regenstein (2005)
Megrim (*Lepidorhombrus boscii*)	Fernandez-Diaz *et.al.*,(2001)
Hake (*Merluccius merluccius*), Dover sole (*Solea vulgaris*)	Gomez-Guillen *et.al.*,(2002),Sarabia *et.al.*, (2000)
Horse mackerel (*Trachurus trachurus*)	Badii & Howell (2006)
Cod (*Gadus morhua*)	Gudmundsson & Hafsteinsson (1997), Fernandez-Diaz *et.al.*, (2001)
Catfish (*Ictalurus punctatus*)	Yang *et.al.*, (2007), Liu *et.al.*, (2008,2009)
Sin croaker (*Johnius dussumieri*), shortfin scad (*Decapterus macrosoma*)	Cheow *et.al.*, (2007)
Black tilapia (*Oreochromis mossambicus*), red tilapia (*Oreochromis nilotica*)	Jamilah & Harvinder (2002)
Bigeye snapper (*Priacanthus macracanthus*), brownstripe red snapper (*Lutjanus vitta*)	Jongjareonrak *et.al.*, (2006)
Yellowfin tuna (*Thunnus albacares*)	Chiou *et.al.*, (2006)
Blue shark (*Prionace glauca*)	Yoshimura *et.al.*, (2000)
Nile perch (*Lates niloticus*)	Muyonga *et.al.*, (2004a)
Grass carp (*Ctenopharyngodon idella*)	Kasankala *et.al.*,(2007)
Skate (*Raja kenojei*)	Cho *et.al.*, (2006)
Atlantic salmon (*Salmo salar*), Harp seal	Arnesen & Gildberg (2007)
Dover sole (*Solea vulgaris*)	Gimenez *et.al.*,. (2005)
Lizardfish (*Saurida spp.*)	Wangtueai, S.and Noomhorm,A (2008)
Cuttlefish (*Sepia pharaonis*)	Aewsiri, *et.al.*, (2008)
Big eye snapper (*Priacanthus hamrur*)	Binsi *et.al.*,.(2009)
Shark	Yoshimura *et.al.*, (2000)
Lumpfish	Osborne *et.al.*,(1990)

2.3.5.2 Marine Fish

Several reports have been published on the utilization of the skin of marine fish species for the extraction of gelatin. Arnesen & Gildberg (2007) studied the characteristics of gelatin from the skin of Atlantic salmon and cod prepared by acid extraction. In three separate experiments the average yields of gelatin from salmon and cod skins were 39.7% (±2.2%) and 44.8% (±0.2%) respectively, on a dry matter basis. Gelatin from salmon contained slightly more hydroxyproline and proline (16.6%) than cod gelatin (15.4%), whereas the content of serine was lower (4.6% versus 6.3%). Salmon gelatin expressed slightly higher gelling temperature (12 ^{0}C) than cod (10 ^{0}C), and higher initial gel strength. During storage at 10 ^{0}C, gel strengths were increased and more so with gels made from cod than from salmon gelatin. Hence, gels made from cod and salmon gelatins extracted at 56 ^{0}C achieved the same gel strength (195 g) after 7 days of storage. Gelatins extracted at a higher temperature (65 ^{0}C) gave lower gel strengths.

Extraction of gelatin from Megrim *(L.boscii)* skins with different organic acids has been reported by *Gomez*-Guillen & Montero(2001). The type of acid used influenced the viscoelastic and gelling properties of gelatin. Acetic and propionic acid extracts produced gelatins with the highest elastic and viscous moduli, melting temperature and gel strength , particularly when pre treated with dilute sodium hydroxide. A study by Gudmundsson & Hafsteinsson (1997), indicated that concentrations of sodium hydroxide, citric and sulfuric acids used in the extraction of gelatin from cod skins affected both yield and quality and the process can be optimized to obtain the desired properties for gelatin. This study also showed that freeze dried gelatin had considerably higher bloom value than air dried gelatin. Zhou & Regenstein (2004) observed that the best extraction of gelatin from the skin of Alaska pollock (*T. chalcogramma*) was possible under optimized process conditions using Response Surface Methodology which yielded good quality gelatin with a gel strength of 460gf, viscosity 6.2 cP and yield of 18%. An alkaline pretreatment followed by an acid neutralization could not only remove non collagenous

proteins and but also result in high gelatin yield with good gel property in a neutral or weak acid medium (Zhou & Regenstein ,2005)

Optimisation study on the extraction of gelatin from dorsal skin of yellowfin tuna (*T. albacares)* using response surface methodology, and comparison of physical properties of yellowfin tuna skin gelatin with those of two mammalian skin gelatins (bovine and porcine) was carried out by Cho *et.al.*, (2005) .The gel strength of yellowfin tuna skin gelatin (426 Bloom) was higher than bovine and porcine gelatins (216 Bloom and 295 Bloom, respectively), while gelling and melting points were lower. Dynamic viscoelastic properties of yellowfin tuna skin gelatin did not change at 20^0C, but increase at 10^0C as a similar pattern with mammalian gelatins.

Fish skin gelatin was extracted from the skin of bigeye snapper (*Priacanthus macracanthus*) and brownstripe red snapper (*Lutjanus vitta*) with yields of 6.5% and 9.4% on the basis of wet weight, respectively (Jongjareonrak *et.al.*, 2006). Both skin gelatins having high protein, low fat and high hydroxyproline content (75.0 and 71.5 mg/g gelatin powder). The bloom strength of gelatin gel from brownstripe red snapper skin gelatin (218.6 g) was greater than that of bigeye snapper skin gelatin (105.7 g).

A comparative study was carried out on the structural and physical properties of gelatin extracted from the skins of different marine species viz., megrim (*L.boscii*), Dover sole (*S.vulgaris*), cod (*G.morhua*), hake (*M.merluccius.L)* and squid (*D.gigas*) by Gomez-Guillen *et.al,* (2002). It was reported that gelatin from flat fish species (sole and megrim) had better gelling ability and thermostability than that obtained from cold water species (cod and hake). The difference in the physical properties is due to the differences in amino acid composition, the α1/ α2 collagen-chain ratio and the molecular weight distribution. The cod gelatin had a lower alanine and imino acid content and a decreased proline hydroxylation degree. Cod and hake gelatins had a low α1/ α2 ratio (~ 1) and hake gelatin showed a significant decrease in β-components and other aggregates. Squid gelatin showed viscoelastic properties intermediate between flat fish species and cold water species.

Skins of two tropical fishes sin croaker (*J. dussumieri*) and shortfin scad (*D. macrosoma*) were used for the preparation of gelatin and their physico chemical characteristics were compared with commercial bovine gelatin (Cheow, *et.al.*, 2007). Shortfin scad gelatin had higher melting and gelling temperatures compared to that from sin croaker. The former can be of potential use as an alternative to mammalian gelatin due to its good visco elastic properties. Gelatins prepared from the fermented skin of skate (*Raja kenojei*) under optimum extraction conditions were found to have similar physicochemical properties and higher yields compared with gelatins produced from the skin of other marine species. Activated carbon treatment and freeze drying improved the colour and eliminated fishy odour (Cho, *et.al,* 2006).

2.3.5.3 Fresh Water Fish

Jamilah & Harvinder (2002) reported the extraction and determination of the physico-chemical characteristics of gelatin from the skins of black (*Oreochromis mossambicus*) and red (*O. nilotica*) tilapia. The gelatins from both the black and the red tilapias were snowy white, shiny and light-textured. The gelatin of black tilapia skin had a strong fishy odour while that of the red tilapia skin had a barely detectable odour. Their pH values were in the vicinity of 3. The bloom strength of gelatin from black tilapia skin was higher (180.8 g) than that from red tilapia skin (128.1 g). The black tilapia skin gelatin was also significantly more viscous, had a higher melting point, and had higher total amino acid content.

Response Surface Method was used to determine the optimum operating conditions for extracting the gelatin from channel catfish skin (Liu *et al.,* 2008). The optimal conditions for maximum gel strength are 68.8 h for the time of treatment with calcium hydroxide solution, 43.2^{0}C for the extraction temperature, 5.73 h for the extraction time with hot water. The gelatin from channel catfish skin showed high gel strength of 276 ± 5 g.

Kasankala *et. al.*, (2007) studied the optimum gelatin extraction conditions from Grass carp fish skin using response surface methodology (RSM). The predicted responses were 19.83% gelatin yield and 267 g gel strength. Gelling and melting points were 19.5^{0}C and 26.8^{0}C, respectively.

Grass carp gelatin showed high contents of imino acids (proline and hydroxyproline of around19.47%).

Type A gelatins were extracted from skins and bones of young and adult Nile perch and analysed to determine their functional and chemical properties (Muyonga *et. al.*, 2004a). Total gelatin yield was highest from adult fish skins and lowest from the bones of young fish while percentage gelatin recovery at 50^0 C was maximum from young fish skins. The gelatins obtained were free of fishy odour. Nile perch skin gelatin had turbidity of 20.5– 158 NTU and ash content of 0.5–1.7% while bone gelatins had turbidity of 109–517 NTU and ash content of 4.4–11.2%. Bloom gel strength was 81–229 and 134–179 g, respectively, for skin and bone gelatins. Gelatin from adult Nile perch skins exhibited higher viscosity and lower setting time than bone and the young fish skin gelatins. Skin gelatins were found to exhibit higher film tensile strength but lower film percent elongation than bone gelatins. Bone and skin gelatins had similar amino acid composition, with a total imino acid content of about 21.5%. Nile perch skin gelatins had a higher content of polypeptides compared to bone gelatins. The differences in functional properties between the skin and bone gelatins appeared to be related to differences in molecular weight distribution of the gelatins.

2.3.5.4 Gelatin from the Bone, Cartilage and Scales of Fish

There are considerable differences between extractability and yield of gelatin from fish skins and bones. Skin and bone gelatins differ in their functional properties and molecular weight distribution. These properties also vary with age of source fish, although the influence of age is less pronounced. Skin collagen easily denatures to give gelatin at low temperature, even from adult fish while bone collagen on the other hand requires a more severe heat treatment. As a result, bone gelatin consists of a high proportion of low molecular weight fractions, which are associated with poor gelling properties. Additional pre-treatment such as liming may be required to obtain bone gelatin with better functional properties.

The properties of gelatin from the bones and skin of Nile Perch was studied by Muyonga *et. al.*,(2004a). Skin gelatins were found to exhibit higher film tensile strength but lower film percent elongation than bone gelatins. Bone

and skin gelatins had similar amino acid composition, with a total imino acid content of about 21.5%. Nile perch skin gelatins had a higher content of polypeptides compared to bone gelatins. Both bone and skin gelatins also contained low molecular weight peptides. The differences in functional properties between the skin and bone gelatins appeared to be related to differences in molecular weight distribution of the gelatins.

Cho *et. al., (2004)* optimsed the process for extraction of gelatin from the cartilage of shark (*I. oxyrinchus*) and examined the functional properties in comparison with porcine gelatins. Shark cartilage gelatin had lower concentration of hydroxyproline and showed higher turbidity than porcine skin gelatins. Foam formation ability, foam stability, water-holding capacity and gel strength of shark cartilage gelatin were lower than the two porcine gelatins, but fat-binding capacity was higher in the shark cartilage gelatin. The lower amount of imino acids with low content of hydroxyproline seems to be the main reason for low gel strength of shark cartilage gelatin. Formation of molecular aggregates due to hot air drying also affected the gel strength of shark bone gelatin.

Wangtueai & Noomhorm (2008) characterized the physicochemical properties of the gelatin from lizardfish scales and the results indicated high protein and low ash content. The lizardfish scales gelatin was found to contain 20.4% imino acids (proline and hydroxyproline).

2.3.5.5 Physical Properties of Fish Skin Gelatin

2.3.5.5.1 Extractability and Yield

Conversion of collagen into soluble gelatin can be achieved by heating the collagen in either acid or alkali. Thermal solubilisation of collagen (in the presence of acid or alkali) is due to the cleavage of a number of intra- and intermolecular covalent crosslinks that are present in collagen. In addition, some amide bonds in the elementary chains of collagen molecules undergo hydrolysis (Bailey & Light, 1989). The extraction process can influence the length of the polypeptide chains and the functional properties of the gelatin. This depends on the processing parameters (temperature, time, and pH), the pretreatment, and the properties and preservation method of the starting raw material.

Fish gelatin has been extracted using a number of different methods. The direct procedures used for preparing fish gelatin typically involve a mild chemical pretreatment of the raw material and mild temperature conditions during the extraction process. In general, a mild acid pretreatment of the fish skin is used prior to gelatin extraction (Karim & Bhat, 2009).

Gomez-Guillen & Montero (2001) previously reported a procedure for extracting gelatin with high gelling capacity from fish skins: the procedure was essentially based on a mild acid pretreatment for collagen swelling, followed by extraction in water at moderate temperatures (45 ^{0}C). The entire process takes about 24 h. Because of the acid lability of the crosslinks found in fish skin collagen, mild acid treatment is sufficient to produce adequate swelling and to disrupt the non-covalent intra- and intermolecular bonds (Montero *et. al.*, 1990; Norland, 1990; Stainsby, 1987). Subsequent thermal treatment above 40^{0}C (well above helix-to-coil transition temperatures for fish gelatins) destroys hydrogen bonding and cleaves a number of covalent bonds, which destabilizes the triple-helix via a helix-to-coil transition and results in conversion to soluble gelatin (Djabourov, *et. al.,* 1993). High-molecular weight polymers may occur in the resulting gelatin through the possible persistence of crosslinks, depending on the nature and degree of solubilization.

Kolodziejska *et. al.*, (2004) showed that it is possible to omit the chemical treatment and to shorten the extraction time from skins of cold-water fish from 12 h to 30 min, but minced raw material must be used instead of whole skins. Because of the structural characteristics of collagen, fish skins are difficult to mince in a meat grinder. However, they can be comminuted easily after treatment with diluted acetic acid (1:6) at temperatures below 15^{0}C for 2 h (Sadowska, *et. al.*, 2003).

Prior to extraction, the methods for preservation of the raw material have been found to affect some of the physical properties of fish gelatin. Fernandez-Diaz, *et. al.*, (2003) reported that gelatin from skins frozen at -12 ^{0}C had lower gel strength values compared to both fresh skins and skins frozen at -20 ^{0}C. Liu *et. al.*, (2008) has observed that that compared to gelatins from fresh and frozen skins, gelatin from dried channel catfish skin exhibited higher gel strength, and it was attributed this to the large α-chain content of gelatin from the dried skins. It was also observed that the gelling

and melting points of dried channel catfish skin gelatin solution were similar to those of fresh skin gelatin solution, but distinctly different from those of frozen skin gelatin.

On an average, the extraction yield of fish gelatin is lower than mammalian gelatin, giving approximately between 6% and 19% (expressed as grams of dry gelatin per 100 g of clean skin). The lower extraction yield of fish gelatin could be due to the loss of extracted collagen through leaching during the series of washing steps or due to incomplete hydrolysis of the collagen (Jamilah & Harvinder, 2002). In addition, it has been reported that some heat-stable proteases endogenous to the skin are involved in the degradation of gelatin molecules (specifically the β and α chains) during the extraction process at elevated temperatures, which contribute to the low Bloom strength (Intarasirisawat, *et. al.*, 2007). Thus, Nalinanon, *et. al.*, (2008) envisaged that the addition of an appropriate protease inhibitor together with the pepsin-aided process might be an effective means to obtain a higher yield with negligible hydrolysis of the peptides. Indeed, they showed that for extraction of gelatin from the bigeye snapper, the pepsin-aided process in combination with a protease inhibitor (pepstatin A) markedly increased the yield from 22.2% to 40.3% (yield was calculated based on the hydroxyproline content of the gelatin in comparison with that of the skin prior to extraction).

Rahman *et. al.*, (2008) have also reported a higher yield (18%) of gelatin from yellowfin tuna skin. The yield and quality of gelatin are not only influenced by the species or tissue from which it is extracted, but also by the extraction process itself (Montero & Gomez-Guillen, 2000). This was further examined in work by Zhou & Regenstein (2004), where they studied the optimization of extraction conditions for pollock skin gelatin. The observed yields for pollock skin gelatin in their studies varied from 3% to 19%, and were most sensitive to the pretreatment temperature and the concentration of H^+. The pretreatment at room temperature led to a high loss of gelatin, although it may have slightly increased the viscosity. They suggested that a low pretreatment temperature should be used during pollock skin gelatin extraction, and this result may possibly be applicable to other cold-water fish.

In comparison, Gudmundsson & Hafsteinsson (1997), using a prolonged extraction of whole cod skins, achieved a yield of gelatin between 11% and 14%, depending on the concentrations of the sodium hydroxide, sulfuric acid, and citric acid solutions used in the preliminary treatment of raw material. Gomez-Guillen, *et. al.*, (2002) reported that the yield of extractions varied slightly among the fish species (sole:8.3%; megrim: 7.4%; cod: 7.2%; hake: 6.5%). They also observed that squid skin requires higher extraction temperatures (80 ^{0}C), but even under these conditions, the yield was only 2.6%, lower than yields from fish skin extracted using a milder procedure.

In the cases of other species, the extraction yield of gelatin from skins ranged from 5.5% to 21% of the starting weight of the raw material (Gime nez *et. al.*, 2005a,b; Grossman & Bergman,1992; Jamilah & Harvinder, 2002; Muyonga *et. al.*, 2004a; Songchotikunpan *et. al.*, 2008). The variation in such values depends on the differences in both the proximate composition of the skins and the amount of soluble components in the skins (Muyonga *et al.*, 2004a), as these properties vary with the species and the age of the fish. In addition, variation in the extraction method can also have an effect on yields. The wide range in gelatin yields could also be attributed to differences in collagen content of the raw material; however, this information is often not available in published data (Songchotikunpan, *et. al.*, 2008). Reporting gelatin yield as dry gelatin weight compared to the weight of wet skin is common, but not very reliable. Water content may vary because of different treatments to the skin (freezing, salting, scraping, draining, etc.). Therefore, gelatin yield should be reported as the amount of dry gelatin compared to the amount of dry matter in skin (Arnesen & Gildberg, 2007).

2.3.5.5.2 Viscosity

Viscosity is the second most important commercial property of gelatin after gel strength (Ward & Courts, 1977). Viscosity is partially controlled by molecular weight and molecular size distribution (Sperling, 1985). The viscosities of for most of the commercial gelatins have been reported to be in

the range of 2.0 to 7.0 cP for most gelatins and upto 13.0cP for specialized ones (Johnston-Banks, 1990). Minimum viscosity for gelatin was observed to be in the pH range of 6-8(Stainsby, 1952). Gelatins within a pH range of 3 show the maximum values for viscosity. Jamilah and Harvinder (2002) reported viscosity values of 3.2cP and 7.12cP for red and black tilapia respectively whereas for channel catfish the optimum value predicted was 3.23 cP (Yang *et. al.*, 2007).

2.3.5.5.3 Clarity

Clarity (i.e. inverse of turbidity) of a gelatin solution can be important for commercial applications and this functional property is frequently assessed for determining the quality of gels. When the protein is treated for a long time at high temperatures, aggregation is activated and turbidity is increased (Johnson & Zabik, 1981). Increase in higher molecular weight aggregates can increase the turbidity (Montero, *et. al.*, 2002). Cho, *et. al.*, (2004) observed high turbidities for gelatin extracted from shark cartilage employing high temperature and long extraction periods.

2.3.5.5.4 Melting and Setting Temperatures

The melting temperature of gelatin has been found to correlate with the proportion of the imino acids proline and hydroxyproline (both with a 5-membered pyrrolidine ring) in the original collagen (Ledward, 1986; Piez & Gross , 1960; Veis,1964) This is typically 24% for mammals and 16–18% for most fish species (Norland, 1990). Cold water fish, for example cod, have a very low hydroxyproline content and coupled with this a very low gelling and melting temperature. Fish gelatin with lower gel melting temperature had a better release of aroma and offered stronger flavour and useful in product development to control the texture and flavour release during mastication. Gomez-Guillen *et. al.*, (2002) correlated the thermal stability of gelatin to the number and stability of Proline rich region in collagen or gelatin molecules, which are high in fresh warm water fish and mammalian species. Gudmundsson (2002) observed that gelatins with high melting temperature formed stronger gels and in this study also it can be seen that Grass carp

which has the highest melting temperature forms the strongest gel among the three gelatin samples.

Setting (gelling) denotes the gelling process which involves the transition from random coil to triple helical structure of gelatins. The imino acid content stabilizes the ordered conformation when gelatin forms the gel network during gelling. A critical amount of regenerated helices are required to form the gel network. Gelling and melting temperatures are also influenced by the change in ionic strength. This suggests that the junction zones and the gel network may be stabilized by both hydrogen bonds and electrostatic bonding (Haug *et. al.*, 2004). Muyonga *et. al.*, (2004a) reported a setting temperature of 19.5 ^{0} C and a setting time of 60 seconds for the gelatin from the skin of adult Nile perch extracted at 50 ^{0}C which is similar to the values observed for Grass carp skin gelatin. Gudmundsson (2002) compared the rheological properties of fish gelatins (tuna, tilapia, cod and megrim) with conventional bovine and porcine gelatins. The gelling and melting points of tilapia (*O..aureus)* (18.2 ^{0} C and 25.8 ^{0} C respectively)were the highest among the fish gelatins and was comparable to low molecular weight porcine and bovine gelatins. Cold water fish gelatins ie., gelatins from the skins of cod and megrim had very low melting and gelling points when compared with gelatins from warm water fish and animal sources mainly due to the low imino acid content, which in turn reduces the propensity for intermolecular helix formation (Gilsenan & Ross-Murphy, 2000).

2.3.5.5.5 Odour

Choi & Regenstein (2000) studied the physicochemical differences between pork and fish gelatin and the effect of melting point on the sensory characteristics of a gelatin–water gel. Quantitative descriptive analysis (QDA) was performed to determine the effect of the melting point on the sensory characteristics of gelatin gels. They noted that flavored fish gelatin dessert gel product had less undesirable off-flavors and off-odors, with more desirable release of flavor and aroma than the same product produced with pork gelatin possessing equal Bloom values, but a higher melting point. The lower melting temperature of fish gelatin seems to assist in the release of fruit aroma, fruit

flavor, and sweetness. In contrast, since pork gelatin melts more slowly than fish gelatin in the mouth, the perceived viscosity of pork gelatin might be expected to be higher than that of the fish gelatin under the same conditions. Choi & Regenstein (2000) further noted that since fish gelatin has a better release of aroma, it might offer new opportunities to product developers. Muyonga *et. al.*, (2004a) has reported that the gelatins prepared from the skin and bone of Nile Perch were found to be free of fishy odour and to have a mild putrid odour with a mean hedonic score of 2–2.5 with activated carbon treatment. Strong fishy odour was reported for freeze dried gelatin prepared from the skin of black tilapia (Jamilah & Harvinder, 2002).

2.3.5.5.6 Colour

The colour of the gelatin depends on the raw material used for the extraction and also whether it is obtained from first stage, second stage or subsequent stages The colour of the gelatin depends on the raw material. However, it does not influence other functional properties (Ockerman &Hansen, 1999). Commercial gelatin is not colourless in solution but has a colour varying from a very pale yellow to dark amber. There can be no doubt that the colour attribute of gelatin has practical significance in that some 60% of world production is consumed by the confectionery industry (Siebert, 1992). In this industry, the products are very often coloured and it stands to reason that, the less the colour variation in the ingredients, the easier it would be to produce a uniform product. Furthermore, in the minds of most people the lack of colour is associated with purity, hence, pale colour is normally more desirable than darker colour. The importance of gelatin colour is recognised by manufacturers (Hoffmann, 1985; Schreiber, 1977).

2.3.5.6 Functional Properties of Fish Skin Gelatin

2.3.5.6.1 Gel strength, Gelling and Melting Points

Gel strength is one of the important properties of gelatin, and the purpose of gelatin was determined by the range of gel strength values. Generally, fish gelatin has lower gel strength than mammalian gelatin (Norland,1990). Especially, characteristics of collagen have influence on the

physical properties of gelatin, because gelatin is derived from collagen. Bovine and porcine gelatins have considerably higher gelling and melting points than most fish gelatins, and the high gelling and melting points expand the range of gelatin application (Choi & Regenstein, 2000; Gilsenan & Ross-Murphy, 2000; Gudmundsson, 2002; Leuenberger, 1991). Gomez-Guillen *et. al.*, (2002) reported that tropical-fish, such as tilapia, was a superior material for gelatin processing (Grossman & Bergman, 1992), however, cold-water fish, cod gelatin has poor physical properties (Gudmundsson & Hafsteinsson, 1997). According to the results of gel strength measured by Choi & Regenstein (2000), fish gelatins showed lower gel strength than porcine skin gelatin (300 Bloom). Cho *et. al.*, (2005) reported gel strength of 426 Bloom for yellow fin tuna skin gelatin which is remarkable for gel strength of fish gelatin. The gelling point (18.7 ^{0}C) and melting point (24.3 ^{0}C) of yellow fin tuna skin gelatin were lower than two mammalian gelatins. This pattern of the gelling and melting points is similar with the other fish gelatins, especially tuna gelatin and tilapia (Gilsenan & Ross- Murphy, 2000; Gudmundsson, 2002).

Gelation is either inhibited or enhanced and the texture of the gel can be very different from those of the gels formed by the components alone (Oakenfull, 1987). With some ingenuity, physical properties of mixed biopolymer systems can be more finely controlled. For this reason, mixed systems are of great technological importance and can be used as one of the approaches to modulate the strength of the gelatin gel (Karim & Bhat,2008). Improvement of gel strength of gelatin using modified starch has been described in several patents (Helmstetter, 1977; Szymanski & Helmstetter, 1975). Haug *et. al.*, (2004) reported that a mixed gelatin-k-carrageenan gel system could be formulated carefully leading to systems with improved gel strength, gelling and melting temperature.

The outcome of blending gelatin with other hydrocolloids can be both positive and negative . Gellan gum, for example, accelerates the gelling speed of gelatin and substantially increases gel firmness, but reduces color and clarity. On the other hand, blending of gelatin with citrus pectin reduced the firmness of the gelatin gel. Carrageenan has an even stronger negative effect on firmness, color, and clarity of gelatin gels (Schrieber & Gareis, 2007).

2.3.5.6.2 Foam Formation Ability and Foam Stability

In general, there have been very limited studies on the emulsifying and foaming properties of fish gelatin compared to the number of studies on its gelation properties. In general, fish gelatin emulsions are moderately stable to creaming. Surh *et. al.*, (2006) have studied whether physically stable oil-in-water emulsions could be produced using fish gelatin, and determined the influence of gelatin molecular weight (low molecular weight fish gelatin [LMW-FG] and high-molecular weight fish gelatin [HMW-FG]) and environmental stresses (pH, salt, and thermal processing) on the stability of such emulsions. They noted that emulsions with monomodal particle size distributions and small mean droplet diameters (0. 43 ~ 0.35 mm for LMW-FG and 0.71 mm for HMW-FG) could be produced at protein concentrations 4.0 wt% for both molecular weight fish gelatins. However, the presence of a small fraction of relatively large droplets (>10 mm) was observed in the emulsions, even at relatively high protein concentrations. Surh *et. al.*, (2006) noted that the number of large droplets and the amount of destabilized oil was less in the HMW-FG emulsions than in the LMW-FG emulsions. This effect may be attributed to the fact that the thickness of an adsorbed gelatin membrane increases with increasing molecular weight. The emulsions of both low- and high-molecular weight fish gelatins were fairly stable when subjected to high salt concentrations (250 mM sodium chloride), thermal treatments (30 and 90 ^{0} C for 30 min), and different pH values (pH 3–8), demonstrating that fish gelatin may have limited use as a protein emulsifier for oil-in-water emulsions.

2.3.5.6.3 Water Holding and Fat Binding Capacities

Water-holding and fat-binding capacities are functional properties that are closely related to texture by the interaction between components such as water, oil and other components. Fat binding capacity depends on the degree of exposure of the hydrophobic residues inside gelatin. Cho *et. al.*, (2004) has observed that the gelatin extracted from shark cartilage had a higher fat binding capacity than porcine skin gelatin which is attributed to the higher content of hydrophobic amino acid tyrosine.

2.3.5.6.4 Texture Profile

Instrumental Texture Profile Analysis (TPA) has been used for many years for the measurement of food textural properties (Bourne, 1982; Sanderson, 1990). The textural parameters obtained from TPA force/deformation curves have been well correlated with sensory evaluation of textural parameters (Bourne & Comstock 1981; Munoz *et. al.*, 1986a), provides more information than ``gel strength" measurements, and is useful for routine analysis of gel texture (Sanderson,1990).

Texture profiling of the gel by instrumental method involves compressing the test sample at least twice and quantifying the mechanical parameters from the recorded force- deformation curves. These are the attributes of food manifested by the reaction of the food to the stress (Szczesniak, 2002). Texture Profile Analysis tests were intended to simulate the action exerted upon the gel by the tongue and teeth, and, therefore, differ from the simpler gel strength test. Gelatin gels are quite soft and flexible, but their textural properties, in general, are very narrow (Johnston-Banks, 1990; *Munoz et al.,*1986a,b;Wolf *et. al.*, 1989). Lau *et. al.*, (2000) has reported that the gel hardness can be increased by the addition of suitable amounts of calcium ions to the polymer solutions and increasing the ratio of gellan to gelatin, whereas brittleness, springiness and cohesiveness were very sensitive to low levels of added calcium (0-10 mM), but less sensitive to higher calcium concentrations and gellan/gelatin ratio. Important parameters in the texture profile of a gel are hardness, cohesiveness, springiness, gumminess, chewiness, fracture force and adhesiveness.

Hardness is defined as the peak force during compression cycle. The peak force during first compression("first bite") of the gel sample is Hardness I and the peak force during second compression ("second bite") of the gel sample is Hardness II. Muyonga *et. al.*,,(2004a) observed that there is a high correlation between hardness of the gel and bloom strength in the case of gelatin from the skin of Nile Perch and hence hardness can be used to compare the gel strengths. Yang *et. al., (2007)* has reported that the gel strength of gelatin produced from the skin of channel catfish showed high correlation with hardness and chewiness.

Cohesiveness is the area of positive force area during the second compression to that during the first compression of the gel sample. It gives a relative and dimensionless measure of how much of gel strength is remained after the deformation of the first compression i.e., visco elasticity. Cohesiveness is a measure of the degree of difficulty in breaking down the gel's internal structure (Sanderson, 1990; Wolf *et. al.*, 1989).

Springiness (sometimes also referred to as ``elasticity'') is a perception of gel ``rubberiness'' in the mouth, and is a measure of how much the gel structure is broken down by the initial compression (Sanderson, 1990). Springiness index is the ratio between the height of the gel sample and the height that the sample recovers during the time that elapses between the end of the first compression and the start of the second compression. High springiness results from the gel structure being broken into a few large pieces during the first TPA compression while low springiness results from the gel breaking into many small pieces (Lau *et. al.*, 2000).

Gumminess by sensorial definition is the energy required to disintegrate a semi-solid food product to a state ready for swallowing. Related to foods with low hardness levels. By instrumental definition it is the calculated parameter of the Product of Hardness x Cohesiveness. Gumminess was significantly different for all the three gel samples. Gumminess was highest for Grass carp gel followed by Rohu and Common carp gel which is again dependant on the hardness of the gels. Gumminess is a desirable attribute in marshmallow type of products where the product gives "a feel in the mouth" sensation while chewing.

Chewiness by sensorial definition is the energy required to chew a Solid food product to a state where it is ready for swallowing. This attribute is difficult to quantify precisely due to complexities of mastication (shear, compression, tearing and penetration). By instrumental definition it is the calculated parameter of Product of Gumminess x Springiness (essentially primary parameters of Hardness x Cohesiveness x Springiness). Fracture force by sensorial definition is the Force at which a material fractures. Related to the primary parameters of hardness and cohesiveness, where brittle materials have low cohesiveness. Not all foods fracture and thus value may relate to hardness if only single peak is present. Brittle foods are never

adhesive. By Instrumental definition it is the first significant break in the first compression cycle. Adhesiveness by sensorial definition is the work necessary to overcome the attractive forces between the surface of the food and the surface of other materials with which the food comes into contact (e.g. tongue, teeth, and palate). By instrumental definition it is the negative area for the first bite, representing the work necessary to pull compressing probe away from sample.

2.3.5.7 Chemical Properties

2.3.5.7.1 Proximate Composition & pH

Gelatin consists of mostly protein and water. So, ash, lipid and other impurity contents are important for quality of gelatin. Usually ash contents up to 2.0% can be accepted in food applications. The Chemical composition of shark cartilage gelatin was 7.98% moisture, 90.9% crude protein, 0.54% crude ash and 0.21% crude lipid as reported by Cho *et. al.*, (2004). A study on the extraction of gelatin from the skin and bone of Nile Perch by Muyonga *et. al.*, (2004a) has showed that the proximate composition of gelatin was found to vary with the type of tissue used as raw material but was unaffected by age of the fish. The skin gelatins were generally low in ash, with most having ash content lower than the recommended maximum of 2.6% .The bone gelatins, however, had much higher ash content (in the range3–10%), indicating that the leaching process was inadequate. The gelatin extracted from Grass carp had 12.3% moisture, 0.2% fat and 0.12% ash (Kasankala *et. al.*, 2007). Jongjajareonarak *et. al.*, (2006) reported a protein content of 87.9% & 88.6% for freeze dried gelatin from the skin of big eye snapper and brown eye snapper respectively. The moisture content of gelatin may be as high as 16 %, however, more normally it is about 10 % to 13 % because at 13 % moisture content the glass transition temperature of gelatin is about 64°C which allows particle size reduction to be a simple operation (McCormick, 1995). In addition, at 13 % moisture content and 25°C gelatin is close to equilibrium with ambient air moisture contents of ca. 46 % RH. At 6 % to 8 % moisture content gelatin is very hygroscopic and it becomes difficult to determine the physical attributes with accuracy (Cole, 2000). The different mineral contents between

the skins of the species might be associated with the various reasons. However the nature of the ash is important. For example, 2 % $CaSO_4$ in gelatin can have excellent clarity in spite of the solubility product of the ash being exceeded (due to the crystal-habit modifying effect of gelatin), however on dilution of the gelatin in a confectionery formulation, the ash can precipitate. Furthermore, ammonia is often used as a pH modifier in gelatin preparation and salts like NH_4Cl are not determinable by pyrolysis (Cole, 2000).

Choi & Regenstein (2000) observed that the gel strength of the fish and pork gelatins decreased markedly below pH 4 and slightly above pH 8. For the melting point also similar dependencies were observed in relation to pH. Crumper & Alexander (1952) observed that the rigidity of pork gelatin is maintained at pH range 4 – 10. Cole (2000) reported that for Type B gelatin, the viscosity is minimal and the gel strength is maximum at a pH of 5; hence from the manufacturers' point of view it is advantageous to manufacture gelatin at this pH. The pH reported for gelatin extracted from the skin of red tilapia was 3.05 and 3.91 for black tilapia (Jamilah & Harvinder, 2002). Minimum viscosity of gelatin has been noted to be in the range of pH 6–8 for many gelatins (Ward & Courts, 1977).The pH effect on viscosity is minimum at the isoionic point and maximum at pH 3 and 10.5.

2.3.5.7.2 Amino Acid Composition

Generally, collagens present in fish skins show a wider variety in amino acid compositions than those of mammalian collagens. Their hydroxyproline and, to a lesser extent, proline contents are lower than those in mammalian collagens, and this is compensated for by higher serine and threonine contents (Balian & Bowes, 1977). In general, fish collagens have lower imino acid contents than mammalian collagens, and this may be the reason for the denaturation at low temperature (Grossman & Bergman, 1992). The source and type of collagen will influence the properties of the resulting gelatins.

Overall, fish gelatins have lower concentrations of imino acids (proline and hydroxyproline) compared to mammalian gelatins, and warm-water fish

gelatins (such as big eye-tuna and tilapia) have a higher imino acid content than cold-water fish such as cod, whiting and halibut) gelatins (Eastoe & Leach, 1977). The proline and hydroxyproline contents are approximately 30% for mammalian gelatins, 22–25% for warm-water fish gelatins (tilapia and Nile perch), and 17% for cold-water fish gelatin (Muyonga *et. al.*, 2004a).

Avena-Bustillos *et. al.*, (2006) reported similar trends in that they found that cold-water fish gelatins have significantly fewer hydroxyproline, proline, valine, and leucine residues than mammalian gelatins, but significantly more glycine, serine, threonine, aspartic acid, methionine, and histidine residues. However, both cold-water fish and mammalian gelatins have the same proportion of alanine, glutamic acid, cysteine, isoleucine, tyrosine, phenylalanine, homocysteine, hydroxylysine, lysine, and arginine residues.

Haug *et. al.*, (2004), conducting a similar comparative study on the rheological properties of fish and mammalian gelatins, found that the main difference between fish and mammalian gelatins is the content of the imino acids, proline and hydroxyproline, which stabilize the ordered conformation when gelatin forms a gel network. The lower content of proline and hydroxyproline gives fish gelatin a low gel modulus, and low gelling and melting temperatures. It should be kept in mind that the super-helix structure of the gelatin gel, which is critical for the gel properties, is stabilized by stearic restrictions. These restrictions are imposed by both the pyrrolidine rings of the imino acids in addition to the hydrogen bonds formed between amino acid residues (Te Nijenhuis, 1997).

The amount of the imino amino acids, proline and hydroxyproline, determines the shrinkage temperature and the denaturing temperature, i.e., the temperature at which the collagen helix unwinds, and as a result, the temperature at which solutions of the extracted gelatin gels. The differences in the physical properties between mammalian gelatin and gelatin from cold water fish species are due to a lower content of the imino acids Proline (Pro) and Hydroxyproline (Hyp). Calf skin gelatin contains approximately 94 Hyp and 138 Pro residues per 1000 total amino acid residues, while cod skin gelatin contains approximately 53 and 102 amino acids of Hyp and Pro, respectively, per 1000 total residues (Piez & Gross, 1960). Gelatins from warm water fish species, like fish gelatin from tilapia, contain c.a 70 and 119

residues of Hyp and Pro, respectively, per 1000 total residues, and have physical properties more equal to those of mammalian gelatins (Sarabia *et. al.*, 2000). Harp seal gelatin also contains almost the same amounts of Hyp and Pro as bovine gelatin, and therefore has properties very similar to those of bovine origin (Arnesen & Gildberg, 2002).

2.3.5.7.3 Molecular Weight Distribution

During gelatin manufacturing, the conversion of collagen to gelatin yields molecules of varying mass, due to the cleavage of inter-chain covalent cross links and the unfavorable breakage of some intra-chain peptide linkages (Zhou *et al.*,, 2006). As a result, the gelatin obtained has a lower molecular weight than native collagen, and consists of a mixture of fragments with molecular weights in the range of 80–250 kDa (Poppe, 1997).

Fish and mammalian gelatins have a polydisperse molecular weight distribution related to the collagen structure and production process. In addition to different oligomers of the alpha subunits, intact and partially hydrolyzed alpha-chains are also present, giving rise to a mixture containing molecules of different molecular weights (Schrieber & Gareis, 2007). The polydispersity, calculated as the ratio of the weighted average molecular weight (Mw) to the number average molecular weight (Mn), of gelatin always has a value over 2 (Schrieber & Gareis, 2007). However, in a rheological study on several types of fish gelatin, Gudmundsson (2002) reported polydispersity values in the range of 1.57–2.21 and isoelectric point (pI) values for megrim (9.5), tilapia (9.1), and cod (8.9).

β-Chain and γ-chain aggregations are present in salmon and pollock skin gelatins as well as in commercial mammalian and fish skin gelatins. Large amounts of β - and γ -chains have been shown to negatively affect some of the functional properties of fish gelatins, such as lowering viscosity, lowering melting and setting points, and resulting in a longer setting time (Muyonga *et. al.*, 2004a). Chiou *et. al.*, (2006) reported that pollock and salmon gelatins had slightly different molecular weight profiles compared to porcine gelatin, and that the fish gelatins had chains with slightly lower molecular weights. In addition, the fish gelatins contained lower molecular weight species that were not present in the porcine gelatin.

Gomez *et. al.*, (2009) has observed that the molecular weight distribution of the tuna-skin gelatin exhibited appreciably higher quantities of β -components (covalently linked α-chain dimers), whereas bovine-hide gelatin showed a certain degradation of α_1-chains being indicative of a greater proteolysis. Both types of gelatin contained α-chains weighing around 100 kDa and β -components weighing around 200 kDa, typical of type I collagen. Nevertheless, there were appreciable differences in a-chain yields during extraction. The fish gelatin exhibited α_1/α_2 ratio of around 2, indicating that the native structure is maintained. In contrast, intensities for the bands for the $\alpha_1$and $\alpha_2$chains in the mammalian gelatin were similar, indicating possible degradation of the α_1-chains during the extraction process.

2.3.5.7.4 Fourier Transform Infrared Spectroscopic (FTIR) Spectra of Gelatin

FTIR spectroscopy has been used to study the changes in the secondary structure of gelatin (Aewsiri *et. al.,* 2008; Muyonga *et.al.,* 2004b). FTIR spectroscopy has been used to study collagen crosslinking (Paschalis *et.al.*,2001), denaturation (Freiss & Lee, 1996), thermal self assembly (Jakobsen *et.al*, 1983; George & Veis,1991) and gelatin melting The spectral changes indicate the changes in collagen secondary structure and have been shown to include changes in the amide A (Milch,1964), amide I (1636 – 1661 cm^{-1}), amide II (1549– 1558 cm^{-1}) (Renugopalakrishnan *et.al.,*1989) and the amide III (1200– 1300 cm^{-1}) regions (Freiss & Lee, 1996). The amide I is the most useful peak for infrared analysis of the secondary structure of protein including gelatin (Surewicz & Mantsch, 1988). Yakimets *et.al.,* (2005) reported that the absorption peak at 1633 cm^{-1} was characteristic for the coil structure of gelatin. Denaturation of collagen to gelatin has been found to lead to a reduction in the intensity of amide A, I, II and III peaks , narrowing the amide bands (Freiss & Lee,1996; Prystupa & Donald,1996). Prystupa and Donald (1996) studied the gelatin melting and found it to be associated with the reduction in the 1678 cm^{-1} peak and 1660/1690 cm^{-1} peak intensity ratio and increase in amide I components occurring around 1613,1629 and 1645 cm^{-1}.

Muyonga *et. al.,* (2004b) studied the FTIR spectra of collagens and gelatin from the skin and bones of adult Nile perch. It was observed that conversion of collagen to gelatin leads to a loss in the triple helical structure and decrease in molecular order.

2.3.5.8 Mammalian Gelatin Vs Fish Gelatin

Gelatin from marine sources (fish skin, bone and fins) has been looked upon as a possible alternative to bovine and porcine gelatin, especially since the outbreak of the BSE in the 80's. Search for new gelling agents to replace mammalian gelatin led to patents for fish gelatin production (Grossman & Bergman 1992; Holzer, 1996) as well as several published methods for fish gelatin production (Gomez-Guillen & Montero, 2001; Gudmundsson & Hafsteinsson, 1997; Nagai & Suzuki, 2000). Recently, harp seal also has been considered as raw material for gelatin production (Arnesen & Gildberg, 2002).

The commercial interest in fish gelatin has this far, however, been relatively low. This is due to sub-optimal physical properties compared to mammalian gelatin. Common problems connected with fish gelatin from cold water species, representing the majority of the industrial fisheries, are low gelling and melting temperature and low gel modulus (Leuenberger, 1991). This makes these gelatins unsuited as mammalian gelatin replacements, especially since they typically gel below 8^0C (Leuenberger, 1991; Norland, 1990). The differences in the physical properties between mammalian gelatin and gelatin from cold water species are due to a lower content of the imino acids Proline and Hydroxyproline. The melting and gelling temperature of gelatin has been found to correlate with the proportion of the imino acids proline and hydroxyproline (both with a 5-membered pyrrolidine ring) in the original collagen (Veis, 1964). This is typically ~24% for mammals and 16–18% for most fish species. Cold water fish, for example cod, have a very low hydroxyproline content and coupled with this a very low gelling and melting temperature. For example, 10% mammalian gelatin forms a gel at approximately room temperature, whereas 10% cod will just about gel at ~2^0C.

The amino acid compositions of mammalian gelatins are remarkably constant when compared to those from different species of fish. Glycine, the

simplest amino acid, accounts for approximately one-third of the total amino acid residues in mammalian gelatins, proline and hydroxyproline for approximately one-fifth, and alanine for approximately one-ninth. In all, these four amino acids account for approximately two out of every three amino acid residues in mammalian collagen used in gelatin manufacturing .Fish collagens show a wider variation in composition. Their hydroxyproline and, to a lesser extent, proline contents are lower than that of mammalian collagens and this is compensated for higher concentrations of serine and threonine (Balian & Bowes 1977).

Calf skin gelatin contains approximately 94 Hydroxyproline and 138 Proline, residues per 1000 total amino acid residues, while cod skin gelatin contains approximately 53 and 102 amino acids of Hydroxyproline and Proline, respectively, per 1000 total residues (Piez & Gross, 1960). Gelatins from warm water fish species, like fish gelatin from tilapia contains c.a 70 and 119 residues of Hydroxyproline and Proline, respectively, per 1000 total residues, and have physical properties more equal to those of mammalian gelatins (Sarabia *et. al.*, 2000). Harp seal gelatin also contains almost the same amounts of Hydroxyproline and Proline as bovine gelatin, and therefore has properties very similar to those of bovine origin (Arnesen & Gildberg, 2002). Quantitatively, however, fish gelatin from cold water fish species is still preferred due to the greater availability of byproducts (e.g. skin and bone) from which it can be manufactured. Collagen from fish has just recently been identified as a potential allergen and could possible become a problem for the use of fish gelatin in commercial products (Hamada *et. al.*, 1999).

Haug *et. al.*, (2004) compared the physical and rheological properties of fish gelatin with bovine gelatin. It was observed that fish gelatin is heterogeneous in molecular compositions and that it mainly contains alpha and beta chains. Fish gelatin gave gels with a considerably lower storage modulus, gelling (4–5 ^{0}C) and melting temperature (12–13 ^{0}C) compared to mammalian gelatin gels which could probably due to the lower content of proline and hydroxyproline in fish gelatin. The degree of chain segment ordering at the gelling temperature in fish gelatin (at 5 ^{0}C) and mammalian gelatin (20 ^{0}C) was almost identical. This clearly showed the importance of the

content of imino acids for the formation of some ordered structures and stabilization of the gelatin gel network.

Rheological characteristics of mammalian and fish skin gelatins were examined by Gilsenan & Ross-Murphy (2000).It was reported that Gelatins from cold water fish have a much higher critical concentration and lower melting point than mammalian samples, due to the lower imino acid content, which in turn reduces the propensity for intermolecular helix formation. The lower content of proline and hydroxyproline gives fish gelatin a low gel modulus, and low gelling and melting temperatures. The super-helix structure of the gelatin gel, which is critical for the gel properties, is stabilized by stearic restrictions. These restrictions are imposed by both the pyrrolidine rings of the imino acids in addition to the hydrogen bonds formed between amino acid residues (Te Nijenhuis, 1997).

Warm water fish gelatins however, have properties quite similar to mammalian samples. Gelatin from the skin of yellowfin tuna (*T.albacares*) had a high gel strength (426 Bloom) in comparison with bovine and porcine gelatins (216 Bloom and 295 Bloom, respectively), while gelling and melting points were lower (Cho *et. al.*, 2005). Studies by Zhou *et. al.*, (2006) showed that the gelling ability of Pollock skin gelatin could be enhanced by mixing it with high quality gelatins from warm water species or mammals. Fish gelatin with lower gel melting temperature had a better release of aroma and offered stronger flavour and useful in product development to control the texture and flavour release during mastication.

Eventhough fish gelatin will not be able to completely replace mammalian gelatin, it is envisaged that it might become a niche product offering unique and competitive properties to other biopolymers, as well as meeting the demand of global halal /kosher market (Karim & Bhat,2008). However, while fish gelatin seems to be a promising alternative, some problems persist, including inferior gelling property and quality variations from batch to batch and from species to species.

2.3.5.9 Optimization of Process Parameters for the Production of Gelatin from Fish Skin using Response Surface Methodology

Response Surface Methods (RSM) have been used for many years in the physical sciences to study the relations between the magnitude of a response and the independent factors influencing the response. The goal of the RSM is to use experimental observations of a response at various factor levels as data to explore and describe the response in some factor region of interest (Menke, 1973). Recently, response surface methodology has been used to evaluate the effectiveness of food manufacturing processes. RSM is a collection of mathematical and statistical techniques widely used to determine the effects of multiple variables and to optimize different biotechnological process. It is a mathematical modeling technique that relates product treatment to outcomes and establishes regression equations that describe inter-relations between input parameters and product properties (Rao, *et. al.*, 2000; Ozdemir & Devres, 2000). The basic principle of RSM is to determine model equations that describe interrelations between the independent variables and the dependent variables (Edwards & Jutan, 1997).

RSM has effectiveness in the optimization and monitoring of food manufacturing processing. In the extraction of gelatin from fish skin many workers have adopted RSM for optimization of process. Cho *et. al.*, (2004) adopted the Central Composite Design for extracting optimization in gelatin processing from the skin of yellowfin tuna. Concentration of NaOH (X_1), treatment time (X_2), extraction temperature (X_3), and extraction time (X_4), were chosen for independent variables. Dependent variables were gel strength, and gelatin content. Optimal conditions obtained were 1.89% (X_1), 2.87 days(X_2), 58.15 ^{0} C (X_3) and 4:72 h(X_4). Yang *et. al.*, (2007) adopted a 2-step response surface methodology involving a central composite design to optimize the extraction of gelatin from channel catfish skin. After the 2nd-step optimization, the most suitable conditions were 0.20 M NaOH pretreatment for 84 min, followed by a 0.115 M acetic acid extraction at 55°C. The optimal values obtained from these conditions were Yield = 19.2%, Gel strength = 252 g, and Viscosity = 3.23 cP. Kasankala *et. al.*, (2007) has optimized the process of extraction of gelatin form Grass carp skin by RSM. The optimum conditions were 1.19% HCl, 24 h pre-treatment time, 52.61^{0}C

extraction temperature and 5.12 h extraction time. The predicted responses were 19.83% yield and 267 g gel strength. Wangtueai & Noomhorm (2008) used RSM with a 4-factor, 5-level Central Composite Design to ascertain the optimum gelatin extraction conditions from lizard fish scales The results showed the optimum conditions for the highest values of the responses when a concentration of NaOH at 0.51%, a treatment time at 3.10 h, an an extraction temperature at 78.5^{0}C and an extraction time at 3.02 h.

2.3.5.10 Gelatin / Collagen based Edible Films

Edible films are thin films prepared based on a biopolymer. Edible films and coating materials are potentially used to extend the shelf-life and improve the quality of almost any food system by serving as mass transfer barriers to moisture, oxygen, carbon dioxide, lipid, flavor and aroma between food components and the surrounding atmosphere (McHugh, 2000). The main biopolymers used in the edible films preparations are polysaccharides (Nisperos – Carreido,1994) and proteins(Gennadios, *et. al.,*1994:Torres ,1994).Among the proteins studied, collagens(Gennadios *et al* 1994) and fish myofibrillar proteins(Cuq, *et. al.,*1995:1997 a,b,c) were studied as bases for edible film preparations.

Edible films may serve as gas and solute barriers, thereby improving the quality and shelf life of muscle foods (Wong *et. al.*, 1994). One example of such a film is gelatin which is reported to have better oxygen barrier properties when combined with other types of films (Gennadios, 2002). In one study, Villegas *et. al.*, *(*1999) subjected cooked ham and bacon to gelatin dips (2%, 4%, and 6%), packaged them in oxygen permeable or vacuum packaging films, and stored them under frozen conditions for seven months. Results from these experiments demonstrated that gelatin dips significantly improved oxidative and color stability of the treated products, as compared to untreated controls. Additional studies have demonstrated that gelatin can be used to carry antioxidants, to reduce oxidation, enhance color stability, to retain flavor, taste, and aroma of foods during refrigerated or frozen storage (Gennadios *et. al.*, 1997). Gelatin films have been used as a delivery system for applying antioxidants to poultry or applied directly to poultry meat surfaces or processed meats to prevent microbial growth, salt rust, grease bleeding,

handling abuse, water transfer, moisture loss, and oil adsorption during frying (Childs,1957; Gennadios *et. al.*, 1997; Klose *et. al.*, 1952) . However, gelatin lacks strength and requires a drying step to form more durable films (Daniels, 1973). The use of collagen-based films has been proposed for processed meats, including hams, netted roasts, roast beef, fish fillets, and meat pastes (Gennadios *et. al.*, 1997). Currently, the meat industry uses collagen films during the processing of meat products. When heated, intact collagen films can form a "skin" or edible film that becomes an integral part of the meat product (Cutter & Miller, 2004; Gennadios *et. al.*, 1997).These commercially available collagen films have been purported to reduce shrink loss, increase permeability of smoke to the meat product, increase juiciness, allow for easy removal of nets after cooking or smoking, and absorb fluid exudate. Protein coatings derived from collagen also have been used to reduce transport of gas and moisture in meats (Baker *et. al.*, 1994; Cutter & Summer, 2002).

Among all proteins, gelatin has been attracted the attention for the development of edible films due to its abundance and biodegradability (Bigi *et. al.*,2002). The use of gelatin in the preparation of edible films or coatings was very well studied and produced many patents, particularly in the field of pharmaceuticals (Gennadios *et. al.*,1994 :Torres ,1994). Collagen and gelatin films have been used for sausage casing (Johnston-Banks, 1990), production of hard and soft capsules, wound dressing and adsorbent pad in the pharmaceutical industry (Digenis *et. al.*,1994).Gelatin edible films, with high puncture strength, low puncture deformation and high water vapor permeability, prepared from bovine and porcine skin have been reported (Sobral *et. al.*,2001). Edible wrappings based on blends of gelatin with other constituents have been marketed (Torres, 1994). Carvalho *et. al.*,(1997a) prepared edible films from bovine hide gelatin and determined its mechanical properties. The drying properties of films of gelatin plasticized with sorbitol, has been studied in detail by Carvalho *et al.*, (1997b) and Menegalli *et. al.*,(1999). Sobral (1999) studied the influence of film thickness on the mechanical properties, water vapour permeability and the colour of the films made from bovine hide and pigskin gelatins, plasticized with sorbitol. Arvanitoyannis *et. al.*,(1997 &1998) elaborated on the thermal and functional

properties of film made from the blend of gelatin and edible starch incorporating plasticizers.

2.3.5.11 Plasticizers in Edible Films

The addition of plasticizer alters some of the functional and physical properties of these films, such as increasing flexibility, moisture sensitivity, as well as other functional properties. Normally, plasticizers such as glycerol and sorbitol are used to modify some of the functional and physical properties of the film. Studies were carried out on the effects of plasticizer on the physical, chemical and functional properties of these films. Sobral *et. al.*, (2002) investigated the effect of glycerol and water on glass transition behavior of Nile tilapia protein films. Also, Bigi *et al.,* (2004) described the relation between the triple-helix content and mechanical properties of gelatin films. Lukasik & Ludescher (2006) investigated the molecular mobility and cross-linked effects in cold and hot cast films plasticized with water and glycerol, by phosphorescence emission. On the other hand, Sionkowska *et. al.*, (2004) studied the molecular interactions in chitosan and collagen blends by Fourier transform infrared (FTIR) spectroscopy. Studies on the effect of plasticizers on the physical properties of pigskin gelatin films showed that plasticizer causes no apparent tendency to re-crystallization in the film structure, but alter other physical properties, such as flexibility, interactions between the macromolecule chains and susceptibility to humidity. Changes observed in the physical properties of the films can be, in principle, attributed to the fact that the plasticizers reduce the interactions between the adjacent chains in the biopolymer affecting thus, the moisture sensitivity and flexibility of the material (Bergo & Sobral, 2007)

Protein films and coatings are often quite stiff and brittle due to the extensive interactions between protein chains through hydrogen bonding, electrostatic forces hydrophobic bonding and/or disulphide cross linking. Relatively small molecular weight hydrophilic plasticizers are often added to the film solutions which mainly compete with the protein chains for hydrogen bonding and electrostatic interactions. The result of plasticizer addition is a reduction in protein chain–to-chain interactions, lowering of protein glass transition temperatures and an improvement in film flexibility. Also film

elongation increases and film strength decreases. Plasticizers reduce the film's ability to act as barrier to moisture, oxygen, aroma and oils. Commonly used plasticizers for edible film preparations are glycerol, sorbitol, propylene glycol, sucrose, fatty acids and monoglycerides. Water is also an important plasticizer in protein based films (Arvanitoyannis, 2002).

2.3.5.12 Fish Skin Gelatin based Films

Studies on the production of films from fish gelatin and their characterization are very recent, and it has been observed that all fish gelatins exhibited excellent film forming properties (Avena-Bustillos *et. al.*, 2006; Gomez- Guillen *et. al.*,2007; Jongjareonrak, *et. al.*, 2006a,2006b). Gelatin films from the skins of Nile perch, a warm-water fish species, were reported to exhibit stress and elongation at break similar to that of bovine bone gelatin (Muyonga *et. al.*, 2004a). Films from tuna skin gelatin plasticized with glycerol presented lower water vapor permeability (WVP) compared to values reported for pigskin gelatin (Gomez- Guillen *et. al.*, 2007).

The gelatin prepared from the skins of the Atlantic halibut (*H. hippoglossus*) was investigated for the development of edible films plasticized with 30 g sorbitol/100 g gelatin. The gelatin extracted from the halibut skins showed a suitable filmogenic capacity, leading to transparent, weakly colored, water-soluble and highly extensible films (Carvalho *et. al.*, 2007)

Water vapor permeability of cold- and warm-water fish skin gelatins films (from Alaska Pollock and salmon) was evaluated and compared with different types of mammalian gelatins. Water vapor permeability of cold-water fish gelatin films (0.93 gmm/m2hkPa) was significantly lower than warm-water fish and mammalian gelatin films This was related to increased hydrophobicity due to reduced amounts of proline and hydroxyproline in coldwater fish gelatins. The hydroxyl group of hydroxyproline is able to attract water due its high hygroscopic character. Lower water vapor permeability of fish gelatin films can be useful particularly for applications related to reducing water loss from encapsulated drugs and refrigerated or frozen food systems (Bustillos *et. al.*, 2006).

Antioxidative activity and properties of bigeye snapper and brownstripe red snapper skin gelatin-based films incorporated with BHT Butylated

Hydroxy-Toluene(BHT) or α-tocopherol were investigated by Jongjareonrak *et. al.*,(2006a). Mechanical properties and color of fish skin gelatin films of both species were generally affected by the incorporation of BHT or α-tocopherol as well as storage time. Water Vapour Permeability (WVP) of films decreased, when both BHT and a-tocopherol were incorporated. Films were more transparent with the incorporation of α -tocopherol and increasing storage time. Oxidation of lard was effectively retarded when covering with fish skin gelatin films regardless of antioxidant incorporation.

Few reports are available on the use fish skin gelatin for the preparation of edible films. Jongjareonrak, *et. al.,* (2006a) prepared transparent and relatively strong edible films from the skin gelatin of brownstripe red snapper (*Lutjanus vitta*) and bigeye snapper (*Priacanthus marcracanthus*). Mechanical properties of film from brownstripe red snapper skin gelatin were generally better than that from big-eye snapper skin gelatin. Gomez-Guillen (2007) observed that the edible films of tuna-fish gelatin were transparent and showed acceptable mechanical properties and barrier properties to water vapour and UV light. The antioxidant properties of the film increased significantly when natural extracts with high polyphenols content were added, producing only minor modifications of the film properties. Carvalho (2008) reported that the gelatin extracted from halibut skins showed suitable filmogenic capacity, leading to transparent, weakly colored and highly extensible films. Bustillos, *et. al* (2006) compared the water vapor permeability of cold- and warm-water fish skin gelatins with different types of mammalian gelatins and observed that fish gelatin films had lower water vapour permeability than mammalian gelatin films and this can be useful particularly for applications related to reducing water loss from encapsulated drugs and refrigerated or frozen food systems. Gelatins from freshwater fish skin, particularly from tilapia, Nile perch, Grass carp, catfish have been found to have superior physical and functional properties than coldwater fish skin gelatins and can be even comparable to gelatins from animal sources.

2.3.5.13 Fish Gel based Products

Gelatin is an important functional biopolymer, and its largest single use in food products is in water gel desserts. Gelatin desserts consist of the mixtures of gelatin powder, sweetener, water, and appropriate flavors and colors, along with a pH balancing of ingredients. The melt-in-the-mouth property is one of the most important characteristics of gelatin-based gel desserts. Some other biopolymers, such as agar and carrageenan, can also form thermally reversible gels with water, but the melting temperature of these gels is higher than the human body temperature. Commercial water gelatin desserts are generally made of gelatins from pork or non religiously slaughtered beef, which are unacceptable to Jews and Muslims. Some consumers are also concerned about BSE in mammalian gelatins. Fish gelatins can satisfy the requirements of these consumer groups. In addition, gelatin desserts made from various gelatins may provide variety in textural and gel melting properties, offering new product development opportunities (Choi & Regenstein 2000).

Zhou & Regenstein (2007) compared water gel desserts from various gelatins using instrumental measurements. Desserts made from Alaska pollock gelatin or gelatin mixtures containing Alaska pollock gelatin were more resistant to the destruction caused by the large deformation than tilapia gelatin and pork gelatins. In addition, the gel dessert made from Alaska pollock gelatin melted at a lower temperature than those from tilapia skin gelatin and pork gelatins, while desserts made from gelatin mixtures reflected the melting properties of the separate gelatins.

The largest single use of Gelatin in food products is in water gel desserts. Gelatin desserts consist of mixtures of gelatin powder, sweetener, water, and appropriate flavors and colors, along with a pH balancing of ingredients. The melt-in-the-mouth property is one of the most important characteristics of gelatin-based gel desserts (Zhou & Regenstein 2007). Some other biopolymers, such as agar and carrageenan, can also form thermally reversible gels with water, but the melting temperature of these gels is higher than the human body temperature. Commercial water gelatin desserts are generally made of gelatins from pork or non-religiously slaughtered beef, which are unacceptable to Jews and Muslims. Some consumers are also

concerned about the risk Bovine Spongiform Encephalopathy in consuming mammalian gelatins. Fish gelatins can satisfy the requirements of these consumer groups. In addition, gelatin desserts made from various gelatins may provide variety in textural and gel melting properties, offering new product development opportunities. The flavored fish gelatin dessert product had less undesirable off taste and off odour and a more desirable release of flavour and aroma than the product made from an equal bloom, higher melting point pork gelatin. (Choi & Regenstein, 2000).

The texture of commercial gelatin desserts is usually evaluated by measuring the Gel strength, which in gelatin industry refers to non fracture rigidity (Wainewright 1977). For the same measurement conditions ie, sample shape and size, maturation temperature and time, and instrumental parameters, the gel strength of a gelatin dessert would depend mainly on the properties of the gelatin and its concentration. Although gel strength is one of the important commercial criteria for gelatin desserts, this parameter may not represent all the textural properties encountered during human consumption of the product, which includes processes using the fingers (sometimes), and the teeth and tongue under both nondestructive and destructive conditions. Previous studies using small strain rheological tests (Gilsenan & Ross-Murphy, 2000; Gudmundsson, 2002; Joly-Duhamel, *et. al.,* 2002;Zhou *et. al.,* 2006) provided useful information on the properties of gelatin gels, but these small strain rheological measurements generally did not correlate well with sensory evaluation (Bourne, 2002; Foegeding, *et. al.,* 2003).On the other hand, the large deformation rheological tests were found to correlate better with the sensory studies on gelled systems (Munoz, *et. al.,* 1986a, 1986b; Barrangou *et al.,* 2006), and have been applied to measure gelatin gel textures (Munoz, *et al.,* 1986a, 1986b; Bot, *et. al.,* 1996; Surowka, 1997). In addition, texture profile analysis (TPA), a method introduced by Szczesniak and co-workers (Friedman, *et. al.,* 1963; Szczesniak *et al.,* 1963) to measure the mechanical textural characteristics of foods, was also found to correlate well with specific sensory characteristics. This method was further developed by Bourne (1968, 1978, 1995, 2002), who adapted the Instron Universal Testing Machine to food studies and clarified the mechanical textural parameters that could be calculated from a TPA curve.

2.3.5.14 Microbiological Quality of Fish Gelatin

Hides, skins and bones used as raw material for gelatin production are usually heavily contaminated with micro-organisms originating from soil and faecal material. These organisms consist of potentially pathogenic and non-pathogenic vegetative cells and spores. The initial washing and degreasing step using hot water will remove a substantial number of the contaminating microbes. The subsequent treatment at low or high pH over substantial periods of time will effectively kill contaminating micro-organisms (Russell *et. al.*, 1994; Russell, 1998). The level of potentially surviving micro-organisms will be further reduced due to the high-temperature-short-time processing step before drying. The combined effect of exposure to high or low pH in combination with heat treatment ensures that viable microorganisms would not be present in the final product (Brown & Booth, 1991; Schreiber & Seybold, 1993). However, as with other food materials gelatin can be contaminated after manufacture. Most countries have microbiological specifications for gelatin, but generally they are not very onerous. Total mesophilic plate counts of 1000 are generally accepted with various countries limiting the presence of Coliforms, E. coli, Salmonella, Clostridia spores, Staphylococci, and sometimes even Pseudomonas(Cole, 2000).

Since gelatin is an excellent nutrient for most bacteria, manufacturing processes should follow the Good Manufacturing Practices to avoid contamination during different stages. Since the gelatin extracts are subjected to harsh conditions during production and a final ultra high-temperature treatment, the bacterial load at this stage is expected to be greatly reduced. Many studies have been carried out recently to assess the bacterial contamination during different stages of gelatin processing and isolate the species. Since gelatin is extracted in the form of liquor by hot water treatment and undergoes a complex series of processing stages before being finally blended and packaged off as dry gelatin product, contamination at any stage affects the quality of the final product. Owing to its proteinaceous nature and the presence of residual sugars (Sharma *et. al.*, 2003a, 2003b), gelatin acts as an excellent medium for the growth of many microorganisms including

enterobacteria. Some of these are capable of producing extracellular proteases in the growth medium, which liberates nutrients for the gelatinase negative organisms. Bacterial contamination of gelatin may affect its safety and/or quality in use. Indeed, some of these contaminants possess pathogenic properties for man and thus are a threat to human health in food and pharmaceutical applications. Furthermore, gelatin contaminants have shown to exhibit gelatinase activity (De Clerck & De Vos 2002) and therefore negatively affect the viscosity and gelling capacity of the product. As a result of this gelatin liquefaction, nutrients may become available for gelatinase negative contaminants, promoting their growth. Therefore, a periodic monitoring of bacterial contamination during gelatin production seems to be needed to trace and evaluate the effects of varying production parameters (e.g. raw materials and chemical treatment) on the contaminating bacterial flora.

Sharma *et. al.*, (2006a) studied the Enterobacterial contamination of gelatin during different stages of its manufacturing. The Enterobacteriaceae-positive samples were processed and these enterobacterial species were isolated and identified as *Proteus mirabilis, Serratia marcescens, E. coli, Salmonella typhi, Klebsiella oxytoca, Klebsiella pneumoniae, Shigella flexneri and Serratia liquefaciens*. Since these enteric bacteria are pathogenic, some of them are gelatinolytic and also have a significant effect on the quality and amino acid content of gelatin.They are of great concern both for the manufacturers as well as for the consumers. Sharma *et. al.*, (2006b) reported the isolates of many known pathogens viz., *Staphylococcus epidermidis,* Staphylococcus *aureus, Pseudomons aeruginosa & Clostridium perfringens* from samples of different stages of gelatin manufacturing. These also exhibited gelatinase activity which caused depletion of the nutritional quality of food grade gelatin.

De Clerck *et. al.*, (2004) examined the bacterial contamination of semifinal gelatin extracts from several production plants and a total 1,129 isolates were obtained from a total of 73 gelatin batches originating from six different production plants. The majority of isolates belonged to members of *Bacillus* or related endospore-forming genera. Representative strains were identified as *Bacillus cereus*, *Bacillus coagulans*, *Bacillus fumarioli*, *Bacillus*

amyloliquefaciens, *Bacillus licheniformis*, *Bacillus pumilus*, *Bacillus sonorensis*, *Bacillus subtilis*, *Bacillus gelatini*, *Bacillus thermoamylovorans*, *Oxybacillus contaminans*, *Anoxybacillus flavithermus*, *Brevibacillus agri*, *Brevibacillus borstelensis*, and *Geobacillus stearothermophilus*. The majority of these species include strains exhibiting gelatinase activity. Moreover, some of these species have known pathogenic properties. These findings are of great concern with regard to the safety and quality of gelatin and its applications.

Hazard Analysis at Critical Control Points (HACCP) and Good Hygienic Practice (GHP) are fundamental for the production of gelatin. The HACCP system includes the determination of Critical Control Points (CCP) and establishment of Critical Limits (CL). CL have to be established for pH, concentration of acid/base and treatment time and temperature/time at appropriate processing steps regarded as CCP's for a safe gelatin production (European Commission ,1998).

2.3.5.15 Safety of Gelatin as a Food Ingredient

Gelatin is regarded as a food ingredient rather than an additive and it is Generally Regarded as Safe (GRAS). In 1993 the FDA reiterated the GRAS status of gelatin and stated that there was no objection to the use of gelatin from any source and any country provided that the hide from animals showing signs of neurological disease were excluded and also specified raw materials were excluded from the manufacturing process. Although, at the beginning of the BSE scare in Europe the popular media brought suspicion on all products of bovine origin as being possible transmitters of the disease to humans, this was a thoroughly unscientific assessment of the dangers of spreading infection. It is now recognized that BSE is a neurological and brain problem and not associated with the hide of the animal. It is also recognized that the processes of manufacturing gelatin make it virtually impossible for the survival of a defective prion, if it were present in the first place.

Hence, today, gelatin retains its GRAS status. Furthermore, the Joint Expert Commission on Food Additives (JECFA) placed no limit on the use of gelatin. Gelatin is an excellent growth medium for most bacteria; hence considerable care needs to be taken, during manufacture, to avoid contamination. This care is evidenced by the use of documented HACCP programs by manufacturers. In the same way to ensure product reproducibility, most companies are implementing ISO 9000 quality management systems.

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3.0 Materials and Methods...

3.1 Materials

3.1.1. Chemicals

Analytical reagents supplied by different companies as detailed below were used for the experiments.

Qualigens

Ammonium molybdate

Boric acid

Chloroform

Ethanol

Glacial acetic acid

Glycerol

Nitric acid

Petroleum ether

Potassium iodide

Sodium chloride

Sodium hydroxide

Sodium meta bisulphate

Sodium sulphate

Sulphuric acid

Trichloro acetic Acid

Merck

Acetonitrile

Coomassie Brilliant Blue R250

Hydrochloric acid

O –Phthalaldehyde

Parafilm M

Perchloric acid

Phenolphthalein

Potassium carbonate

Sodium dodecyl sulphate

Thio barbituric acid reagent

Sisco Research Laboratories (SRL)

2-mercaptethanol

Ammonium per sulphate

Ferric chloride

Glycine

L-Leucine

Methanol

Standards of Arsenic, Lead, Copper, Zinc, Cadmium and Chromium

Tryptophan

SD. Fine Chemicals

Bromophenol blue

Potassium bromide

Sodium hypochlorite

Tri sodium citrate

Sigma chemicals

Acrylamide /BIS Acrylamide

Amino acid standards

Gelatin from Bovine Skin Type B ~ 225 Bloom

Gelatin from Bovine Skin Type B ~ 75 Bloom

Gelatin from cold water fish skin

Gelatin from Porcine Skin Type A ~ 175 Bloom

Gelatin from Porcine Skin Type A ~ 300 Bloom

Gelatin standards

L -4- hydroxyproline

Wide Range Sigma Marker (Molecular weight 6500 – 200,000 Da)

3.1.2. Equipment and Glassware

Amino acid analyzer	:	HPLC- LC 10 AS, Schimadzu with FL6A fluorescence Detector and Shimadzu CR 6A Chrompac recorder
Atomic Absorption Sctrophotometer	:	Varian AA 420 ,USA

Bio-rad Tetra Mini Protean II unit with gel documentation system	:	Bio-Rad Laboratories, Hercules, CA
Bloom jars	:	Schott Duran, Germany
Bowl Chopper	:	Tecator 1094
Centrifuge	:	REMI Cooling centrifuge, Model CPR 24, Remi Instruments, India
Circulating water bath	:	(Haake D3, Germany)
Deionised water generation system	:	ELGA Purelab Ultra, UK.
Flake ice machine	:	F90 compact unit, Icematic, Italy
Freeze Drier	:	Martin Christ, Gamma 1-16 LSC, Germany
Gas Permeability Apparatus		Davenport,UK
Glass wares	:	Borosil Glass ware, India
Homogenizer	:	Ultra Turrax, T20 B IKA Labortechnik, Germany
Hot air oven	:	Beston hot air oven, India
Infrared Spectrophotometer	:	Nicolet Avatar 360 ESP
Micrometer Screw Gauge	:	Reston Equipment, India
Microwave Digester	:	Anton Paar, Germany
pH meter	:	Cyberscan 510 pH meter. Eutech Instruments, Singapore
Spectrocolorimeter	:	Hunter lab Miniscan ® XE plus, UK
Spectrophotometer	:	Genesys 5, Spectronic Instruments, USA
Texture Analyser & Tensile Strength Tester	:	Lloyd Instruments, Model LRX Plus, U.K

Vacuum Chamber	: Heraeus Vacutherm –Germany
Viscometer	: Brookfield DV E Model, England
Water Bath	: Julabo TW 20, Germany
Weighing Balance	: Sartorius Electronic Balance, Germany

3.1.3 Fish Skin

The raw materials for the study were the skins of three cultured freshwater fishes viz., Rohu (*Labeo rohita* – Hamilton Buchanan), Common carp (*Cyprinus carpio)* and Grass carp (*Ctenopharyngodon idella*). Fish samples were procured from different freshwater farms located in Central Kerala. The size description of the species collected is given in Table 3.1.

Table 3.1 Size description of the selected fish species

Species	Average size
Rohu	Length:55 ± 2.8 cm , Weight : 2500 ± 120g
Common carp	Length:30 ± 3.5 cm, Weight : 1500 ± 65g
Grass carp	Length: 62 ± 2.2 cm, Weight : 2610 ± 140g

The fish was brought to the laboratory in iced condition. The samples were then filleted and the skinless boneless fillets were used for the preparation of value added products. The skin was collected, cleaned by removing scales, washed and blast frozen and stored at -18 ^{0} C with a maximum storage of less than two months before use.

3.2 Methods

3.2.1 Preparation of the Fish Skin for Gelatin Extraction (Zhou and Regenstein, 2004)

Frozen skins were thawed at 4 °C for about 20 h, chopped into small pieces (about 2 to 3 cm), and washed with tap water (1:6 w/v) for 10 min. Washing was repeated 3 times. The cleaned fish skins were drained using cheesecloth for 5 min, and the cheesecloth containing the skins were squeezed by hand to remove liquid.

3.2.2. Process of Gelatin Extraction

3.2.2.1 Pretreatment of fish skins prior to main extraction

The gelatin extraction procedure followed was essentially as described by Grossman and Bergman (1992) with slight modifications. The cleaned and drained fish skins were given a pretreatment with an alkaline solution followed by an acid solution. The detailed steps were as follows: Cleaned skins (c.a 30.00 g) were taken in conical flask and treated with different concentrations of sodium hydroxide (1:6 w/v) for variable times. Then, the samples were rinsed with tap water and drained using cheesecloth. The above treatment was repeated for 2 times. Afterwards the samples were treated with different concentrations of sulphuric acid (1:6 w/v) for variable times. The samples were then rinsed with tap water and drained using cheesecloth. The acid treatment was also repeated two times. The treated samples were squeezed manually using cheesecloth to remove excess water prior to the extraction. The conditions followed for the pretreatment are given in Table 3.2

Table 3.2 Process variables adopted for the pretreatment of fish skins.

Process variables	Range		
NaOH concentration (mol/L)	0.1	0.15	0.2
H_2SO_4 concentration (mol/L)	0.1	0.15	0.2
Pretreatment time (minutes)	40	50	60

3.2.2.2 Gelatin extraction

The pretreated fish skins were taken in flasks for gelatin extraction with varying volumes of deionized water. The flasks were covered with parafilm and the extraction was carried out in a water bath for variable times at variable extraction temperatures as given in Table 3.3. Finally, the gelatin solutions were filtered through 4 layers of cheesecloth, and freeze dried prior to further work.

Table 3.3 Process variables adopted for the extraction of gelatin.

Process variables	Range		
Skin/water ratio	1:4	1:5	1:6
Extraction time	6H	8H	10H
Extraction temperature	40 ^{0}C	50 ^{0}C	60 ^{0}C

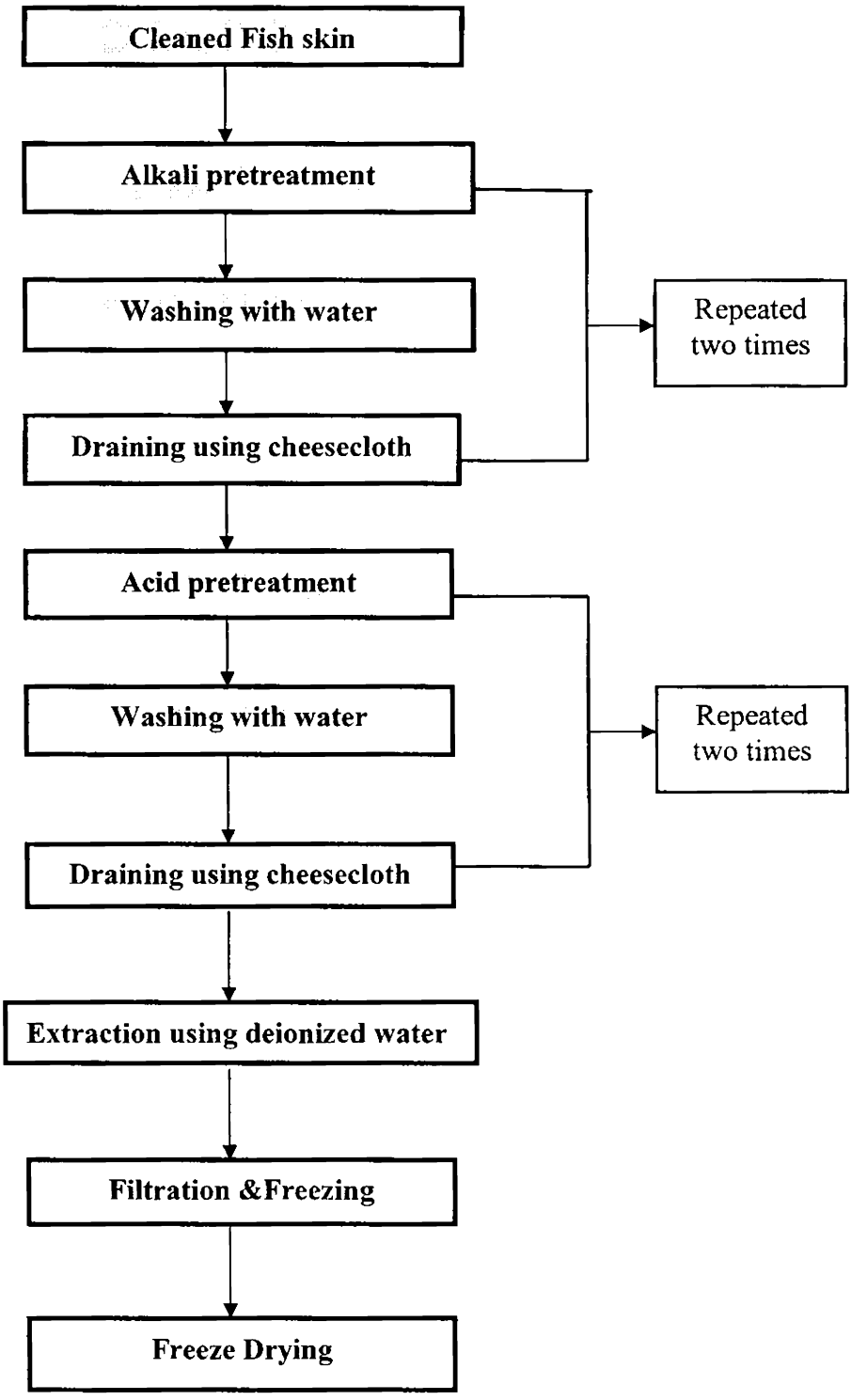


Fig.3.1 Flow chart for pretreatment and extraction of gelatin from fish skin

3.2.3 Experimental Design for Process Optimization

Optimization is the method of choice when seeking a best alternative from a specified set of alternatives. The experimental design for optimization is a two stage process. In the first stage called screening, the objective is to efficiently determine the critical control variables from a large number of potential variables. In the second stage of optimizing, the objective is to determine the optimum values for the critical control factors so that the desired quality objectives are met.

3.2.3.1 Screening Experimental Design

Screening was done to determine the critical variables for the extraction of gelatin from the skin of Rohu, Common carp and Grass carp with a fractional factorial design. Fractional factorial design consists of an appropriately chosen small fraction of the full factorial design which permits the study of a large number of variables in an economical number of trials. A fractional factorial design was used for screening. Six important factors (independent variables) that affect the extraction of gelatin from fish skin and their ranges between model levels described as −1 and +1 were selected for the screening experiments (Table 3.4). The design used in the study is a resolution three design (2 6-3 /iii) in which the main effects are confounded with two factor interactions. The importance of these factors was evaluated based on the responses on two dependent variables selected. These selected dependent variables were gel strength (Bloom) and yield (%) which can be rated as the most commercially important physical properties of the extracted gelatin. A total of eight groups of extraction experiments were conducted using different combinations of these six factors (Table 3.4).

From the screening experiments, four factors were identified as critical variables that had a significant effect on the extraction of gelatin from the skin of Rohu, Common carp and Grass carp. These were Alkali pretreatment concentration (mol/L), Acid pretreatment concentration (mol/L), Pretreatment time (min), and Extraction temperature (°C).

Table 3.4 Independent Variables and their Levels in the 6 Factor, 2 Level Fractional Factorial (2 6-3 /Iii) Screening Design*

Independent variables	Symbol	Levels		
		-1	0	+1
NaOH concentration (mol/L)	X1	0.1	0.15	0.2
H_2SO_4 concentration (mol/L)	X2	0.1	0.15	0.2
Pretreatment time (minutes)	X3	40	50	60
Skin/water ratio	X4	1:4	1:5	1:6
Extraction time (hours)	X5	6	8	10
Extraction temperature (^{0}C)	X6	40	50	60

*No. of variables: 6, Levels: 2, Observations: 8, Resolution: 3 : Wt. of sample: 30g for each run, Pretreatment ratio 1: 6.

Table 3.5 Fractional Factorial Screening Design in Coded Units

Standard Order	X1	X2	X3	X4	X5	X6
1	-1	-1	-1	+1	+1	+1
2	+1	-1	-1	-1	-1	+1
3	-1	+1	-1	-1	+1	-1
4	+1	+1	-1	+1	-1	-1
5	-1	-1	+1	+1	-1	-1
6	+1	-1	+1	-1	+1	-1
7	-1	+1	+1	-1	-1	+1
8	+1	+1	+1	+1	+1	+1

3.2.3.2 Optimization of Experimental Design

Once the important variables are determined by the screening, Response Surface Methodology was used for optimizing the process of extraction of gelatin. The independent variables and their levels used in the Design are given in table 3.6. A 4 factor, 5 levels Central Composite Rotatable Design was formulated which is given in Table 3.7. Here five levels were assigned for each factor instead of the two level design for screening experiments. To study the effect of the selected independent variables on the responses, a total of 31 runs which included seven centre point runs using the Central Composite Design were carried out Experimental data were statistically analyzed using the software Design-Expert 6.0.11, (Stat-Ease, Inc., Minneapolis MN, USA).

Table 3.6 Independent variables and their levels in the 4 factor, 5 level Central Composite Rotatable Design for optimization of the extraction conditions of gelatin from fish skin

	Symbol		Code level				
Independent variables	**coded**	**uncoded**	**-2**	**-1**	**0**	**1**	**2**
NaOH concentration(mol/L)	X1	X1	0.05	0.1	0.15	0.2	0.25
H_2SO_4 concentration(mol/L)	X2	X2	0.05	0.1	0.15	0.2	0.25
Pretreatment time(minutes)	X3	X3	30	40	50	60	70
Extraction temperature (^{0}C)	X4	X4	30	40	50	60	70

Table 3.7 Central Composite Design for Optimizing the Extraction Condition of Fish Skin Gelatin

Standard Order	X1	X2	X3	X4
01	-1	-1	-1	-1
02	1	-1	-1	-1
03	-1	1	-1	-1
04	1	1	-1	-1
05	-1	-1	1	-1
06	1	-1	1	-1
07	-1	1	1	-1
08	1	1	1	-1
09	-1	-1	-1	1
10	1	-1	-1	1
11	-1	1	-1	1
12	1	1	-1	1
13	-1	-1	1	1
14	1	-1	1	1
15	-1	1	1	1
16	1	1	1	1
17	-2	0	0	0
18	2	0	0	0
19	0	-2	0	0
20	0	2	0	0
21	0	0	-2	0
22	0	0	2	0
23	0	0	0	-2
24	0	0	0	2
25	0	0	0	0

26	0	0	0	0
27	0	0	0	0
28	0	0	0	0
29	0	0	0	0
30	0	0	0	0
31	0	0	0	0

3.2.4 Gelatin Water Dessert Preparation (Zhou & Regenstein, 2007)

Gelatin water desserts were prepared by dissolving gelatins or their mixtures in a flavored orange drink (prepared from orange flavour instant drink mix, Kraft Foods Ltd., Thailand) heated 45 – 50 ^{0}C to compare the sensory and physical properties of gel desserts from various sources such as bovine , porcine and fish skin gelatin. The gelatin desserts prepared had the same gelatin concentration (3%w/w). Gelatin concentration lower than 3% resulted in desserts which are very soft and disintegrated immediately after formation, particularly in the case of fish skin gelatins. Gelatin concentration above 3% produced hard gels which is an undesirable feature for the desserts. Hence 3% concentration of gelatin was found to be the optimum concentration for the preparation of desserts. The final composition of desserts is shown in Table 3.8. The dessert solutions were then poured separately into 2 different containers: (1) standard bloom jars (112.5 g) for gel strength determination; (2) cylindrical plastic molds having diameter of 38 mm and a height of 22 mm for texture profile analyses. All samples were then matured at 2 - 4 ^{0}C for 20 - 24 hours before measurements were made.

Table 3.8 Composition and pH of Gelatin Desserts*

	BG	PG	RG	CG	GG
Gelatin (g)	3.0	3.0	3.0	3.0	3.0
Water (g)*	87.0	87.0	87.0	87.0	87.0
Sugar (g)*	9.0	9.0	9.0	9.0	9.0
Others (g)*	<1.0	<1.0	<1.0	<1.0	<1.0
Final pH	3.8	3.7	3.6	3.7	3.7

*BG =Bovine Skin Gelatin (225B); PG = Porcine Skin Gelatin (300B); RG = Rohu Skin Gelatin; CG = Common carp Skin Gelatin ; GG = Grass carp Skin Gelatin

*The amount of water and sugar are calculated based on the ingredient label of the flavored orange drink. The word "others" is based on the ingredient label of the flavored orange drink, and refers to those compounds providing appropriate orange flavor and color, and are used to balance the pH, which include synthetic flavours (E102, E110, E171), acidity regulator (E330), minerals(E341)(0.57%), stabilizer(E415), vitamins(0.32%), Ferrous citrate(0.07%),edible salt and the total amount of these compounds is less than 1.0 g in 100 g of the final gelatin desserts.

3.2.5 Preparation of Gelatin Films (Sobral *et al.*, 2001)

The gelatin films were prepared according to the casting technique described by Sobral *et.al.*, (2001) with slight modifications. This consists of dehydrating a filmogenic solution, conveniently applied on a support. The filmogenic solutions of gelatin were prepared under the following conditions: 7.5g of gelatin / 105 ml distilled water (6.67% w/w) was mixed with 1.5g glycerol as plasticizer at natural pH of the solution. The plasticizer used was 20% (w/w) of the gelatin. Initially the gelatin was hydrated at room temperature in water and solubilized later in a water bath with digital control (±0.5 ^{0}C) kept at 55 ^{0} C. After complete solubilisation, remaining water and glycerol were added, and the solution was kept in water bath under agitation for 30 minutes. The filmogenic solutions were degassed under vacuum and 25 ml of the solution was applied on High Impact Polypropylene trays of size 24 X 14 cm. The films were dried overnight at ambient temperature and manually peeled off from the surface.

3.2.6 Analytical Methods

3.2.6.1 Determination of yield (Muyonga *et. al.*, 2004a).

The yield was calculated by taking 10 ml of gelatine solution in duplicate which was centrifuged, filtered and evaporated and used for solid concentration determination .The following equation was used for gelatine yield calculation:

$$\text{Yield (\%)} \quad \frac{C \times V}{M} \times 100$$

where C = light liquor concentration (g/ml), V = liquor volume, M = weight of sample (g) used for extraction.

3.2.6.2 Determination of pH (BS 757, 1975)

A 1% (w/v) solution of gelatin was prepared in distilled water at 60 ^{0} C, cooled to room temperature and the pH was measured using Cyberscan 510 pH meter.

3.2.6.3 Determination of Colour

Gelatin solutions (6.67% w/v) were used for the measurement of color. Colour analysis was performed with a Hunter lab Miniscan ® XE plus spectrocolorimeter (Hunter Associates Laboratory, Inc. Reston, Virginia, USA). Measurements were recorded using the L* a* b* colour scale (CIE,1986). Six repetitions of the different colour parameters were recorded.

3.2.6.4 Viscosity *(Cho et. al., 2006)*

Gelatin solutions at a concentration of 6.67%(w/v) were prepared by dissolving the dry powder in distilled water and heating at 60 ^{0} C. Viscosity (cP) of 10 ml of the solution was determined using Brookfield digital viscometer (Model DV E Brookfield Engineering, USA) equipped with a No.1 spindle at 30 ± 0.5 ^{0} C

3.2.6.5 Clarity (ISO 7027:1999)

The clarity / turbidity of a 6.67 % gelatin solution was measured as the absorbance at 620 nm of a 6.67 % gelatin solution in 1 cm cuvettes against water using a spectrophotometer.

Results of spectrophotometric measurements can be expressed as absorbance (E) or transmission (T). Conversion of results is possible using the following formulas:

$E = \log 1 / T = 1 / 10E$

Procedure

7.50 g (± 0.01) gelatin was weighed into a 150 ml bottle and 105 ml (± 0.2) water was added. The solution was then stirred to moisten the gelatin completely, after which the bottle was covered with rubber stopper and allowed to stand at room temperature for 1 - 4h.

The bottle was then placed in a water bath at 65°C for about 20 min. for dissolving the sample. The bottle was shaken to dissolve the gelatine completely and to achieve a homogeneous solution. The completely dissolved sample was transferred to the cuvettes, and allowed to cool to room temperature. The absorbance at 620 nm was measured at room temperature against deionized water.

3.2.6.6 Foam Formation Capacity and Foam Stability (Cho *et. al.*, 2004)

One gram of gelatin was placed in 50 ml distilled water and allowed to swell. The swollen sample was kept at 60 ^{0}C and the foam was prepared by homogenizing at 10,000 rpm for 5 min. in the Ultra Turrax homogeniser. The homogenized solution was poured into a 250 ml flask. The foam formation ability was calculated as the ratio of volume of foam to the initial volume of liquid. The foam stability was calculated as the ratio of the initial volume of foam to the final volume of foam after 30 min.

3.2.6.7 Water holding and Fat binding capacities (Cho *et. al.*, 2004)

One gram of gelatin was taken in a centrifuge tube and weighed (tube with gelatin). For measuring water-holding capacity and fat-binding capacity, 50 ml distilled water or 10 ml sunflower oil was added respectively and held at room temperature for 1 h. The gelatin solutions were mixed in vortex mixer for 5 s every 15 min for one hour. The solutions were then centrifuged at 450g for

20 min. The upper phase was removed and the centrifuge tube was drained for 30 min on a filter paper after tilting to a 45^0 angle. Water holding and Fat binding capacities were calculated as the weight of the contents of the tube after draining divided by the weight of the dried gelatin, and expressed as the percentage of weight of dried gelatin.

3.2.6.8 Determination of Melting point (Wainewright, 1977)

Gelatin solutions 6.67% (w/w) were prepared and a 5-mL aliquot of each sample was transferred to a small culture test tube of 12 × 75 mm size. The samples were degassed in vacuum chamber (Heraeus vacutherm – Germany). The tubes were then covered with parafilm and heated in a water bath at 60 ^{0} C for 15 minutes. It was then cooled immediately in ice chilled water and matured at 10 ^{0} C for 16-18 hours. 5 drops of a mixture of 75% chloroform and 25% methyl red dye was placed on the surface of the gel. The gels were then put in a water bath (circulating bath – Haake D3) at 10 ^{0} C and the water heated at the rate of 0.2 ^{0} C per minute. The temperature at which the drops began to move freely down the gel was taken as the melting point.

3.2.6.9 Determination of Setting point and Setting time

Determination of setting point and setting time of gelatin was carried out as described by Muyonga *et. al.*, (2004a) but with slight modifications Gelatin solutions of 10% (w/w) were prepared as described in Section 3.2.6.8 and transferred to thin wall (12 mm × 75 mm) test tubes. The dissolved samples were transferred to water bath held at 40 ^{0}C (circulating bath – Haake D3). The water bath was then cooled slowly at the rate of 0.2 ^{0} C per minute. A thermometer was inserted into the sample and lifted out at 30 seconds intervals. The temperature of the mixture at which the gelatin solution no longer dripped from the tip of the thermometer was recorded as the setting temperature.

Setting time was determined on samples prepared in the same way as those for the determination of the setting temperature. Samples were transferred to a water bath maintained at 10 ^{0} C (circulating bath – Haake D3). A rod was inserted in the gelatin solution and observed at intervals of 15 seconds. The time at which the rod could not detach from the gelatin sample was recorded as the setting time.

3.2.6.10 Gel strength determination (Jelly strength, Bloom) (BS 757: 1975)

Definition

The gel strength (Bloom) is the mass in grams necessary to depress a standard plunger 4 mm into the gel having a gelatin concentration of 6.67 %(w/v) and matured at 10.0°C for 17 h.

Principle

A 6.67 % solution of the gelatin sample is prepared in a wide-mouthed test bottle at 60°C, cooled to 10^0C and kept for 17 h for maturation at this temperature. The resulting gel is tested using a Texture Analyzer.

Equipment

Texture Analyzer (Lloyd Instruments, Model LRX Plus, U.K).

Plunger: AOAC plunger, with 12.70 mm (0.500 inches) diameter, plane surface and sharp edge, no measurable radius.

Bloom jars (Schott Duran): The standard Bloom jar has a capacity of approximately 155 ml, internal diameter of 59 mm +/- 1 mm, overall height 85 mm and a flat bottom to ensure it does not rock on a flat surface.

Thermostatic water bath: held at 65 ± 2 °C.

Balance: with a sensitivity of 0.01 g.

Procedure

7.50 ± 0.01 g gelatin was weighed into the Bloom bottle and 105 ± 0.2 ml deionized water was added and stirred. The bottle was covered with a rubber stopper and the sample was allowed to stand at room temperature for 4 hours. The bottles were then placed in water bath at 45 °C for about 20 min with occasional shaking for complete dissolution. The bottles were allowed to cool for about 15 min. at room temperature, and then placed in chilled condition at 2- 4 ^{0} C for 17 h. For determining the gel strength, the plunger of the Texture Analyzer was set to move a distance 4 mm into the gel with a speed of 0.5 mm/sec. The sample bottle was removed form the chill condition and immediately placed at the centre of the platform of the Texture Analyzer so that the plunger contacts the sample as nearly at its midpoint as possible and the measurement was taken. The value given by the Texture Analyzer was the gel strength (Bloom).

3.2.6.11 Determination of Odour (Muyonga *et. al.*, 2004a).

Determination of odour by sensory evaluation was conducted using a seven member panel. Samples for sensory evaluation were prepared by dissolving 0.5 g of gelatin in 7 ml of distilled water, to obtain a solution containing approximately 6.67% gelatin. The samples were prepared in screw cap test tubes with and dissolved as described for the Bloom samples in Section 3.2.6.10. The samples were then held in a water bath at 50^0 C, with the screw caps lightly closed. Panelists were instructed to remove the screw caps, sniff the contents and identify the odour they perceived as well as indicate the odour intensity, using a five point scale (0 = no odour, 1 = very mild and only perceivable on careful assessment, 2 = mild but easily perceivable, 3 = strong but not offensive, 4 = strong and offensive, 5 = very strong and very offensive,)

3.2.6.12 Texture Profile Analysis (Muyonga *et. al.*, 2004a)

TPA was measured using a Lloyds Texture Analyzer (Lloyd Instruments, Model LRX Plus, U.K.). The samples for Texture Profile Analysis were prepared in the same method as described in Section 3.2.6.10 for Bloom determination. The gel samples were then poured into cylindrical plastic containers with a diameter of 30 mm and a height of 40 mm and stored in a chilled room at 9–10 ^{0}C for 17 h. Before testing, the samples were equilibrated to room temperature for 30 min. The samples were removed from the plastic moulds and sections (20 mm length) cut off and tested by imparting a 50% strain, double compression, using 50 mm diameter cylindrical probe. Pre-test, test and post-test speed were set at 1 mm/s and trigger force at 5 g. The Hardness, Springiness index, Cohesiveness, Chewiness, Gumminess, Fracture Force, Adhesiveness and stiffness were determined as described by Pye (1996). From the TPA curve the mechanical textural parameters were calculated. Hardness is defined as the peak force (unit: g) in the first cycle; cohesiveness is defined as the ratio of the positive force area during the second cycle divided by the positive force area in the first cycle (A2/A1, a dimensionless quantity); gumminess is defined as hardness X cohesiveness (the unit for gumminess: g). Texture Profile Analysis result was tabulated using Nexygen Software.

3.2.6.13 Determination of Moisture (Method 934.01: AOAC, 2000)

5-10 g sample was weighed into pre-weighed clean petri dish. Dishes were placed in a hot air oven at 105±1° C for 6 hours. Dishes were cooled in desiccators and weighed to a constant weight. Moisture loss was calculated as

$$\% \text{ Moisture} = \frac{\text{Loss in weight x 100}}{\text{Weight of the sample}}$$

3.2.6.14 Determination of Crude Protein (Method 954.01: AOAC, 2000)

0.3 to 0.5 g of the moisture free gelatin sample was transferred into a digestion flask of 50 ml capacity. A few glass beads, a pinch of digestion mixture (8 parts K_2SO_4 & 1 part $CuSO_4$) and 10 ml concentrated sulphuric acid were added to the flask. It was digested over a burner until the solution turns colorless. The digest was transferred quantitatively into a 100 ml standard flask and made up to the mark. The 2 ml of well-mixed made-up solution was transferred to the reaction chamber of the Micro-Kjeldahl distillation apparatus, 2 drops of phenolphthalein indicator and 40% sodium hydroxide were added till the indicator changes to pink. Distillation was done for 4 minutes and ammonia liberated was absorbed into 2% boric acid containing a drop of Tashiro's indicator. The amount of ammonia liberated was determined by titration with N/50 sulphuric acid. Percentage Crude protein was determined as:

% Crude protein = nitrogen content x 5.4 (Nitrogen conversion factor as per Eastoe & Eastoe, 1952)

3.2.6.15 Determination of Crude Fat (Method 991.36:AOAC, 2000)

About 1-2 g of accurately weighed moisture free sample was taken in a thimble plugged with cotton and was extracted with petroleum ether (40-60°C BP) in a Soxhlet apparatus for about 10 hrs, at a condensation rate of 5-6 drops per min. Excess solvent was evaporated and the fat was dried at 100°C to a constant weight. The crude fat was determined as ; $\% \text{ Crude fat} = \frac{\text{Weight of fat x 100}}{\text{Weight of the sample}}$

3.2.6.16 Determination of Ash Content (Method 942.05:AOAC, 2000)

About 2-3 g of the moisture free sample was transferred into a previously heated, cooled and weighed silica crucible. The sample was charred at low red heat. Then the crucible was placed in a muffle fumace at 550° C for about 6 hours until a white ash was obtained. Crucible was cooled in a desiccator and weighed. Ash content was calculated as

$$\% \text{ Ash} = \frac{\text{Weight of residue x 100}}{\text{Weight of the sample}}$$

3.2.6.17 Estimation of Arsenic, Lead, Copper, Zinc, Cadmium and Chromium using Atomic Absorption Spectrophotometer (AOAC, 2000)

Reagents

1. Nitric acid
2. Perchloric acid
3. 1&2 mixed in 9:4 ratio
4. Stock solutions of Arsenic, Lead, Copper, Zinc, Cadmium and Chromium prepared by diluting concentrated solution of 1000 mg/L (SRL)

Procedure

1 g gelatin sample was used for the experiment. To the sample containing flask, 7 ml of nitric acid and perchloric acid (9:4) mixture was added, covered with a watch glass and left at room temperature overnight. The samples were then digested using a microwave digester (Anton Paar). The completely digested samples were allowed to cool at room temperature, filtered using glass and carefully transferred and made up into a clean 50 ml volumetric standard flask. The samples were analysed using Varian spectra AA 220, AAS equipped with deuterium background corrector, for the determination of Arsenic, Lead, Copper, Zinc, Cadmium and Chromium.

3.2.6.18 SDS-Polyacrylamide Gel Electrophoresis (SDS - PAGE)

Electrophoretic separation of gelatin proteins were separated by SDS-PAGE technique as described by Laemmli (1970). It is based on the principle that in the presence of 10% SDS and 2-mercaptoethanol, proteins dissociate into their sub units and bind large quantities of the detergent which mask the

charge of the proteins and giving a constant charge to mass ratio so that the proteins move according to their molecular weight in an electric field. In this discontinuous buffer system, the separating (resolving gels) and stacking gels are made up in the electrode buffer, Tris-glycine. During electrophoresis, the leading ion is chloride while the trailing ion is glycine. In this experiment, 7.5% gel concentration was used for the effective separation.

Reagents

1. Tris-HCl : 0.5M, pH 6.8
2. Tris-HCl : 1.5M, pH 8.8
3. SDS: 10%
4. Acrylamide /BIS: 30% T, 2.67% C
5. Sample Buffer:

   Distilled water: 3.8 ml

   Tris-HCl : 0.5M, pH 6.8,1 ml

   Glycerol: 0.8 ml

   10% SDS : 1.6 ml

   2-mercaptoethanol: 0.4 ml

   1% bromophenol blue: 0.4 ml

6. Electrode Buffer:

   Tris base: 9g

   Glycine : 43.2g

   SDS: 3g

   These reagents were dissolved in 600ml distilled water.

   Working solution: Dilute 100ml from stock to 500ml with distilled water.

7. Separating gel (7.5%):

   10% SDS : 100µl

   Acrylamide :2.5ml

   APS 10% : 50µl

   Distilled water: 4.85ml

   Tetramethylethylenediamine(TEMED) : 5 µl

   Tris-HCl : 1.5M, 2.5ml

8. Stacking Gel (4%)

   10% SDS : 100µl

   Acrylamide :1.33ml

APS 10% : 50µl

Distilled water:6.1ml

TEMED : 10 µl

Tris-HCl : 0.5M, 2.5ml

9. Ammonium per sulphate (APS): 10%

Procedure

The apparatus used was Bio-rad Tetra Mini Protean II unit (Bio-Rad Laboratories, Hercules, CA) with gel documentation system. Wide range sigma marker (mol. wt: 6500-200,00Da, S8445, Sigma Aldrich) was used in electrophoresis. Dry gelatin was dissolved in distilled water at 60^0C to attain final concentration of 2 mg/ml of gelatine. 0.1 ml of the sample was taken in a micro centrifuge tube and added 0.1 ml of the sample buffer, heated in boiling water bath for 4 minutes, cooled and kept frozen pending analysis.

The separating gel was prepared without TEMED and APS. Degassed for 15 minutes to remove the air bubbles. Added TEMED and APS with intermittent shaking after each addition immediately transferred the solution to the apparatus. Added a little water on the top of the gel to level it and kept for 45 minutes. Prepared stacking gel after keeping the comb over the apparatus at 45 ^{0}, poured the gel slowly, and pressed the comb slowly and evenly. Marked the wells and kept for 45 minutes for setting. After removing the comb the whole apparatus was transferred to sandwich clamp assembly into the inner cooling core. Rinsed the apparatus and wells with electrode buffer and filled the inner chamber of the apparatus completely and the outer chamber to the optimum level. Injected 10 µl of the sample into the wells. The electrode lid was placed at proper position and connection was established. The power of 200V was supplied. Electrophoresis was carried out for 45 minutes approximately until the dye reaches the bottom. Subsequently, the gel was removed and was placed in a big petridish containing the stain Coomassie Brilliant Blue R250 to stain the protein bands. Kept for 30 minutes and transferred the gel to 7% acetic acid for destaining which was changed intermittently till complete destaining occurred.

3.2.6.19 Estimation of Amino Acids

Total amino acids in gelatin samples were determined as per the procedure of Ishida *et. al.*, (1981).

Reagents

1. HCl : 6N
2. HCl : 0.05M
3. Buffer A: Dissolve tri sodium citrate (58.8g) in 2 L of double distilled water, add 210 ml ethanol of 99.5%, adjust the pH to 3.2 by adding 60% perchloric acid and make up to 3 L using double distilled water.
4. Buffer B: Dissolve tri sodium citrate, 58.8 g and boric acid, 12.4 g in double distilled water, adjust the pH to 10 by adding 4N NaOH, and make up the volume to 1L using double distilled water.
5. O-Phthaladehyde (OPA) Buffer: Dissolve 122.1 g of Na_2CO_3, 40.7 g of H_3BO_3 and 56.4 g of K_2SO_4 in double distilled water and make up the volume to 3L.
6. O-Phthalaldehyde solution (OPA): Dissolve 400 mg OPA, 7 ml ethanol, 1 ml of 2-Mercaptoethanol and 2ml of 30% w/v Brij-35 in 500ml OPA buffer.
7. Sodium hypochlorite solution: 4% w/v Sodium hypochlorite in OPA buffer. ie., 0.3ml Sodium hypochlorite in100ml OPA buffer.

Total amino acids

Principle

The amount of each amino acid present within a given protein does not vary from molecule and can provide useful information about the nature of the protein molecule. The sample was hydrolysed with 6N HCl at 1100 C (24h) so that the released amino acids can be assayed.

Sample preparation

100 mg gelatin sample was taken in a heat stable test tube; added 10ml 6N HCl and heat sealed the tube after filling with pure nitrogen gas. The

hydrolysis was carried out at 110^0C for 24 hrs. After the hydrolysis, the contents were quantitatively transferred into a round bottom flask through Whatman filter paper. No 42 and washed the filter paper 2-3 times with distilled water. The contents were flash evaporated 2-3 times to remove traces of HCl. The residue was dissolved and made up to 10 ml with 0.05 M HCl.

HPLC Analysis

The sample was filtered through a membrane filter of 0.45 µm and inject 20 µl of this to an amino acid analyzer (HPLC- LC 10 AS) equipped with cation exchange column packed with a strongly acidic cation exchange resin i.e., styrene di vinyl benzene co polymer with sulphinic group. The column used was Na type i.e., ISC- 07/S 1504 Na having a length of 19 cm and diameter 5 mm.

The instrument was equipped with Shimadzu FL 6A fluorescence detector and Shimadzu CR 6A Chrompac recorder. The mobile phase of the system consists of two buffers, Buffer A and buffer B. A gradient system can be followed for the effective separation of amino acids. The oven temperature can be maintained at 60^0 C. The total run was programmed for 62 min. The amino acid analysis was carried out with non-switching flow method and fluorescence detection after post–column derivatization with o-phthalaldehyde.In the case of proline and hydroxyl proline, imino group is converted to amino group with hypochlorite. Amino acid standard (Sigma chemical Co., St. Lousis, USA) was also run to calculate the concentration of amino acids in the sample.

Quantification of amino acids

The standard and the sample were analyzed under identical conditions. The elution time of the amino acids of the sample was compared and identified with those of the standard. Quantification of amino acid was done by comparing the respective peak areas in the chromatogram of the sample and the standard. The amino acid content was calculated as follows,

$$\text{mg amino acid/gm tissue} = \frac{\mu\text{mol}*\text{mol.wt}*\text{volume made up}*1000*100}{1000*1000*20*\text{wt. of sample}}$$

The amount of each amino acid is expressed as mg amino acid/ gm tissue and mg amino acid/ ml serum.

3.2.6.20 Fourier Transform Infrared Spectroscopy (FTIR)

Spectral analysis of the fish skin gelatins were carried out as per the method described by Muyonga et.al., (2004b) using a Nicolet Avatar 360 ESP Infrared Spectrophotometer at a scanning range from 400 to 4000 cm $^{-1}$ at data acquisition rate of 4cm cm $^{-1}$ per point. FTIR spectra were obtained from discs containing 2 mg sample in approximately 100 mg potassium bromide (KBr). All spectra were obtained Background was subtracted using the Omnic software. Triplicate samples of gelatins were scanned for 32 times and the averaged spectrum was used for the analysis. The self deconvolution provided information on the number and location of components.

3.2.6.21 Methods for the Testing of Physical Properties of Gelatin Films.

Conditioning of the films

For characterization of functional properties the prepared films were conditioned at 22 ^{0} C and 58% of relative humidity in desiccators for 4 days.

3.2.6.21.1. Determination of Colour

Colour analysis of gelatin films was performed with a Hunter lab Miniscan ® XE plus spectrocolorimeter (Hunter Associates Laboratory, Inc. Reston, Virginia, USA). Measurements were recorded using the L* a* b* colour scale (CIE,1986). Six repetitions of the different colour parameters were recorded.

3.2.6.21.2. Determination of Thickness (IS: 1060-part I- 1966)

Thickness or Caliper is the perpendicular distance between the two principal surfaces of the gelatin film. Caliper of kraft liner for a particular grammage should be uniform across the sheet.

Apparatus : Micrometer Screw Gauge

Report : Corrected values of average, minimum and maximum obtained on each test specimen. The thickness of film is measured in mm or mils of points. (1 mil = 1 point = 1/1000 inch = 0.025mm).

3.2.6.21.3. Determination of Tensile Strength and Elongation at Break: (IS:2508-1984)

Tensile strength has been defined as the force parallel to the plane of the specimen required to produce failure in a specimen of specified width and length under specified condition of loading.

Apparatus: Tensile Strength Machine

The machine used should be able to maintain a constant rate of traverse of one grip. The load scale should be accurate to within 1% or 0.1 N whichever is less. The load range should be such that the breaking load of the test pieces should fall between 15% and 85% of the full scale reading.

Preparation of samples

Samples were cut in lengthwise and crosswise direction, five numbers each with a minimum length 50 mm longer than the gauge length. The thickness was measured using a micrometer.

Gauge length of the Specimens: 50±1mm length x 15mm width

Traverse speed of machine: 500 mm/min.

The conditioned specimen was clamped between the grips of the machine. Machine was then switched on at the pre adjusted speed. The load and elongation at break were recorded.

Calculation

The tensile strength at break calculated in Kg/cm^2 from the original area of cross section. i.e., kgf/cross section area in cm^2. The mean of five results is expressed for the lengthwise and crosswise samples (MD and CD).

Cross Section area = width X thickness in cm.

Elongation at break is expressed as percentage of the original length between the reference lines. The mean value of the five results is expressed from MD & CD samples.

$$\% \text{ ELB} = \frac{L_2 - L_1 \times 100}{L_1}$$

Where L_1 is the original length

and L_2 is the length at the time of break

Breaking Length

$$\text{Breaking length (m)} = \frac{\text{Tensile strength in kg X Length of the strip in meters}}{\text{Wt. of strip in kg}}$$

3.2.6.21.4. Determination of Water Vapour Transmission Rate (ASTM 1989: E96-80)

This is an important property of the film under 3 mm thickness, to be considered in the selection of barrier materials for hygroscopic foods. It is measured as the quantity of water vapour in gms that will transmit from one side to the other of the film of an area of one sq. meter in 24 h. when the relative humidity difference between the two sides is maintained at 90±2% at 37°C.

Apparatus: Test Dishes

Shallow aluminium dishes of as large a diameter as a can was used. A wax seal between the test piece and the dish was given so as to prevent the transmission of water vapour at or through the edges of the sheet.

Method

WVTR was determined by sealing the open end of the dish containing the desiccant (fused Calcium chloride) by the test specimen and exposing the dish to the desired RH and temperature conditions. For standard test this condition is 37°C and 92% RH, when the desiccant used exerts 2% RH. Increase in weight of the desiccant after a known period of time gives the amount of water vapour transmitted by the specimen.

$$WVTR = \frac{Qx24x90}{A\ t\ (H_1-H_2)}\ g/m^2/24\ h.\ at\ 90\pm2\%\ RH\ \&\ 37°C.$$

Where;

Q - Quantity of water vapour pass through the test material of area **A** m^2 for **t** hours when the relative humidity on either side maintained at H_1 and H_2.

3.2.6.21.5 Determination of Gas Transmission Rate: (ASTM 1987: D1434)

The permeability of films by gases is described as the volumetric rate of transmission of the gas, under known pressure differential, through a known area of film and is usually expressed as the transmission rate in ml per square meter per 24 hrs per atmosphere ($ml/m^2/24$ hrs. atmos). The permeability of plastic materials to different gases is of considerable significance in many

applications. It can often be desirable to achieve a certain degree of permeability to certain gases, rather than to produce an entirely impermeable pack.

The phenomenon of gas permeability is dependent on the physical nature of the film, its density, degree of crystallinity and thickness and on the other the size and mobility of the gas molecules. The degree of polarity of both plastic materials and gas molecules as well as their tendency to be either hydrophobic or hydrophilic do influence the permeability of films with respect to particular gases.

Apparatus

Gas Permeability Apparatus (Davenport-designed in general accordance with B.S.2782, method 514A, Procedure 2 and ASTM D 1434)

Procedure

Unscrew the bolts holding down the upper half of the permeability cell and remove it. As supplied, the apparatus will have the 'X' volume controlling insert correctly fitted in the lower half of the cell. A dried circular filter paper (Whatmann No.1) is placed on the top of the insert and the sample of film spread over the filter paper. The upper part of the film permeability cell is then replaced. The bolts are then reinserted and tightened up with the box spanner.

The test gas is now turned on and the cell 'flushed out ' with a brisk stream of gas for a few seconds, after the flow may be reduced to a slow rate, to ensure that no air can diffuse back in to the cell (1 bubble/second through liquid paraffin). The lower part of the cell is then evacuated (using vacuum pump capable of giving a vacuum at least as low as 0.2 mm Hg. A vacuum gauge also be connected between the apparatus and the vacuum pump-Tipping Mc Leod gauge) as rapid as possible and as soon as the gauge indicates that the pressure is 0.2 mm Hg or lower. The apparatus is tilted to the left until the mercury runs out of the reservoir into the manometer, partially filling it. Return the apparatus to the normal position and immediately set the movable scale to a convenient starting point, start a stopwatch and begin to take readings, at suitable time intervals.

Calculation

GTR = $\frac{273xpVx24x10^4}{A \times T \times P}$ where,

GTR = Gas transmission rate in ml/m^2/24 hrs at 1 atmosphere pressure difference.

p = Rate of pressure change in capillary in cm Hg per hour.

V = Total volume in ml of the space between the lower surface of the film and the top of the mercury column in the capillary.

This total volume expressed as,

(a) The volume of cell cavity (i.e. 5,10,15 or 20)

(b) The volume of capillary tube above the mercury level half way through test; as the area of cross section of the capillary is 0.018 cm^2, this volume will be 0.018 X, when X is the length of the capillary above the mercury at the half way point in cm.

(c) The 'free space' volume of filter paper - can be taken as 0.24 ml.

A = Area of the specimen - 23.77cm^2

T = Temp. in °K (273+°C)

P = Pressure difference =1 atmosphere (76cm Hg)

i.e., = $\frac{273 \times pV \times 24 \times 10^4}{23.77 \times 76 (273+°C)}$

3.2.7. Microbiological Analyses

3.2.7.1 Total Aerobic Count at 30°C (AFNOR, 1982: NFV 59- 101)

Reagents and culture media

Diluent : Dissolve in 1000 ml of distilled water Sodium monohydrogen phosphate (Na_2HPO_4, 12 H_20) 9.0 g and Potassium dihydrogen phosphate (KH_2PO_4) 1.5 g. Adjust the pH so that the final value after sterilization will be 7.0 ± 0.1 at 25°C, dispense 180 ml in flasks, sterilize at 121°C for 20 min, store at 4°C.

Plate count agar: Dissolve in 1000 ml of distilled water: Tryptone 5.0 g ,Yeast extract 2.5 g ,Dextrose 1.0 g ,Agar 15.0 g. Adjust the pH so that the final value after sterilization will be 7.0 ± 0.2, dispense 15 ml in tubes, sterilise at 121°C for 20 min, store for one month maximum at 4°C. Before use, regenerate for 20 min in boiling water bath, then cool to 45°C.

Procedure

Preparation of 1/10 test solution (S1): Under aseptic conditions, weigh out 20 g of gelatin, transfer to 180 ml of diluent, and shake to disperse. Leave the gelatin to absorb the diluent for 1 hour at room temperature, then place the flask in the 45°C water bath and shake gently to assure dissolution (maximum 1 hour) and obtain the test solution (S1).

Inoculation: Transfer 1 ml of (S1) in each of two sterile Petri dishes and add the content of a media tube. Homogenise and leave to cool on a flat surface and incubated at 30°C for 72 ± 3 h.

Result: After incubation, count the colonies in each plate. The arithmetic average of the counts were carried out and multiplied by 10 (inverse of the dilution factor of (S1) solution).

3.2.7.2 Coliforms (30°C), (AFNOR, 1982: NFV 59- 102).

Reagents and culture media

Diluent : Dissolve in 1000 ml of distilled water Sodium monohydrogen phosphate (9g) & Potassium dihydrogen phosphate (1.5 g). Adjust the pH to 7.0 ± 0.1 at 25°C, dispense 180 ml in flasks, sterilize at 121°C for 20 min, store at 4°C. Ingredients of Selective liquid culture mediumis given in Table 3.9.

Table 3.9 Ingredients of Selective liquid culture medium

Lactose: 10.0 g	Thiamine:0.001 g
Sodium glutamate:6.35 g	Nicotinic acid : 0.001 g
Sodium formate:0.25 g	Pantothenic acid : 0.001 g
L-cystine: 0.02 g	Magnesium sulphate : 0.10 g
L (-) aspartic acid: 0.024 g	Ammonium ferric citrate: 0.01 g
L (+) arginine : 0.02 g	Calcium chloride : 0.01 g
Potassium monohydrogen phosphate : 0.90 g	Ammonium chloride : 2.5 g
Bromocresol purple : 0.01 g	Distilled water: 1000 ml

Adjust the pH to 6.7 ± 0.1 at 25°C. Dispense 50 ml in flasks containing Durham tube. Sterilise at 116°C for 10 min, store for one month maximum at 4°C.

Procedure

Under aseptic conditions, weigh out 20 g of gelatin, transfer it in 180 ml of diluent, and shake to disperse. Leave the gelatin to absorb the diluent for 1

hour at room temperature, then place the flask in the 45°C water bath and shake gently to assure dissolution (maximum 1 hour) and obtain the test solution (S1).

Inoculation

Transfer 10 ml of (S1) in a flask containing 50 ml of the liquid culture medium. Mix the inoculum carefully into the culture medium, avoiding introduction of air into the Durham tube and incubated at 30°C and for 48 ± 2 hours.

Result

After the incubation period, the flasks were examined to detect the presence/absence of gas in the Durham tube. The presence of gas is always accompanied by an abundant culture of micro-organisms forming cloudiness and/or yellowing of the medium, which indicates the presence of at least one "coliform" in the quantity of gelatin inoculated and the result is given as: Presence of 30°C developing coliforms in 1 g of gelatin.

3.2.7.3 Coliforms (44.5°C), (AFNOR, 1982: NFV 59- 103).

Reagents and culture media

Diluent : Dissolve in 1000 ml of distilled water Sodium monohydrogen phosphate (9 g)and Potassium dihydrogen phosphate (1.5 g). Adjust the pH to 7.0 ± 0.1 at 25°C, dispense 180 ml in flasks, sterilize at 121°C for 20 min, store at 4°C. Ingredients for Selective liquid culture medium (double strength): lactose - sodium glutamate-ammonium chloride (LGA) is given in Table 3.10.

Table 3.10 Ingredients for Selective liquid culture medium-LGA

Lactose : 20.0 g	Sodium glutamate : 12.70 g
Sodium formate : 0.50 g	L-cystine : 0.04 g
L (-) aspartic acid : 0.048 g	L (+) arginine : 0.04 g
Thiamine : 0.002 g	Nicotinic acid : 0.002 g
Pantothenic acid : 0.002 g	Magnesium sulphate : 0.20 g
Ammonium ferric citrate : 0.02 g	Calcium chloride : 0.02 g
Potassium monohydrogen phosphate : 1.80 g	Bromocresol purple : 0.02 g
Ammonium chloride : 5.0 g	Distilled water : 1000 ml

Adjust the pH to 6.7 ± 0.1 at 25°C, dispense 100 ml in flasks containing Durham tube, sterilise at 116°C for 10 min, store at 4°C.

Procedure

Preparation of the 1/10 test solution (S1): Under aseptic conditions, weigh out 20 g of gelatin, transfer it in 180 ml of diluent, and shake to disperse. Leave the gelatin to absorb the diluent for 1 hour at room temperature, then place the flask in the 45°C water bath and shake gently to assure dissolution (maximum 1 hour) and obtain the test solution (S1).

Inoculation ; Transfer 100 ml of (S1) in a flask containing 100 ml of the double strength liquid culture medium.Mix the inoculum carefully into the culture medium, avoiding introduction of air into the Durham tube and incubated at 44.5°C for 48 ± 2 hours.

Result

After the incubation period, examine the flask to detect the presence/absence of gas in the Durham tube. The presence of gas is always accompanied by an abundant culture of micro-organisms forming cloudiness and/or yellowing of the medium, which indicates the presence of at least one "coliform" in the quantity of gelatin inoculated and the result is given as: Presence of 44.5°C developing coliforms in 10 g of gelatin.

3.2.7.4 Sulphite-Reducing Anaerobic Spores (37°C), (AFNOR, 1982: NFV 59- 106).

Reagents and culture media

Diluent: Dissolve in 1000 ml of distilled water Sodium monohydrogen phosphate (9 g) and Potassium dihydrogen phosphate (1.5g) .Adjust the pH so that the final value after sterilisation will be 7.0 ± 0.1 at 25°C, dispense 180 ml in flasks, sterilize at 121°C for 20 min, store at 4°C. Ingredients for Beef extract-yeast extract-sulphite-iron agar is given in Table 3.11.

Table 3.11 Ingredients for Beef extract-yeast extract-sulphite-iron agar

Tryptone : 10.0 g	Sodium chloride : 5.0 g
Beef extract : 3.0 g	Cysteine hydrochloride : 0.3 g
Yeast extract : 6.0 g	Soluble starch : 5.0 g
Glucose :2.0 g	Sodium metabisulphite : 1.0 g
Ammonium ferric citrate : 1.0 g	Agar : 12.0 g
Distilled water : 1000 ml	

Adjust the pH to 7.6 ± 0.2 at 25°C, dispense 20 ml in tubes, sterilise at 115°C for 30 min, store for 1 week only at 4°C. Before use, regenerate for 20 min in boiling water bath, then cool to 60°C.

Procedure

Preparation of the 1/10 test solution (S1): Under aseptic conditions, weigh out 20 g of gelatin, transfer it in 180 ml of diluent, and shake to disperse. Leave the gelatin to absorb the diluent for 1 hour at room temperature, then place the flask in the 45°C water bath and shake gently to assure dissolution (maximum 1 hour) and obtain the test solution (S1).

Treatment of the test solution: Pour 25 ml of (S1) in a tube and place in water bath to pasteurise at 80°C for 10 min and cool rapidly in 45°C water bath.

Inoculation

Transfer 5 ml of the pasteurised solution (6.2) into each of 2 tubes of culture medium at 60°C prepared and regenerated as previously described. Mix carefully the inoculum with the medium using circular movements without letting any air into the culture medium and incubated the tubes for 72 ± 3 hours at 37°C.

Result

Verify the absence of gas production and proteolytic action in the culture medium, then, count the black halo surrounded colonies which are present in the 2 tubes, and report.

3.2.7.5 Clostridium perfringens Spores (AFNOR, 1982: NFV 59- 107).

Reagents and culture media

Diluent : Dissolve in 1000 ml of distilled water Sodium monohydrogen phosphate (9. g) and Potassium dihydrogen phosphate (1.5 g) .Adjust the pH to 7.0 ± 0.1 at 25°C, dispense 180 ml in flasks, sterilize at 121°C for 20 min, store for one month maximum at 4°C. The ingredients of Enrichment broth (Thioglycolate resazurine double strength medium) is given in Table 3.12.

Table 3.12 Ingredients for Enrichment broth

Tryptone : 30.0 g	Sodium thioglycolate :1.0 g
Yeast extract : 10.0 g	L-cystine : 1.0 g
Dextrose : 11.0 g	Resazurine : 0.002 g
Sodium chloride : 5.0 g	Agar : 1.0 g
Distilled water : 1000 ml	

Adjust the pH to 7.1± 0.2 at 25°C. Dispense 10 ml in tubes, sterilise at 121°C for 15 min, store at 4°C in the dark. Before use regenerate for 5 min in a boiling water bath (10 min if pink colour on more than 1/3 high), then cool to 45°C. Ingredients for lactose-sulphite specific medium (LS medium) is given in Table 3.13.

Table 3.13 ingredients for LS medium

Trypsic casein peptone : 5.0 g	Yeast extract : 2.5 g
Lactose : 10.0 g	Sodium chloride : 2.5 g
Cystein hydrochloride : 0.3 g	Distilled water : 1000 ml

Adjust the pH to 7.1 ± 0.1 at 25°C, dispense 8 ml in tubes containing Durham tubes, sterilise at 121°C for 15 min, store at 4°C. Before use, regenerate 5 min in a boiling water bath, then cool to 45°C; prepare separately the two following aqueous solutions:

Sodium bisulphite 12.0 g/l

Ferric ammonium citrate 10.0 g/l

The complete LS medium, prepared immediately before use, contains: Basic medium regenerated 8.0 ml, sulphite solution 0.5 ml and ferric solution 0.5 ml.

Procedure

Preparation of the 1/10 test solution (S1): Under aseptic conditions, weigh out 20 g of gelatin, transfer it in 180 ml of diluent, and shake to disperse. Leave the gelatin to absorb the diluent for 1 hour at room temperature, then place the flask in the 45°C water bath and shake gently to assure dissolution (maximum 1 hour) and obtain the test solution (S1).

Enrichment inoculation : Transfer 10 ml of (S1) in a tube containing 10 ml of the double strength enrichment broth described previously. Mix slowly and place in water bath to pasteurize at 80°C for 10 min, then cool quickly to 45°C.
Enrichment incubation: Cover the broth with a melted paraffin layer, and after solidifying, place the tube in the 46°C regulated water bath. Incubate for 24 hour (and eventually 48 hours).
LS broth inoculation: Subculture the enrichment tube that exhibits production of gas under the paraffin after 24 hours (or eventually 48 hours). Perforate the paraffin and transfer 1 ml into a tube of complete LS medium. Incubate in the 46°C water bath for 24 hours.

Result

After the specified LS incubation period, the simultaneous presence of gas in the Durham tube and of a black iron sulphide precipitate indicate the presence of at least one Clostridium perfringens spore in the amount of seeded gelatin. The result is given as: Presence of Clostridium perfringens spores in 1g of gelatin.

3.2.7.6.Staphylococcus aureus (AFNOR, 1982: NFV 59- 105).

Reagents and culture media

Diluent : Dissolve in 1000 ml of distilled water Sodium monohydrogen phosphate (9 g) and Potassium dihydrogen phosphate (1.5 g) .Adjust the pH so that the final value after sterilisation will be 7.0 ± 0.1 at 25°C, dispense 180 ml in flasks, sterilize at 121°C for 20 min, store at 4°C. the ingredients for Selective liquid enrichment broth - highly salted lactose broth is given in Table 3.14.

Table 3.14 Ingredients for Selective liquid enrichment broth

Beef extract : 3.0 g	Lactose : 7.5 g
Tryptone : 10.0 g	Sodium chloride : 75.0 g
Agar : 0.5 g	Distilled water : 1000 ml

Adjust the pH to 7.4 ± 0.1 at 25°C, dispense 190 ml in flasks, sterilise at 121°C for 20 min, store for 1 month maximum at 4°C.

Isolation medium - Baird-Parker medium

1) Base medium: In 1000 ml of distilled water dissolve, Tryptone (10 g), Yeast extract (1 g), Beef extract (5 g), Glycine (12 g), Lithium chloride (5 g), Sulphamezathine solution (27.5 ml), Agar (15 g) and adjust the pH to 7.2 ± 0.2 at 25°C, dispense 90 ml in flasks, sterilise at 121°C for 2 min, store at 4°C.

2) Sulphamezathine solution: In 100 ml of distilled water dissolve Sulphamezathine (0.2 g) & 10.0 ml (0.1 M) Sodium hydroxide solution. Store for 1 month maximum at 4°C.

3) Potassium tellurite solution:In 100 ml of distilled water dissolve 1.0 g Potassium tellurite and Sterilise by filtration, store for 1 month maximum at 25°C.

4) Sodium pyruvate solution: in 100 ml of distilled water dissolve 20 g Sodium pyruvate and sterilise by filtration, store for 1 month maximum at 25°C

5) Egg yolk emulsion: commercial preparation - 20 %,

6) Complete Baird-Parker medium

Base medium (1) 90 ml

Potassium tellurite solution (3) 1 ml

Sodium pyruvate solution (4) 5 ml

Egg yolk emulsion (5) 5 ml

Melt the base medium, then cool to about 50°C in a water bath, add successively the solutions, with effective mixing after each addition, and cool at 45°C in a water bath, pour 15-20 ml of the complete medium into sterile Petri dishes and allow to solidify. Dry the surface of the medium, cover removed an dishes turned downwards, in a 50°C regulated oven for 30 min, store the dishes for 24 hours maximum at 4°C.

Coagulase test

1) Brain-heart broth: In 1000 ml distilled water dissolve, Peptone (10 g), Calf brain extract (12.5 g), Beef heart extract (5 g) Dextrose (2 g), Sodium chloride (5 g), Sodium monohydrogen phosphate (2.5 g). Adjust the pH to 7.4 ± 0.2 at 25°C, dispense 10 ml in tubes, sterilize at 121°C for 20 min, store for 1 month maximum at 4°C.

2) Rabbit plasma: rehydrated commercially available rabbit plasma.

Procedure

Preparation of the 1/10 test solution (S1)

Under aseptic conditions, weigh out 20 g of gelatin, transfer it in 180 ml of diluent, and shake to disperse. Leave the gelatin to absorb the diluent for 1 hour at room temperature, then place the flask in the 45°C water bath and shake gently to assure dissolution (maximum 1 hour) and obtain the test solution (S1). Inoculation

Transfer 10 ml of (S1) in a flask containing 190 ml of the selective liquid enrichment broth. Mix the inoculum carefully into the culture medium and incubated at 37°C and for 48 ± 2 hours.

Isolation

After incubation transfer a loopful of the flask (6.3.) and streak on the surface of a dish of isolation medium. Return the dish and incubate at 37°C for 24-48 hours. From plate examine for the presence of characteristic colonies black, shining and convex, 1-1.5 mm diameter after 24h and 1.5-2.5 mm diameter after 48 hours incubation, surrounded by a clear partially opaque zone.

Select at random five suspect-colonies, pick each with a sterile inoculating needle and inoculate tubes of brain-heart broth. After incubation for 24 hours at 37°C add 0.1 ml of the culture in each tube to 0.3 ml of rabbit plasma. Incubate for 4-6 hours at 37°C and examine the tubes for plasma coagulation. Coagulase reaction is positive if a large quantity of coagulum is formed (about 3/4 of the volume). Check negative reaction with 0.1 ml of sterile brain-heart broth.

Result

The result is given as: Presence/absence of *S. aureus* in 1 g of gelatin

3.2.7.7 Salmonella (AFNOR, 1982: NFV 59- 104).

Reagents and culture media

Pre-enrichment medium - Buffered peptone water Dissolve in 1000 ml of distilled water: Peptone 10.0 g Sodium chloride 5.0 g Sodium monohydrogen phosphate 9.0 g Potassium dihydrogen phosphate 1.5 g Adjust the pH so that the final value after sterilisation will be 7.0 ± 0.1 at 25°C, dispense 225 ml in flasks, sterilise at 121°C for 20 min, store at 4°C.

Enrichment media-

(I) Rapapport-Vassiliadis broth - green malachite-magnesium chloride broth

Solution A: In 1000 ml of distilled water dissolve: Tryptone (5 g) Sodium chloride (8 g) Potassium dihydrogen phosphate (1.6 g)

Solution B: 400.0 g Magnesium chloride dissolved in 1000 ml of distilled water.

Solution C: 0.4 g Green malachite oxalate dissolved in 100 ml of distilled water. Complete medium (RV 10 formula): Take Solution A 1000 ml, Solution B 100 ml and Solution C 10 ml and adjust the pH to 5.2 ± 0.1 at 25°C, dispense 10 ml in tubes, sterilise at 115°C for 15 min, store at 4°C.

(II) Selenite-cystine broth

Base medium: In 1000 ml of distilled water dissolve Tryptone (5g), Lactose (4 g), Sodium monohydrogen phosphate (10 g), Sodium monohydrogen selenite (4 g).

Dissolve the first three ingredients in water and boil for 5 min, cool and add the selenite salt.

Cystine solution: In 100 ml sterile distilled water in a sterile flask dissolve L-cystine 0.1 g 1 mol/l sodium hydroxide solution 15.0 ml. Do not sterilise.

Complete medium: Under sterile condition, add cystine solution (10 ml), to the cooled base medium (1000 ml) and adjust the pH to 7.0 ± 0.2 at 25°C, dispense 20 ml in sterile tubes.

Isolation media :

Phenol red-brilliant green-agar (PRBG agar): In 1000 ml of distilled water dissolve, Tryptone (10 g) Beef extract (5 g) Yeast extract (3 g) Lactose (10 g) Dextrose (10 g) Sodium hydrogen phosphate (1.0 g) Sodium dihydrogen phosphate (0.6 g) Phenol red (0.09 g) Brilliant green (0.005 g) Agar 14.0g. Adjust the pH to 6.9 ± 0.2 at 25°C, boil gently to dissolve the components, do not sterilise, cool to 45°C. Dispense 15 ml in Petri dishes, cool to room temperature and dry the plates in the oven before use, store 24 hours maximum at 4°C.

Procedure

Pre-enrichment

Weigh 25 g of gelatin and transfer into 225 ml of pre-enrichment medium, and shake to disperse. Allow to stand for 1 hour at room temperature. Then place the flask in the 45°C water bath and shake gently to assure the dissolution is

complete and incubate at 37°C for at least 16 hours but not more than 20 hours.

Enrichment

Seed 10 ml of Rapapport-Vassiliadis broth with 0.1 ml of the culture (6.1), and incubate at 42°C for 24 to 48 hours. Seed 20 ml of selenite broth with 2 ml of the culture and incubate at 37°C for 24 to 48 hours. 6.3. Isolation After 24 h, and after 48 h, transfer a loopful of the two flasks and streak on the surface of the PRBG agar and of the optional agar. Return the plates and incubate at 37°C for 24 to 48 hours.

Confirmation

Characteristic colony was submitted to confirmatory analysis, by means of purification sub-culture, biochemical identification, and serological confirmation.

Result

The result is given as Presence/absence of salmonella in 25 g of gelatin.

3.3. Statistical Analysis

Results are expressed as mean or mean log ± SD for biochemical & microbiological parameters. Statistical analysis between the means using ANOVA and Dunken's' multiple test were carried out to test the significance of variance. Statistical package used in the study is SPSS, 10.

4.0 Results and Discussion....

4.1. Optimization of Process Parameters for the Extraction of Gelatin from Fish Skin

4.1.1 Screening Factors for Gelatin Extraction and the Effect of Factors on the Responses during Screening Experiments

To study a large number of factors efficiently, reduced factorial designs were employed. With this design the important factors can be efficiently evaluated using a small fraction of the experiments required for a full factorial design (Araujo and Brereton 1996). The six important factors (independent variables) that affect the extraction of gelatin from fish skin and their ranges between model levels described as −1 and 1 were selected for the screening experiments. The following were selected as independent variables: Alkali concentration (mol/L), X_1, (0.1 to 0.2); Acid concentration (mol/L), X_2, (0.1 to 0.2); Pretreatment time (min),X_3, (40 to 60); skin/water ratio (w/w), X_4, (1:4 to 1:6); Extraction time (hours), X_5, (6H to 10H); Extraction temperature (◦C), X_6, (40 to 60); The importance of these factors were evaluated based on the responses on two dependent variables selected. These selected dependent variables were gel strength (Bloom) and yield (%) which can be rated as the most commercially important physical properties of the extracted gelatin. A total of eight groups of extraction experiments were conducted using different combinations of these six factors and the responses are shown in Tables 4.1.1(a), 4.1.1(b) and 4.1.1(c). The range of responses of dependent variables obtained for the independent variables based on screening are summarized and re-arranged into Tables 4.1.1(d), 4.1.1(e) and 4.1.1(f) respectively.

Table 4.1.1(a) Experimental results for Rohu gelatin using Fractional Factorial screening design ($2^{6-3/iii}$) in coded units*

	Independent variables						Dependent variables	
No.	X1	X2	X3	X4	X5	X6	Gel strength(B)	Yield (%)
1	-1	-1	-1	+1	+1	+1	123.29 (1.83)	13.60(0.39)
2	+1	-1	-1	-1	-1	+1	219.04(2.79)	14.61±0.38
3	-1	+1	-1	-1	+1	-1	207.05 (3.56)	14.18(1.68)
4	+1	+1	-1	+1	-1	-1	202.70 (2.39)	14.05(0.84
5	-1	-1	+1	+1	-1	-1	200.39(2.72)	10.75(1.19)
6	+1	-1	+1	-1	+1	-1	189.71(2.17)	7.32(1.06)
7	-1	+1	+1	-1	-1	+1	172.13(1.53	13.74±0.60)
8	+1	+1	+1	+1	+1	+1	119.83(1.15)	15.34(1.37)

*Values in brackets are standard deviations of triplicate samples. Independent variables and their ranges X_1: Alkali concentration, 0.1 to 0.2 mol/L; X_2: Acid concentration, 0.1 to 0.2 mol/L :X_3 pretreatment time, 40 to 60 min; X_4: Skin/water ratio, 1/4 to 1/6 w/v. X_5: Extraction time, 6H to 10H X_6: Extraction temperature, 40 to 60 °C

Table 4.1.1(b) Experimental results for Common carp gelatin using Fractional factorial screening design ($2^{6-3/iii}$) in coded units*

	Independent variables						Dependent variables	
No.	X_1	X_2	X_3	X_4	X_5	X_6	Gel strength(B)	Yield (%)
1	-1	-1	-1	+1	+1	+1	100.43(2.54)	11.32(3.09)
2	+1	-1	-1	-1	-1	+1	203.51(2.39)	13.03±0.99
3	-1	+1	-1	-1	+1	-1	207.26(4.67)	9.70(2.59)
4	+1	+1	-1	+1	-1	-1	200.97(2.72)	14.71(0.73)
5	-1	-1	+1	+1	-1	-1	202.51(4.32)	12.49(0.47)
6	+1	-1	+1	-1	+1	-1	189.05(2.62)	12.36(0.49)
7	-1	+1	+1	-1	-1	+1	160.68(1.88)	12.75(0.66)
8	+1	+1	+1	+1	+1	+1	125.83(4.06)	12.95(0.45)

*Values in brackets are standard deviations of triplicate samples. Independent variables and their ranges X_1: Alkali concentration, 0.1 to 0.2 mol/L; X_2: Acid concentration, 0.1 to 0.2 mol/L :X_3 pretreatment time, 40 to 60 min; X_4: Skin/water ratio, 1/4 to 1/6 w/v. X_5: Extraction time, 6H to 10H X_6: Extraction temperature, 40 to 60 °C

Table 4.1.1 (c) Experimental results for Grass carp gelatin using Fractional factorial screening design ($2^{6-3/iii}$) in coded units*

	Independent variables						Dependent variables	
No.	X_1	X_2	X_3	X_4	X_5	X_6	Gel strength(B)	Yield (%)
1	-1	-1	-1	+1	+1	+1	80.74 (5.02)	7.51(0.30)
2	+1	-1	-1	-1	-1	+1	239.87 (1.68)	11.03(0.25)
3	-1	+1	-1	-1	+1	-1	110.10 (1.32)	8.43(0.47)
4	+1	+1	-1	+1	-1	-1	211.63 (2.62)	10.27(1.96)
5	-1	-1	+1	+1	-1	-1	234.83(3.96)	10.75(0.96)
6	+1	-1	+1	-1	+1	-1	256.46 (3.03)	12.42(0.72)
7	-1	+1	+1	-1	-1	+1	163.37(9.14)	10.39(0.38)
8	+1	+1	+1	+1	+1	+1	113.45 (1.56)	9.10(0.08)

*Values in brackets are standard deviations of triplicate samples. Independent variables and their ranges X_1: Alkali concentration, 0.1 to 0.2 mol/L; X_2: Acid concentration, 0.1 to 0.2 mol/L: X3 pretreatment time, 40 to 60 min; X_4: Skin/water ratio, 1/4 to 1/6 w/v. X_5: Extraction time, 6H to 10H X_6: Extraction temperature, 40 to 60 °C

Table 4.1.1(d). Screening Experiment - Range of Responses for independent factors (Rohu Gelatin)*

Results	Level	X_1	X_2	X_3	X_4	X_5	X_6
Gel Strength	-1	803.59	787.6	761.09	702.54	732.42	808.87
	+1	639.56	655.55	682.06	740.61	710.73	634.28
	Range	164.03*	132.05*	79.03*	38.07	21.69	174.59*
Yield	-1	49.85	46.27	56.43	53.15	52.26	46.3
	+1	53.73	57.31	47.15	50.43	51.32	57.28
	Range	3.88*	11.04*	9.28*	2.72	0.94	10.98*

* Indicates significant ($P < 0.05$) differences among the 2 levels.

From Table 4.1.1(d) it can be observed that for the extraction of gelatin from Rohu skin, the range of responses for gel strength can be ranked in the order $X_6 > X_1 > X_2 > X_3 > X_4 > X_5$ and for yield, it is $X_2 > X_6 > X_3 > X_1 > X_4 > X_5$ .This shows the significance of effect of various independent factors on the responses i.e., dependent variables. From the above range of responses, two independent factors viz., X_4 (skin/water ratio) and X_5 (extraction time) were found to have minimum effect on the extraction of gelatin from Rohu skin since these have minimum values of range for gel strength and yield among the six independent factors.

Table 4.1.1(e). Screening Experiment - Range of Responses for independent factors (Common carp Gelatin)*

Results	Level	X_1	X_2	X_3	X_4	X_5	X_6
Gel Strength	-1	667.21	756.83	796.13	708.51	695.51	796.05
	+1	719.37	629.75	590.45	678.07	691.07	590.53
	Range	52.16*	127.08*	205.68*	30.44	4.44	205.52*
Yield	-1	46.26	47.84	48.76	49.26	49.2	52.99
	+1	53.05	51.47	50.55	50.05	50.10	46.32
	Range	5.9*	3.63*	1.79*	0.79	0.9	6.67*

* Indicates significant ($P < 0.05$) differences among the 2 levels.

From Table 4.1.1(e) it can be observed that for the extraction of gelatin from Common carp skin, the range of responses for gel strength can be ranked in the order $X_3 > X_6 > X_2 > X_1 > X_4 > X_5$ and for yield, it is $X_6 > X_1 > X_2 > X_3 > X_5 > X_4$. This shows the significance of effect of various independent factors on the responses i.e., dependent variables. The ranking order of the range of response in this case varies slightly from that of Rohu gelatin; however, as in the case of Rohu gelatin, two independent factors viz., X_4 (skin/water ratio) and X_5 (extraction time) were found to have minimum effect on the extraction of gelatin since these have minimum values of range for gel strength and yield among the six independent factors.

Table 4.1.1 (f). Screening Experiment - Range of Responses for independent factors (Grass carp Gelatin)*

Results	Level	X_1	X_2	X_3	X_4	X_5	X_6
Gel Strength	-1	584.38	794.24	816.70	604.68	744.14	786.36
	+1	775.08	565.21	542.75	754.78	615.31	573.10
	Range	190.70*	229.03*	273.95*	150.10	128.83	213.26*
Yield	-1	37.09	42.28	37.25	41.72	41.88	42.45
	+1	42.82	37.64	42.67	45.40	38.04	37.47
	Range	5.73*	4.64*	5.42*	3.68	3.84	4.98*

*Indicates significant ($P < 0.05$) differences among the 2 levels.

From Table 4.1.1(f) it can be observed that for the extraction of gelatin from Grass carp skin, the range of responses for gel strength can be ranked in the order $X_3 > X_2 > X_6 > X_1 > X_4 > X_5$ and for yield, it is $X_1 > X_3 > X_6 > X_2 > X_5 > X_4$. The ranking order of the range of responses is different from that observed for Rohu and Common carp skin gelatin. Here also, two independent factors viz.,

X_4(skin/water ratio) and X_5(extraction time) were found to have minimum effect on the extraction of gelatin since these have minimum values of range for gel strength and yield among the six independent factors

From the above data for the range of responses, four independent factors were identified as critical variables among the six which were selected for screening that had a significant effect on the extraction of gelatin from the skin of Rohu, Common carp and Grass carp. These were Alkali pretreatment concentration (mol/L),: Acid pretreatment concentration (mol/L), ; Pretreatment time (min), and Extraction temperature (°C) hereinafter designated as coded units X_1, X_2, X_3 & X_4 respectively. The two factors viz., extraction time and skin/water ratio were found to have no significant influence on the gel strength and yield in the given set of experimental conditions. Hence in further experiments conducted for the optimization of extraction conditions and response surface model building they were set at median values of 8 hours and 1:5 (w/v) respectively.

The screening experiments can provide the information as to which steps are crucial to the efficiency of extraction. The degree of conversion of collagen into gelatin (yield) and gel strength is related to the severity of the pretreatments viz., alkali and acid pretreatment, pretreatment time and the extraction temperature. The observations were in good agreement with the report of Montero & Gomez-Guillen (2000), Yang *et. al.*,(2007).

4.1.2 Response Surface Model building and Optimization of Extraction Conditions

Once the important variables were determined by the screening, Response Surface Methodology was used for optimizing the process of extraction of gelatin. A 4 factor, 5 level Central Composite Rotatable Design was formulated (Section 3, Table 3.6). The independent variables and their levels used in the Design are given in Section 3, Table 3.5. For optimization experiments, five levels were assigned for each factor instead of the two level designs for screening experiments. A total of 31 runs which included seven centre point runs using the Central Composite Design were carried out to study the effect of the selected independent variables on the responses.

Experimental results of the 4 factor, 5 level central composite design are shown in Tables 4.1.2(a), 4.1.2(b) and 4.1.2(c). The quadriatic response surface analysis was based on multiple linear regression taking into account of all main, quadriatic and interaction effects. The predicted values are listed together with the experimental data. The analysis of variance for the response surface model is given in Tables 4.1.2 (d), 4.1.2.(e) and 4.1.2.(f). Since the experimental design had seven replicate run at the centre point, the residual sum of squares could be partitioned between pure error and lack of fit components. The *p* values for the lack-of-fit test were large which indicate that the quadriatic models are adequate.The *p* values for the significance of regression were very small and indicate that at least some of the parameters in the models are not zero. For all the responses, both linear and quadriatic terms contribute significantly to the models. Interaction does not contribute significantly for both responses. The values of R^2 suggest that the models can explain a high percentage of the variability in the observed data. Thus the analysis of variance shows the predicted models are statistically valid.

Table 4.1.2(a) Central Composite Design for Optimizing the Extraction Conditions of Rohu Skin Gelatin in coded units together with experimental data and their predicted value*

Stand-ard Order	X_1	X_2	X_3	X_4	Gel Strength (B)		Yield (%)	
					Expt	Pred	Expt	Pred
01	-1	-1	-1	-1	123.12	129.39	13.37	14.91
02	1	-1	-1	-1	215.98	212.93	14.83	15.92
03	-1	1	-1	-1	208.71	212.83	13.56	12.86
04	1	1	-1	-1	214.94	218.14	13.11	12.74
05	-1	-1	1	-1	201.07	206.43	11.27	12.06
06	1	-1	1	-1	192.02	197.34	7.79	7.28
07	-1	1	1	-1	170.83	163.80	13.47	12.55
08	1	1	1	-1	121.15	127.65	14.25	13.95

09	-1	-1	-1	1	121.55	117.18	14.05	14.91
10	1	-1	-1	1	219.69	212.93	14.83	15.92
11	-1	1	-1	1	209.47	212.62	16.08	15.49
12	1	1	-1	1	200.18	205.93	14.73	15.36
13	-1	-1	1	1	197.39	194.28	11.59	12.06
14	1	-1	1	1	187.72	182.39	8.06	9.53
15	-1	1	1	1	171.74	178.76	14.42	14.68
16	1	1	1	1	119.19	127.64	16.88	17.17
17	-2	0	0	0	125.2	117.18	13.37	12.82
18	2	0	0	0	221.44	225.14	14.18	13.59
19	0	-2	0	0	202	200.62	12.89	12.86
20	0	2	0	0	220.99	218.14	14.31	15.16
21	0	0	-2	0	202.7	194.28	9.39	9.43
22	0	0	2	0	189.38	199.95	6.1	5.37
23	0	0	0	-2	173.82	184.37	13.32	14.10
24	0	0	0	2	119.14	126.71	14.88	14.20
25	0	0	0	0	205.62	215.60	14.03	14.09
26	0	0	0	0	221.44	215.60	14.83	14.09
27	0	0	0	0	220.99	215.60	13.11	14.09
28	0	0	0	0	219.69	215.60	14.83	14.09
29	0	0	0	0	200.18	215.60	14.73	14.09
30	0	0	0	0	215.98	215.60	14.18	14.09
31	0	0	0	0	214.94	215.60	14.31	14.09

*X_1 = alkali pretreatment concentration (mol/L); X_2 = Acid pretreatment concentration (mol/L); X_3 = Pretreatment time (min); X_4 = Extraction temperature (°C) ; Expt. =Experimental yield ; Pred.= Predicted yield

Table 4.1.2(b) Central Composite Design for Optimizing the Extraction Conditions of Common carp Skin Gelatin in coded units together with experimental data and their predicted value*

Standa Orde	X1	X2	X3	X4	Gel Strength(B)		Yield (%)	
					Expt.	Pred.	Expt.	Pred.
01	-1	-1	-1	-1	103.12	105.34	9.09	9.21
02	1	-1	-1	-1	205.18	208.41	14.01	15.13
03	-1	1	-1	-1	211.11	208.49	7.51	7.59
04	1	1	-1	-1	203.84	205.88	14.75	14.71
05	-1	-1	1	-1	199.1	197.61	12.86	12.49
06	1	-1	1	-1	192.02	184.05	11.98	12.08
07	-1	1	1	-1	159.33	167.08	12.01	12.48
08	1	1	1	-1	121.15	118.72	13.46	13.06
09	-1	-1	-1	1	100.12	105.33	14.85	13.41
10	1	-1	-1	1	200.77	198.61	13.05	13.03
11	-1	1	-1	1	199.59	198.68	12.55	11.79
12	1	1	-1	1	198.44	196.07	15.42	14.71
13	-1	-1	1	1	201.07	207.41	12.66	12.49
14	1	-1	1	1	188.09	196.06	12.18	12.08
15	-1	1	1	1	162.82	167.08	12.95	12.48
16	1	1	1	1	128.31	130.73	12.67	13.06
17	-2	0	0	0	98.06	105.33	10.01	11.31
18	2	0	0	0	204.59	208.49	12.04	13.03

19	0	-2	0	0	200.07	198.68	9.03	9.69
20	0	2	0	0	200.64	205.87	13.96	14.71
21	0	0	-2	0	207.37	207.42	11.96	12.49
22	0	0	2	0	187.05	195.54	12.91	12.95
23	0	0	0	-2	159.88	151.38	13.29	13.38
24	0	0	0	2	128.03	136.52	12.71	12.76
25	0	0	0	0	156.72	160.21	12.11	12.08
26	0	0	0	0	150.90	160.21	11.89	12.08
27	0	0	0	0	167.34	160.21	12.67	12.08
28	0	0	0	0	153.01	160.21	12.01	12.08
29	0	0	0	0	162.67	160.21	11.52	12.08
30	0	0	0	0	158.88	160.21	11.04	12.08
31	0	0	0	0	160.11	160.21	11.91	12.08

*X_1 = alkali pretreatment concentration (mol/L); X_2 = Acid pretreatment concentration (mol/L); X3 = Pretreatment time (min); X_4 = Extraction temperature (°C) ; Expt. =Experimental yield ; Pred.= Predicted yield

Table 4.1.2(c) Central Composite Design for Optimizing the Extraction Conditions of Grass carp Skin Gelatin in coded units together with experimental data and their predicted value *

Standard Order	X1	X2	X3	X4	Gel Strength (B)		Yield (%)	
					Expt.	Pred.	Expt.	Pred.
01	-1	-1	-1	-1	88.12	82.41	7.51	6.88
02	1	-1	-1	-1	241.39	240.96	11.29	11.03
03	-1	1	-1	-1	111.22	103.77	8.22	8.43
04	1	1	-1	-1	240.44	233.80	11.71	10.27
05	-1	-1	1	-1	234.11	234.83	11.79	10.75
06	1	-1	1	-1	255.28	257.59	13.14	12.78
07	-1	1	1	-1	173.37	167.33	10.77	10.58
08	1	1	1	-1	115.15	109.12	9.09	9.14
09	-1	-1	-1	1	80.39	82.47	7.22	7.51
10	1	-1	-1	1	229.07	223.87	11.01	11.03
11	-1	1	-1	1	98.64	103.77	8.97	8.43
12	1	1	-1	1	209.26	207.94	11.06	10.27
13	-1	-1	1	1	231.28	234.83	10.58	10.75
14	1	-1	1	1	259.91	257.59	12.42	12.78
15	-1	1	1	1	161.3	167.33	10.4	10.58
16	1	1	1	1	103.1	109.12	9.19	9.14
17	-2	0	0	0	78.71	82.40	7.81	7.51
18	2	0	0	0	201.16	203.83	10.79	11.03
19	0	-2	0	0	101.45	103.77	8.11	8.43
20	0	2	0	0	134.18	134.62	8.04	9.14
21	0	0	-2	0	239.1	234.83	9.89	9.62
22	0	0	2	0	244.2	245.67	11.7	12.04
23	0	0	0	-2	155.44	157.10	10.01	11.42
24	0	0	0	2	192.09	198.22	9.03	10.20

25	0	0	0	0	191.09	188.06	9.28	9.44
26	0	0	0	0	180.22	188.06	10.54	9.44
27	0	0	0	0	195.31	188.06	9.01	9.44
28	0	0	0	0	191.11	188.06	9.27	9.44
29	0	0	0	0	178.02	188.06	10.11	9.44
30	0	0	0	0	185.50	188.06	10.29	9.44
31	0	0	0	0	186.21	188.06	9.89	9.44

*X_1 = alkali pretreatment concentration (mol/L); X_2 = Acid pretreatment concentration (mol/L); X_3 = Pretreatment time (min); X_4 = Extraction temperature (°C) ; Expt. =Experimental yield ; Pred.= Predicted yield

Table 4.1.2 (d) Rohu Gelatin- Analysis of Variance (ANOVA) for Response Surface Quadratic Model*

Source of variation	Degrees of Freedom	Gel Strength		Yield	
		Sum of squares	*P* value*	Sum of squares	*P* value*
Regression	14	33154.19	. 001	146.52	.001
Linear	4	20904.67	. 002	108.17	.002
Square	4	8848.62	. 01	35.04	.01
Interaction	6	2335.06	.080	5.49	0.260
Residue Error	15	2510.11	-	2.81	-
Lack of Fit	10	3334.31	0.060	0.79	0.12
Pure error	5	1175.8	-	0.84	-
Total	29	34598.46	-	151.51	-
R^2	-	95.82%	-	96.71%	-
R^2_{adj}	-	92.90%	-	90.09%	-

* Significant for $P < 0.05$.

Table 4.1.2 (e) Common carp Gelatin- Analysis of Variance (ANOVA) for Response Surface Quadratic Model*

Source of variation	Degrees of Freedom	Gel Strength		Yield	
		Sum of squares	*P* value	Sum of squares	*P* value
Regression	14	31484.16	0.001	140.52	.001
Linear	4	25383.54	0.001	110.11	.001
Square	4	4096.05	0.04	26.20	.02
Interaction	6	2199.41	0.07	5.19	0.34
Residue Error	15	1267.44	-	2.51	-
Lack of Fit	10	3081.06	0. 08	0.66	0.23
Pure error	5	1186.38	-	0.83	-
Total	29	32946.44	-	144.01	-
R^2	-	95.56%	-	97.58%	-
R^2_{adj}	-	90.92%	-	95.09%	-

* Significant for $P < 0.05$.

Table 4.1.2 (f) Grass carp Gelatin- Analysis of Variance (ANOVA) for Response Surface Quadratic Model*

Source of variation	Degrees of Freedom	Gel Strength		Yield	
		Sum of squares	P value	Sum of squares	P value
Regression	14	46792.67	0.001	56.91	0.001
Linear	4	34582.96	0.002	50.12	0.001
Square	4	8676.71	0.04	4.77	0.01
Interaction	6	2403.80	0.08	1.07	0.07
Residue Error	15	1701.98	-	1.47	
Lack of Fit	10	3470.54	0.23	0.43	0.32
Pure error	5	1231.44	-	0.64	-
Total	29	47365.45	-	57.43	-
R^2	-	98.79%	-	99.09%	-
R^2 adj	-	95.86%	-	98.43%	-

*Significant for $P < 0.05$.

4.1.3. Numerical Optimization and Assignment of Optimization Parameters for obtaining Solutions with Desirability Values

Determining the overall optimum conditions in a multi response situation requires the use of desirability functions and in this study the optimization method developed by Derringer and Suich (1980), described by Myers and Montgomery (2002) was used. Here a one sided desirability function was used with the responses to be maximized. The programme uses five possibilities for a goal to construct the desirability indices viz., maximum, minimum, in target, in range and is equal. Tables 4.1.3(a), 4.1.3(b) and 4.1.3(c) lists the optimization parameters for the independent factors and responses. Among the independent factors the goal for alkali and acid

concentration to be used in the process is set as minimum and for the other two factors the goal is set in range. For the responses the goal is set as maximum. The limits for each goal are set by the software based on the response surface model constructed in the previous section. The parameter called weights gives added emphasis to upper and lower limits or emphasize a target value. Here the weights are given as one, with which the desirability will vary from zero to one in a linear fashion. Importance is a relative scale for weighing each of the resulting desirability in the overall desirability of the final product, which, in this case is gelatin. Here the importance is set as three for all the factors. A desirability value near to one is good.

The resultant solutions obtained using the response optimizer is given Tables 4.1.3(d), 4.1.3(e) and 4.1.3(f). The optimization solutions for all the three products viz., gelatin from the skin of Rohu, Common carp and Grass carp gives a composite desirability value above 0. 8 based on the set parameters. The responses predicted by the solutions are within the range of the experimental values obtained in the response surface model. Higher values for responses can be obtained by altering the goal of the independent factors particularly alkaline and acid concentrations used in the process.

The response surface plots based on the above optimization is illustrated In Figures 4.1.3(a) to 4.1.3(f). Since alkali and acid concentrations had the most significant effects on the responses, the response surface plots were set with other two factors viz., pretreatment time and extraction temperature at the median values of the lower and upper limits i.e., 50 minutes and 50 ^{0} C to determine the interaction of alkali and acid concentration . In the case of Rohu gelatin extraction, increase in the concentration of NaOH and H_2SO_4 results in the increase in gel strength and the effect is more pronounced in the case of the change in NaOH concentration (Fig.4.1.3 a). The influence of these factors on yield shows the reverse trend (Fig.4.1.3b). For Common carp gelatin, the same trend can be observed (Fig 4.1.3c & Fig.4.1.3d). For the extraction of Grass carp gelatin it can be seen that increase in gel strength and yield was significantly influenced by the concentration of NaOH only under the given set of optimization parameters (Fig.4.1.3.e &4.1.3 f). The results suggest that alkali concentration is the most important factor affecting the gel strength and yield in all the three

extraction processes under a given set of optimization parameters. Zhou and Regenstein (2004) observed that higher acid concentration resulted in lower gel strength during the extraction of Pollock skin gelatin whereas too low or too high acid concentration resulted in poor yield. In the acid pretreatment process for the extraction of Grass carp gelatin by Kasankala, *et. al.,* (2007) it was observed that increase in HCl concentration caused the increase of yield and gel strength upto the optimum point after which the increase in acid concentration resulted in the decrease of the above factors. Higher acid concentration can produce gelatin with shorter fragments which can negatively affect the gel strength. Cho, *et. al.,* (2005) reported that extraction temperature was the most important factor that affected the responses in the optimization of extraction of gelatin from the skin of yellowfin tuna.

Table 4.1.3 (a). Rohu Skin Gelatin- Optimization Parameters in the response optimizer

Name	Goal	Limit		Weight		Importance
		Lower	Upper	Lower	Upper	
NaOH	minimize	0.1	0.2	1	1	3
H_2SO_4	minimize	0.1	0.2	1	1	3
Pretreatment Time	is in range	45	55	1	1	3
Ext. Temp.	is in range	45	55	1	1	3
Gel Strength	maximize	119.14	221.44	1	1	3
Yield	maximize	6.1	16.88	1	1	3

Table 4.1.3(b). Common carp Skin Gelatin- Optimization Parameters in the response optimizer

Name	Goal	Limit		Weight		Importance
		Lower	Upper	Lower	Upper	
NaOH	minimize	0.1	0.2	1	1	3
H_2SO_4	minimize	0.1	0.2	1	1	3
Pretreatment Time	is in range	40	60	1	1	3
Ext. Temp.	is in range	40	60	1	1	3
Gel Strength	maximize	98.06	211.11	1	1	3
Yield	maximize	7.51	15.42	1	1	3

Table 4.1.3 (c). Grass carp Skin Gelatin- Optimization Parameters in the response optimizer

Name	Goal	Limit		Weight		Importance
		Lower	Upper	Lower	Upper	
NaOH	minimize	0.1	0.2	1	1	3
H_2SO_4	minimize	0.1	0.2	1	1	3
Pretreatment Time	is in range	40	60	1	1	3
Ext. Temp.	is in range	40	60	1	1	3
Gel Strength	maximize	98.64	259.9	1	1	3
Yield	maximize	7.22	13.14	1	1	3

Table.4.1.3.(d). Rohu Skin Gelatin- Optimization solutions obtained using the response optimizer

No.	NaOH	H_2SO_4	Pretreatment Time	Extraction Temperature	Gel Strength	Yield	Composite Desirability
1	0.10	0.10	51.35	49.33	189.12	13.060	0.808

Table.4.1.3(e).Common carp Skin Gelatin- Optimization solutions obtained using the response optimizer

No.	NaOH	H_2SO_4	Pretreatment Time	Extraction Temperature	Gel Strength	Yield	Composite Desirability
1	0.10	0.10	60.00	57.97	183.007	12.8779	0.845

Table.4.1.3 (f) Grass carp Skin Gelatin- Optimization solutions obtained using the response optimizer

No.	NaOH	H_2SO_4	Pretreatment Time	Extraction Temperature	Gel Strength	Yield	Composite Desirability
1	0.10	0.10	60.00	40.00	230.84	11.4333	0.874

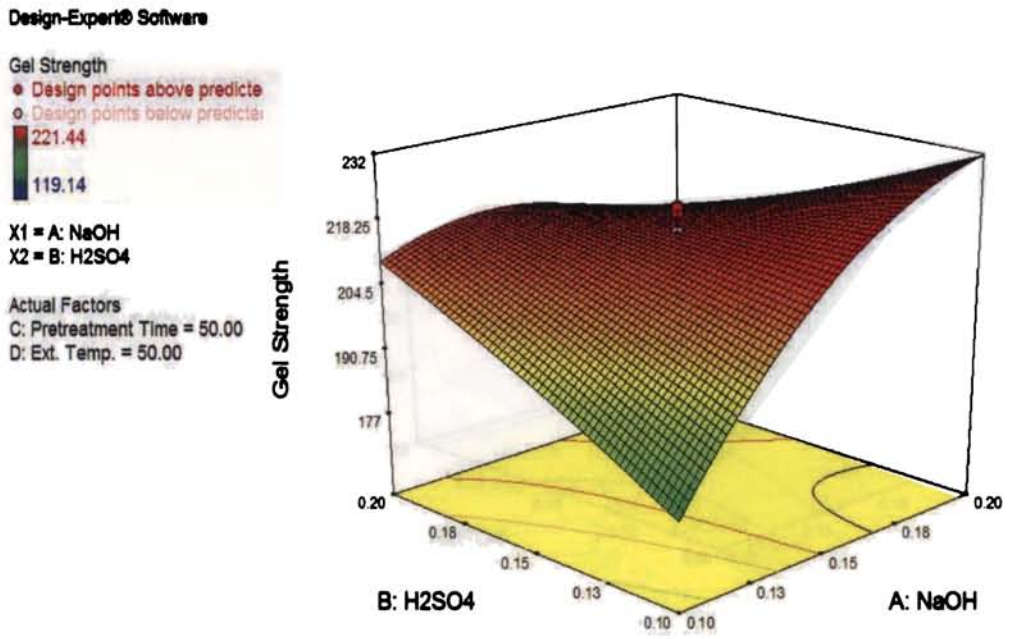


Fig. 4.1.3 (a) –Gel strength

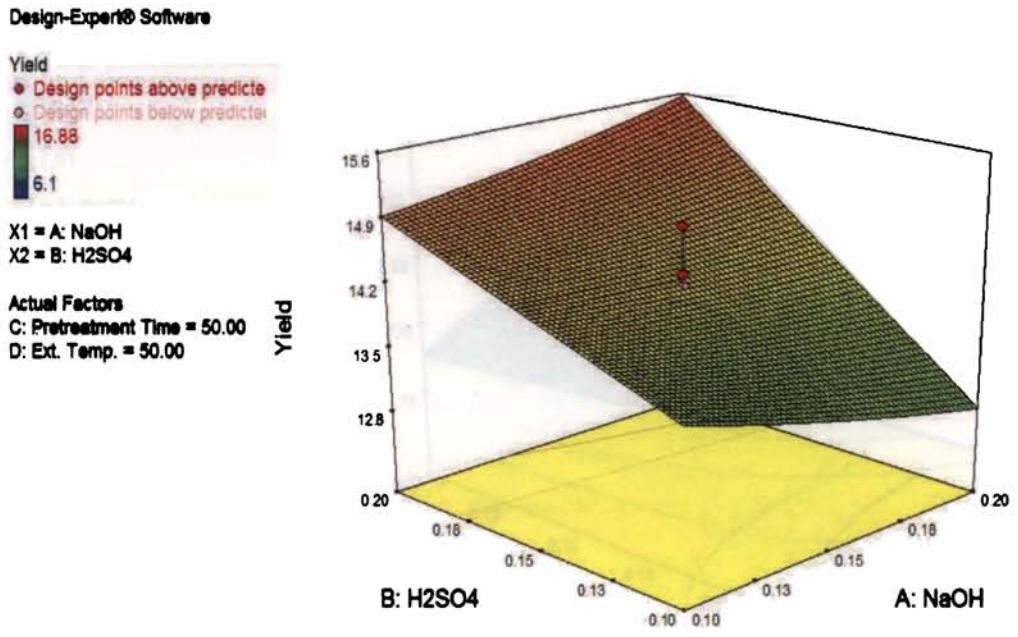


Fig. 4.1.3 (b) - Yield

Three-dimension response surface plots of Rohu skin gelatin extraction

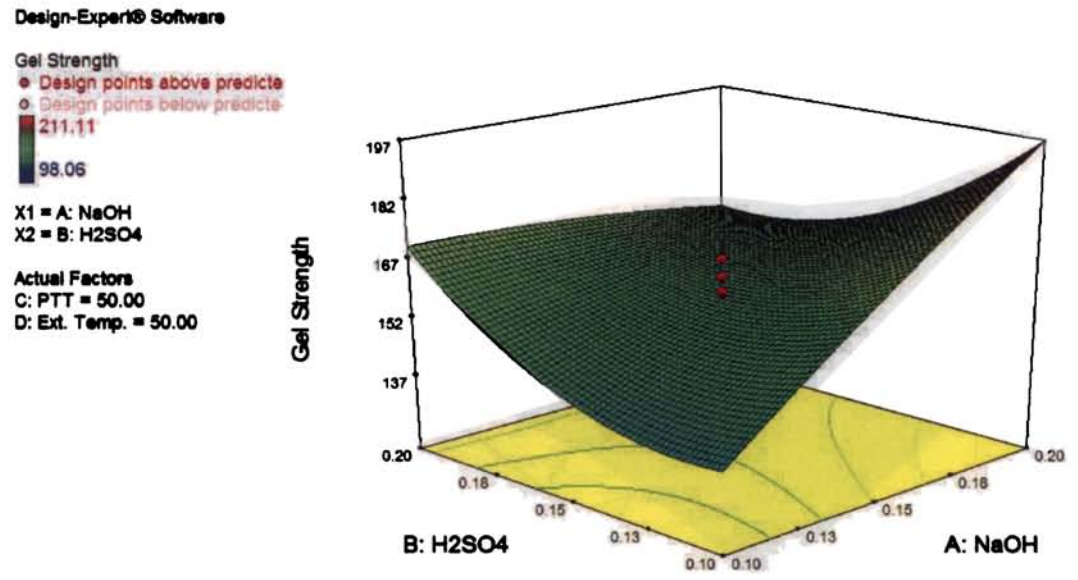


Fig. 4.1.3(c) – Gel strength

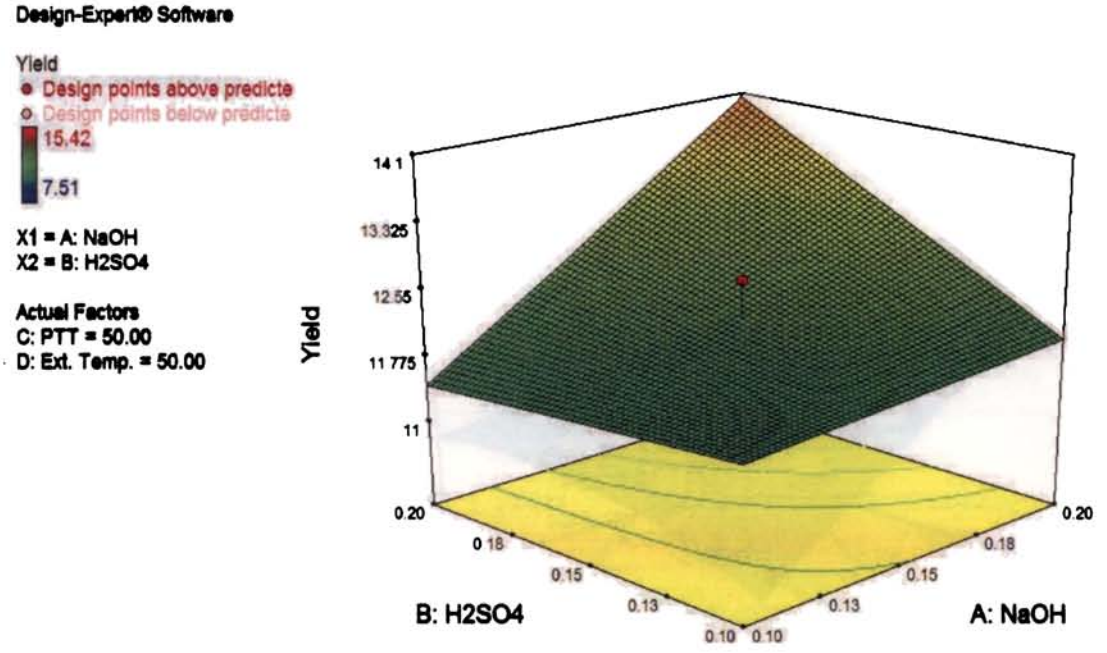


Fig. 4.1.3 (d) - Yield

Three-dimension response surface plots of Common carp skin gelatin extraction

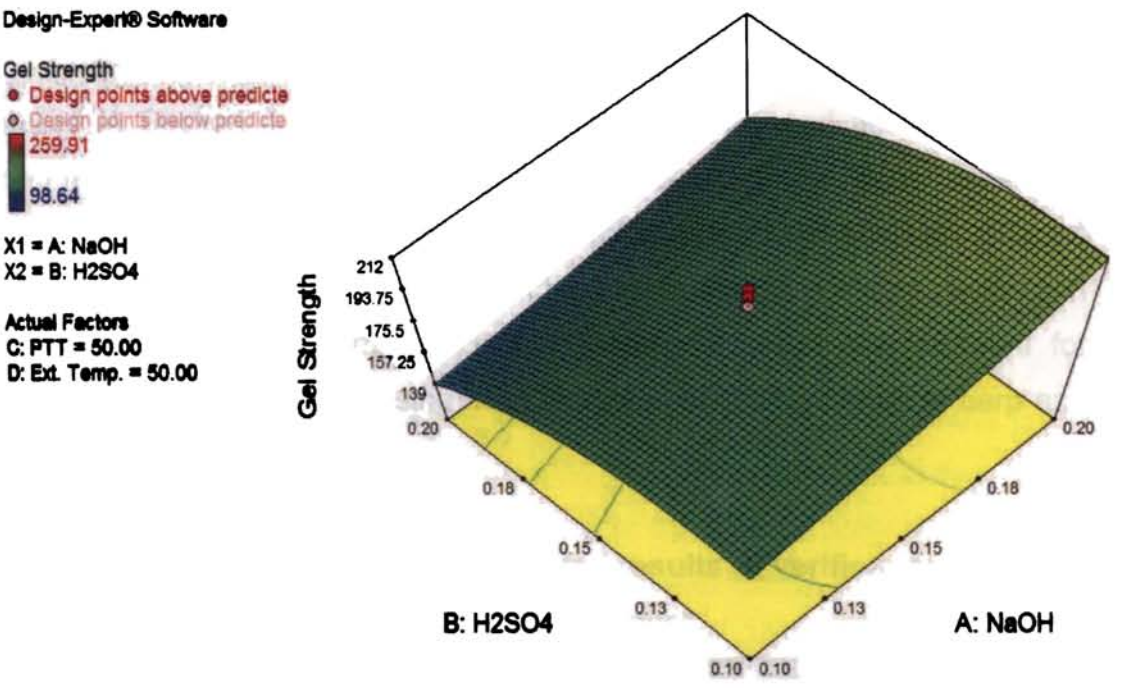


Fig. 4.1.3 (e) – Gel strength

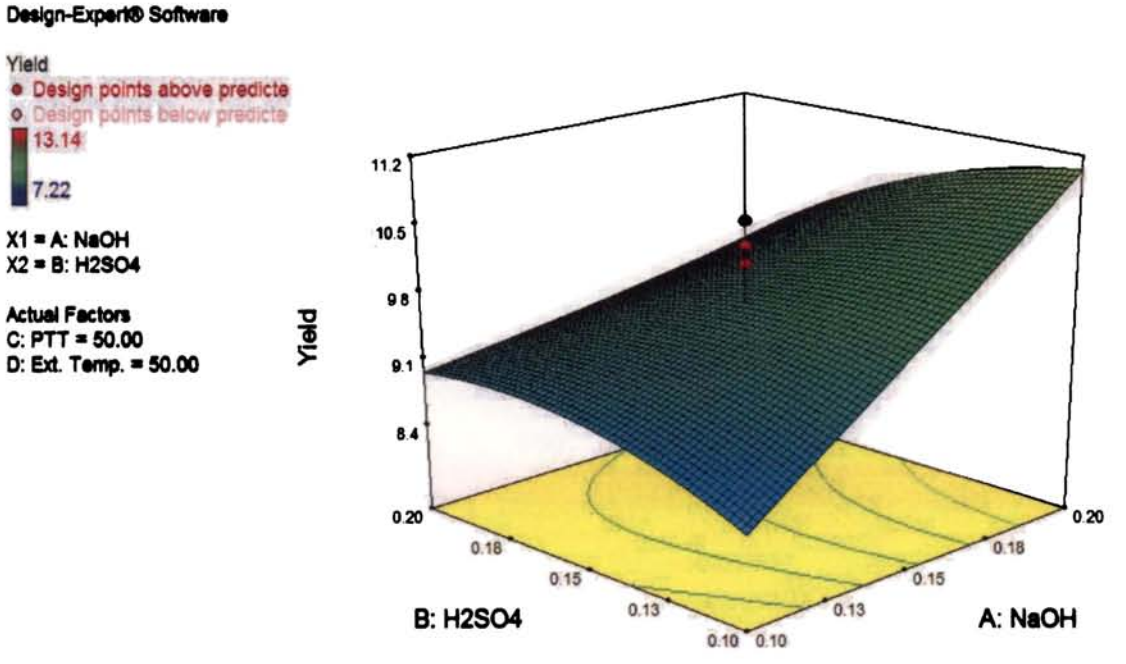


Fig. 4.1.3 (f) - Yield

Three-dimension response surface plots of Grass carp skin gelatin extraction

4.1.4 Verification of Predicted Values

Verification experiments were conducted under optimal conditions detailed in Section 4.1.3 to compare predicted values and actual values of responses (Table 4.1.4). Actual values repeated three times were gel strength (Bloom) and yield against predicted values of gel strength (Bloom) and yield. Both actual values and predicted values almost coincided with each other. Therefore, the estimated response surface model was adapted for optimization of gelatin processing from the skins of Rohu, Common carp and Grass carp.

Table 4.1.4 Experimental and predicted results of verification under optimized conditions*

	Responses	Predicted values	Experimental values
Rohu	Gel strength(Bloom)	189.12	188.6 (3.41)
	Yield (%)	13.06	13.20 (0.90)
Common carp	Gel strength(Bloom)	183.01	181.69 (2.82)
	Yield (%)	12.88	12.10 (0.71)
Grass carp	Gel strength(Bloom)	230.84	228.74 (3.19)
	Yield (%)	11.43	10.62 (0.28)

*Values in brackets are standard deviations of triplicate samples.

4.2 Quality characteristics of Gelatin Extracted from the Skin of freshwater fishes

4.2.1 Skin Yield from Freshwater Fish

The yield of skin without scales obtained from the target species viz., Rohu, Common carp and Grass carp is given in Table 4.2.1. The yield percentage of skin was almost same for Rohu and Grass carp at the given size range. The yield of skin from Common carp was lower than that of the other two species which could be attributed to the deeper body shape of this species. Processing data from commercial channel catfish processing in the U.S indicate that yield of skin is 6% of the initial fish weight (Prinyawiwatkul, *et. al.,* 2002).

Table 4.2.1 Yield percentage during different stages of separation of skin from the carcass of Rohu, Common carp and Grass carp*

Species	Average size	Whole fish (%)	Skinless fillets (%)	Head (%)	Viscera & frames (%)	Skin without scales (%)
Rohu (*L.rohita*)	Length - 55 ± 2.8 cm Weight– 2500 ± 120g	100	42 (1.2)	19.4 (0.8)	30.9 (1.4)	7.2 (0.5)
Common carp (*C.carpio*)	Length - 30 ± 3.5 cm Weight – 1500 ± 65g	100	37.2 (1.8)	20.1 (1.3)	36.2 (2.1)	6.2 (0.7)
Grass carp (*C.idella*)	Length-62 ± 2.2 cm Weight– 2610 ± 140g	100	42.3 (1.5)	21.2 (1.6)	28.5 (2.3)	7.8 (1.1)

*Values in brackets are standard deviations of triplicate samples.

4.2.2 Physical Properties of Gelatins

4.2.2.1 Yield

Yield of gelatin as percentage of the skin is given in Table 4.2.2. The maximum yield was observed for Rohu (12.93%) followed by Common carp(12%) and Grass carp (10.57%). The gelatin yield was significantly lower in Grass carp than the other two sources. The gelatin yields have been reported to vary among the fish species, mainly due to the differences in collagen content, the compositions of skin as well as the skin matrix. Leaching of collagen during the washing treatments of skin during processing could result in the lower yield of gelatin. Insufficient denaturation of soluble collagen during the extraction can also result in lower yield. The acid pretreatment during the extraction removes the non collagen protein after the skin sample swells in the acid solution. The hot water extraction hydrolyses and solublises the gelatin which is then separated by filtration. In this study it was observed that the maximum swelling of the skins during pretreatment with alkali and acid was for Rohu and Common carp skins, which indicate that a better yield can be expected due to the opening of cross links during the swelling. Further, the high degree of cross linking via covalent bonds can cause the decrease in solubility of collagen and might lead to the lower content of extractable gelatin (Foegeding, *et.al.,* 1996). The yields of skin gelatin reported for different species are: sole 8.3%, megrim 7.4%, cod 7.2%, hake 6.5% (Gomez-Guillen, *et. al.,* 2002), red and black tilapia 7.8% and 5.4%, respectively (Jamilah & Harvinder, 2002), young and adult Nile perch 12.5% and 16%, respectively (Muyonga *et. al.,* 2004a), big eye snapper and brown stripe red snapper 6.5% and 9.4%, respectively (Jongjareonrak, *et. al.*, 2006). The yield observed for the species in this study is comparatively better which offers scope for commercially viable extraction of gelatin.

4.2.2.2 Viscosity

Table 4.2.2 shows the viscosity of the gelatins from different species. The viscosity for the samples were in the range of 5.96 – 7.07 and was significantly higher ($p<0.05$) for Grass carp gelatin followed by Rohu and Common carp gelatins. Viscosity is the second most important commercial property of gelatin after gel strength (Ward & Courts, 1977). Viscosity is partially controlled by molecular weight and molecular size distribution (Sperling, 1985). The viscosities of most of the commercial gelatins have been reported to be in the range of 2.0 to 7.0 cP and upto 13.0 cP for specialized ones (Johnston-Banks, 1990). Minimum viscosity for gelatin was observed to be in the pH range of 6-8 (Stainsby, 1952). Gelatins within a pH range of 3 show the maximum values for viscosity. Jamilah & Harvinder (2002) reported viscosity values of 3.2cP and 7.12cP for red and black tilapia respectively whereas for channel catfish the optimum value predicted was 3.23 cP (Yang, *et. al.*, 2007). The results here indicate that high viscosity gelatin can be prepared from Rohu, Common carp and Grass carp. Low viscosity (and high gel strength) is required for poured confectionery, and high viscosity for film forming applications.

4.2.2.3 Clarity

Clarities of gels are shown in Table 4.2.2. Grass carp gel has a significantly higher clarity (2.25%), followed by Rohu (1.77%) and Common carp (1.38%) gels. Clarity of a gelatin solution can be important for commercial applications and this functional property is frequently assessed for determining the quality of gels. When the protein is treated for a long time at high temperatures, aggregation is activated and turbidity is increased (Johnson and Zabik, 1981). Increase in higher molecular weight aggregates can increase the turbidity (Montero, *et. al.,* 2002). Here for the three types of gels the extraction time was same but the extraction temperatures were different (See Tables 4.1.3 d - f, in Section 4.1). The extraction temperature was highest for Common carp (58^0 C) followed by Rohu (49^0 C) and Grass carp (40^0 C) which explains the increase in clarity in the reverse order. The minimum clarity and corresponding high turbidity in Common carp may be due to the formation of aggregates of higher

molecular weight during the higher temperature extraction, decreasing the gelatin's solubility by exposing many hydrophobic residues. Cho, *et. al.,* (2004) observed high turbidities for gelatin extracted from shark cartilage employing high temperature and long extraction periods.

4.2.2.4 Melting Temperature

Significant differences were observed in the melting temperatures of the three gelatins. The melting point of gelatin obtained from Grass carp (29.1^0 C) was significantly higher ($p< 0.05$) than that of Rohu (28.13^0 C) and Common carp (28.27^0 C). The melting points are higher than that reported for many other species viz., 8-10 ^{0}C for cod skin gelatin (Gudmunsson & Hafsteinsson, 1997); 24.3 ^{0}C for yellow fin tuna gelatin (Cho, *et. al.,* 2005); 21.4 – 26.5 ^{0}C for gelatin from the skin and bone of Nile perch (Muyonga, *et al.,* 2004a); 22.5 to 28. 9 ^{0}C for tilapia skin gelatin (Jamilah & Harvinder 2002).

The melting temperature of gelatin has been found to correlate with the proportion of the imino acids proline and hydroxyproline (both with a 5-membered pyrrolidine ring) in the original collagen (Ledward, 1986; Piez & Gross, 1960; Veis, 1964). This is typically 24% for mammals and 16–18% for most fish species (Norland, 1990). Cold water fish, for example cod, have a very low hydroxyproline content and coupled with this a very low gelling and melting temperature. Fish gelatin with lower gel melting temperature had a better release of aroma and offered stronger flavour and useful in product development to control the texture and flavour release during mastication. Here it can be seen the imino acid content of Grass carp gelatin was maximum (20.80%) followed by Common carp (19.50%) and Rohu (19.49%) gelatins (Table 4.2.8) The comparatively high amount of imino acid content can be a contributory factor for the high melting point characteristics of gelatins from these species. Proline plays a major role in promoting the formation of polyroline II helix (Ross-Murphy, 1992). Gomez-Guillen, *et. al.,* (2002) correlated the thermal stability of gelatin to the number and stability of Proline rich region in collagen or gelatin molecules, which are high in fresh warm water fish and mammalian species. Gudmundsson, (2002) observed that gelatins with high melting temperature formed stronger gels and in this

study also it was observed that Grass carp which had the highest melting temperature formed the strongest gel among the three gelatin samples.

4.2.2.5 Setting Temperature & Setting Time

The setting (gelling) temperature observed for the gels from Rohu, Common carp and Grass carp skins were in the range of 17.9 ^{0}C to 20.5 ^{0}C with significant differences between the gels ($p<0.05$). Common carp had the lowest setting temperature and the highest was for Grass carp. Also the Grass carp gel showed a significantly faster setting time ($p<0.05$) of 68.6 seconds when compared to the other two gels. Muyonga, *et. al.,*(2004a) reported a setting temperature of 19.5 ^{0}C and a setting time of 60 seconds for the gelatin from the skin of adult Nile perch extracted at 50 ^{0}C which is similar to the values observed for Grass carp skin gelatin. Gudmundsson, (2002) compared the rheological properties of fish gelatins (tuna, tilapia, cod and megrim) with conventional bovine and porcine gelatins. The gelling (setting) and melting points of tilapia gelatin (18.2 ^{0}C and 25.8 ^{0}C respectively) were the highest among the fish gelatins and was comparable to low molecular weight porcine and bovine gelatins. Cold water fish gelatins i.e., gelatins from the skins of cod and megrim had very low melting and gelling points when compared with gelatins from warm water fish and animal sources mainly due to the low imino acid content, which in turn reduces the propensity for intermolecular helix formation (Gilsenan & Ross-Murphy, 2000). Setting (gelling) temperature denotes the gelling process which involves the transition from random coil to triple helical structure of gelatins. The imino acid content stabilizes the ordered conformation when gelatin forms the gel network during gelling. A critical amount of regenerated helices are required to form the gel network. Gelling and melting temperatures are also influenced by the change in ionic strength. This suggests that the junction zones and the gel network may be stabilized by both hydrogen bonds and electrostatic bonding (Haug *et. al.,* 2004).

In this study, the gelling and melting temperatures observed for gelatins from the skin of Rohu, Common carp and Grass carp are similar, if not better than many of the gelatins from animal sources and can possibly substitute the same in many applications without extensive modifications. There is future

scope for developing binary blends of these gelatins with animal gelatins that are completely compatible and commercially useful in many applications.

4.2.2.6 *Odour*

The gelatins prepared from the skins of Rohu, Common carp and Grass carp were found to have a mild but easily perceivable fishy odour. The hedonic scores (See Section 3.2.6.11) were in the range of 2.3 -2.6 with Grass carp gelatin showing significantly higher score ($p< 0.05$) than Rohu and Common carp gelatins. Muyonga, *et. al.,* (2004a) reported that the gelatins prepared from the skin and bone of Nile Perch by activated carbon treatment were found to be free of fishy odour and to have a mild putrid odour with a mean hedonic score of 2–2.5. Strong fishy odour was reported for freeze dried gelatin prepared from the skin of Black Tilapia (Jamilah & Harvinder, 2002). Choi & Regenstein, (2000) observed that fish gelatins had less off odour and better aroma than pork gelatins on sensory evaluation. Activated carbon treatment at the final stages of extraction can further reduce the odour and improve the acceptability of the gelatins mentioned in this study.

Table 4.2.2 Physical properties of Gelatins extracted from the skin of Rohu, Common carp and Grass carp*

	Rohu	Common carp	Grass carp
Yield (%)	12.93 (0.55)	12.00 (0.50)	10.57 (0.13) [a]
Viscosity (cP)	6.06 (0.04)	5.96 (0.12)	7.07 (0.10) [a]
Clarity (%)	1.77 (0.37)	1.38 (0.19)	2.25 (0.07) [a]
Melting Temperature(^{0}C)	28.13 (0.05) [a]	28.27 (0.05) [b]	29.1 (0.08) [c]
Setting Temperature(^{0}C)	18.52 (0.10) [a]	17.96 (0.15) [b]	20.50 (0.20) [c]
Setting Time (Seconds)	106.00 (3.74)	103.00 (2.45)	68.60 (1.96) [a]
Odour Score	2.30 (0.12)	2.40 (0.11)	2.60 (0.10) [a]

*Values in brackets are standard deviations of triplicate samples.
[a-c] Means within a row with different letters are significantly different ($p<0.05$)

4.2.2.7 Colour

Instrumental colour measurements of the freeze dried gelatin powders are shown in Table 4.2.3. The gelatins from the skin of Rohu, Common carp and Grass carp had a snowy white appearance and were light-textured. Lightness (L*) value was highest for Grass carp gelatin (92.53) and Common carp gelatin showed significantly lower value ($p< 0.05$) for 'L* than the other two gelatin samples. The a * values for the three gelatin samples showed negative values indicating a shift of colour towards green and it was significantly higher for Common carp gelatin (-0.41). The b* values were positive indicating the degree of yellowness. Common carp gelatin had significantly low b* value (1.82) than the other samples. However all the gelatin samples appeared to be in white colour in the visual observation. Similar colour values were observed for freeze dried gelatins from the skin of tilapia (Jamilah & Harvinder, 2002). This could be a positive attribute, since it is easier to incorporate these gelatins into any food system without imparting any strong colour attribute to the product.

The colour of the gelatin depends on the raw material used for the extraction and also whether it is obtained from first stage, second stage or subsequent stages of extraction (Ockerman & Hansen, 1999). However, it does not influence other functional properties .Commercial gelatin is not colourless in solution but has a colour varying from a very pale yellow to dark amber. There can be no doubt that the colour attribute of gelatin has practical significance, in that some 60% of world production is consumed by the confectionery industry (Siebert, 1992). In this industry, the products are very often coloured and it stands to reason that, the less the colour variation in the ingredients, the easier it would be to produce a uniform product. Furthermore, in the minds of most people the lack of colour is associated with purity; hence, pale colour is normally more desirable than darker colour. The importance of gelatine colour is recognised by manufacturers (Hoffmann, 1985; Schreiber, 1977).

Table 4.2.3 Colour of Gelatin from the skin of Rohu, Common carp and Grass carp*

	L*	a*	b*
Rohu	91.89 (0.62)	-0.35 (0.02)	2.76 (0.21)
Common carp	90.15 (0.64) [a]	-0.41 (0.03) [a]	1.82 (0.45) [a]
Grass carp	92.53 (0.63)	-0.36 (0.02)	2.70 (0.22)

*Values in brackets are standard deviations of triplicate samples.
Means within a column with superscript a are significantly different ($p<0.05$)

4.2.3 Functional properties of Gelatins

4.2.3.1 Gel strength

Gel strengths of gelatins from Rohu, Common carp and Grass carp skins are shown in Table 4.2.4. Significant differences were observed for the bloom strengths of Rohu (188.63B), Common carp (181.31B) and Grass carp (231.18B) skin gelatins. Gel strengths of Rohu and Common carp skin gelatins were significantly lower than that of Grass carp skin gelatin. The lower gel strength is probably due to its lower amount of imino acids (Hydroxyproline + Proline), which stabilize gelatin structures. As shown in Table 4.2.8, the amount of imino acids in Rohu, Common carp and Grass carp skin gelatins were 19.49, 19.50 and 20.80% respectively. The amount of proline was highest in Common carp, but the amount of hydroxyproline was lowest. Hydroxyproline is believed to be the major determinant of stability due to its hydrogen bonding ability through its hydroxyl group, although proline is also important (Burjandze, 1979; Ledward, 1986).Rohu, Common crap and Grass carp gelatins had significant amounts of serine and threonine (See Table 4.2.8) with free hydroxyl groups which also may have contributed to the gel strength. This study appears to confirm the role of hydroxyproline as the major determinant for gel strength.

Gel strength is one of the most important functional properties of gelatin and fish gelatin typically has less gel strength than mammalian gelatin (Gilsenan & Ross-Murphy, 2000). Gel strength is a function of complex interactions determined by amino acid composition and the ratio of α-chain and the amount of β-component. Gel structure of gelatin is more stable when

the imino acid (Hydroxyproline + Proline) content is higher, and the amount of aggregates of higher molecular weight is less (Gomez-Guillen, *et al.*, 2002). It is well known that the hydrogen bonds between the water molecules and free hydroxyl groups of amino acids in gelatin are essential for the gelatin gel strength (Babel, 1996). A higher content of other amino acids with free hydroxyl groups viz., serine, threonine and tyrosine can contribute more hydrogen bonds, apart from the imino acid group which can also contribute to the gel strength (Arnesan & Gildberg, 2002). The gelatins from the skins of Rohu, Grass carp and Common carp have medium gel strengths which are of commercial significance, considering the potential applications in edible film preparations. The gel strengths obtained in this study is in reasonable agreement with that reported by Jamilah & Harvinder (2002) for Tilapia fish (180.76 blooms) and Muyonga, *et. al.* (2004a) for Nile perch fish (229 g), but lower than that reported by Cho *et. al.* (2005) for Yellowfin tuna skin gelatin (426 blooms), Grossman & Bergman, (1992) for Tilapia (263 g) and Kasankala, *et. al.,(2007)* for Grass carp (267 g) which are tropical fish. Lower gel strengths were reported for gelatins from the skins of other tropical species viz., sin croaker (124.94g) and shortfin scad (176.92g) by Cheow, *et al.,*(2006). The differences in gel strength among the various species could be explained by differences in manufacturing process used and the intrinsic properties of collagen which varies among fish species. Gudmunsson & Hafsteinsson, (1997) suggested that the gel strength may be dependent on the isoelectric point and may also be controlled, to a certain extent, by adjusting the pH. More compact and stiffer gels can be formed by adjusting the pH of the gelatin close to its isoelectric point, where the proteins will be more neutral and thus the gelatin polymers are closer to each other. Studies by Zhou, *et. al.,* (2006*)* showed that the gelling ability of Pollock skin gelatin could be enhanced by mixing it with high quality gelatins from warm water species or mammals. Fish gelatin with lower gel melting temperature had a better release of aroma and offered stronger flavour and useful in product development to control the texture and flavour release during mastication.

4.2.3.2 Foam Formation Ability (FA) and Foam Stability (FS)

Foam formation abilities of Rohu, Common carp and Grass carp gelatins are shown in Table 4.2.4. Foam formation ability of Common carp gelatin was 2.44 (the ratio of foam volume/liquid volume), significantly lower than Rohu (2.51) of Grass carp (2.83) gelatins. Foam stability (the ratio of the initial volume of foam/ final volume after 30 min) of Common carp was 1.90, significantly less than Rohu (1.86) and Grass carp (1.78) gelatins, demonstrating the lower stability of Common carp gelatin. Thus, Common carp has the lowest foam formation ability and the foam stability among the three gelatins. Foam formation ability is an important functional property of gelatin for commonly used foods such as marshmallows. The reduced foam formation and stability may be due to aggregation of proteins which interfere with interactions between the protein and water needed for foam formation (Kinsella, 1977). Cho, *et. al.,* (2004) reported foam formation ability of 2.6 and 2.9 and foam stability of 1.5 and 1.4 for gelatins from shark cartilage and porcine skin respectively

4.2.3.3 Water Holding (WHC) and fat-binding (FBC) Capacities

Water-holding capacity and fat-binding capacity of the three gelatins are shown in Table 4.2.4. Significant differences are observed in the fat binding capacities of the gelatins. Rohu skin gelatin had the highest fat-binding capacity(457.3%) and Common carp skin gelatin had the lowest water-holding capacity (176%). Fat binding capacity depends on the degree of exposure of the hydrophobic residues inside gelatin. As shown in Table 4.2.8, the hydrophobic amino acid, tyrosine, made up 0.48% of Rohu gelatin which was higher than that of Common carp and Grass carp skin gelatins at 0.21% and 0.22% respectively. The high amount of tyrosine is probably responsible for the high fat binding capacity of Rohu skin gelatin. Cho, *et. al.*, (2004) has observed that the gelatin extracted from shark cartilage had a higher fat binding capacity than porcine skin gelatin which is attributed to the higher content of tyrosine. The water holding capacity of the Grass carp gelatin was

significantly higher than that of Rohu and Common carp gelatins .Water-holding capacity is believed to be affected by the amount of hydrophilic amino acids. The amounts of hydroxyproline in Rohu, Common carp and Grass carp were 7.90, 7.78 and 11.06 %, respectively, showing that Grass carp gelatin had the maximum hydroxyproline content and a correspondingly high value for water holding capacity. Water-holding and fat-binding capacities are functional properties that are closely related to texture by the interaction between components such as water, oil and other components.

Table 4.2.4 Functional Properties of Gelatin from the skin of Rohu, Common carp and Grass carp*

	Rohu	Common carp	Grass carp
Gel strength (Bloom)	188.63 (2.64) [a]	181.31 (2.08) [b]	230.18 (0.88) [c]
Foam Formation Ability (FA)	2.55 (0.03) [a]	2.45 (0.04) [b]	2.85 (0.03) [c]
Foam Stability(FS)	1.83 (0.02)	1.91 (0.01) [a]	1.80 (0.02)
Water Holding Capacity (%)	184.33 (3.30)	176.00 (4.90)	227.11 (3.74) [a]
Fat Binding Capacity (%)	457.33 (6.55) [a]	333.20 (5.10) [b]	364.00 (2.94) [c]

*Values in brackets are standard deviations of triplicate samples.
[a-c] Means within a row with different letters are significantly different ($p<0.05$)

4.2.3.4 Textural Properties

The textural properties of the three gelatins are given in Table 4.2.5. In the three gel samples examines hardness I & II differs significantly ($p< 0.05$) with the highest values observed for Grass carp gel. Grass carp gel had a hardness I value of 3.85 Kgf.The corresponding values for Rohu and Common carp were 2.5 Kgf and 2.05 Kgf respectively. Muyonga *et al.*,(2004a) observed that there is a high correlation between hardness of the gel and bloom strength in the case of gelatin from the skin of Nile Perch and hence hardness can be used to compare the gel strengths. Yang *et al.*, (2007) has reported that the gel strength of gelatin produced from the skin of channel

catfish showed high correlation with hardness and chewiness. In this study also it has been found that a higher hardness indicates high gel strength since Grass carp gelatin had the highest gel strength and hardness. Common carp gelatin was found to have the lowest hardness and correspondingly the lowest gel strength.

Cohesiveness is the ratio of positive force area during the second compression to that during the first compression of the gel sample. It gives a relative and dimensionless measure of how much gel strength remained after the deformation of the first compression i.e., visco elasticity. Cohesiveness is a measurement of the degree of difficulty in breaking down the gel's internal structure. A value of 1 indicates total elasticity and a value of 0 imply that the sample does not recover at all, indicating total loss of elasticity. Significantly higher value for cohesiveness ($p < 0.05$) was observed for Grass carp gelatin (0.57) than the other two gels. All the three gels had medium cohesiveness values indicating medium elastic properties.

Springiness index is the ratio between the height of the gel sample and the height that the sample recovers during the time that elapses between the end of the first compression and the start of the second compression. Springiness index was significantly lower for Common carp gelatin (0.85) than the other two gels. High springiness results from the gel structure being broken into a few large pieces during the first TPA compression while low springiness results from the gel breaking into many small pieces (Lau, *et. al.,* 2000).

Gumminess by sensorial definition is the energy required to disintegrate a semi-solid food product to a state ready for swallowing. By instrumental definition it is the calculated parameter of the Product of Hardness x Cohesiveness. Gumminess was significantly different for all the three gel samples. Gumminess was highest for Grass carp (1.74 Kgf) gel followed by Rohu (1.17 Kgf) and Common carp (0.87 Kgf) gel which is again dependant on the hardness of the gels. Gumminess is a desirable attribute in marshmallow type of products where the product gives "a feel in the mouth" sensation while chewing.

Chewiness by sensorial definition is the energy required to chew a Solid food product to a state where it is ready for swallowing. This attribute is difficult to quantify precisely due to complexities of mastication (shear, compression, tearing and penetration). By instrumental definition it is the calculated parameter of Product of Gumminess x Springiness (essentially primary parameters of Hardness x Cohesiveness x Springiness). Chewiness was highest for Grass carp (16.93 Kgf.mm) gel followed by Rohu gel (10.15 Kgf.mm) and Common carp gel (8.87 Kgf.mm).

Fracture force by sensorial definition is the Force at which a material fractures. Related to the primary parameters of hardness and cohesiveness, brittle materials have low cohesiveness. Not all foods fracture and thus value may relate to hardness if only single peak is present. Brittle foods are never adhesive. By Instrumental definition it is the first significant break in the first compression cycle. Fracture force for Grass carp gel (2.59 Kgf) was significantly higher ($p< 0.05$) than the other two gels indicating that the gel is a highly chewable gel with low brittleness. This can be useful for product formulations involving soft gel capsules.

Adhesiveness by sensorial definition is the work necessary to overcome the attractive forces between the surface of the food and the surface of other materials with which the food comes into contact (e.g. tongue, teeth, palate). Work required to pull food away from a surface. By instrumental definition it is the negative area for the first bite, representing the work necessary to pull compressing probe away from sample. Gelatins from Rohu, Common carp and Grass carp had very low values (0.02 – 0.03 Kgf.mm) for this parameter, implying their chewability.

The above detailed texture attributes of the gelatins of Rohu, Common carp and Grass carp gives an indication that these are useful in food applications for the preparation of the products like fruit gums where gelatin helps in thermo reversible gel formation, provide taste and color neutrality, gives easy pouring ability due to low viscosity and excellent clarity. In addition gelatin gives unique texture and excellent mouth feeling, chewability and attractive appearance

Another potential application could be in the production of marshmallows where gelatin offers high degree of gel firmness, good foam

formation and stabilization and thermo reversible gel formation. Also helps in appetizing appearance of the product, optimal consistency and excellent mouth feeling.

Since the gelatins from Rohu, Common carp and Grass carp have medium gel strength, these can be used in the manufacture of soft gel capsules where the ideally required bloom strength is in the range of 150 to 200 Bloom.

Table 4.2.5 Texture Profile Analysis of Gelatin from the Skin of Rohu, Common carp and Grass carp*

Parameters	Source of skin Gelatin		
	Rohu	Common carp	Grass carp
Hardness 1 (Kgf)	2.50 (0.07) [a]	2.05 (0.06) [b]	3.85 (0.29) [c]
Hardness 2 (Kgf)	2.05 (0.16) [a]	1.71 (0.11) [b]	3.15 (0.38) [c]
Cohesiveness	0.47 (0.04)	0.51 ± 0.05	0.57 (0.04) [a]
Springiness Index	0.92 (0.04)	0.85 (0.02) [a]	0.97 (0.05)
Gumminess (Kgf)	1.17 (0.13) [a]	0.87 (0.11) [b]	1.74 (0.16) [c]
Chewiness (Kgf.mm)	10.15 (1.02) [a]	8.87 (0.36) [b]	16.93 (1.03) [c]
Fracture Force (Kgf)	0.96 (0.35)	1.13 (0.20)	2.59 (0.54) [a]
Adhesiveness (Kgf.mm)	0.03 (0.001)	0.03 (0.001)	0.02 (0.001) [a]

*Values in brackets are standard deviations of triplicate samples.
[a-c] Means within a row with different letters are significantly different ($p<0.05$)

4.2.4 Chemical Properties of Gelatins

4.2.4.1 Proximate Composition

The proximate composition of the skin of fishes used as the raw material for gelatin extraction is given in Table 4.2.6. Grass carp skin contain significantly lower amount ($p< 0.05$) of protein (16.61%) than the other two skins. The protein content of the skin indicates the maximum possible yield of gelatin. Here the maximum yield of gelatin was obtained from Rohu skin and the lowest yield was from Grass carp skin (Refer Table 4.2.2). Subcutaneous accumulation of fat was significantly higher for Grass carp and Rohu skins when

compared with Common carp skin. Skin from the adult fish contains more lipid than the skin of young fish probably because the fish accumulate subcutaneous fat as they age. Here the samples of three species chosen for this study are of almost the same age group as they are cultured species. Ash content was significantly higher for Common carp skin (2.38%), probably because the species has more scales in the skin than the other two, the remnants of which could have increased the ash content of the skin.

A study on the extraction of gelatin from the skin and bone of Nile Perch by Muyonga, *et. al.*, (2004a) has showed that the proximate composition of gelatin was found to vary with the type of tissue used as raw material but was unaffected by age of the fish. The skin gelatins were generally low in ash, with most having ash content lower than the recommended maximum of 2.6% .The bone gelatins however had much higher ash content in the range3–10%.

Table 4.2.6 Proximate Composition of Fresh Raw Skin of Carps*

	Rohu	Common carp	Grass carp
Moisture (%)	78.14(1.19)	76.71(1.22)	79.05 (1.04) [a]
Protein (%)	18.58 (1.09)	18.30 (0.92)	16.61 (0.95) [a]
Lipid(% dwb)	3.02 (0.68)	2.66 (0.55) [a]	3.67 (0.51)
Ash (%)	1.93 (0.11) [a]	2.38 (0.17) [b]	1.50 (0.18) [c]

*Values in brackets are standard deviations of triplicate samples.
[a-c] Means within a row with different letters are significantly different ($p<0.05$)

Table 4.2.7 Proximate Composition and pH of Carp Skin Gelatin*

	Source of skin Gelatin		
	Rohu	Common carp	Grass carp
Moisture (%)	8.10 (0.12) [a]	8.48 (0.11) [b]	7.24 (0.20) [c]
Protein (%)	90.43 (0.70)	89.71 (0.59)	91.54 (0.75) [a]
Lipid (% dwb)	0.57 (0.07) [a]	0.62 (0.06) [b]	0.41 (0.03) [c]
Ash (%)	1.18 (0.04) [a]	1.11 (0.02) [b]	1.10 (0.07) [c]
pH	4.08 (0.04)	4.05(0.06)	4.42 (0.04) [a]

*Values in brackets are standard deviations of triplicate samples.
[a-c] Means within a row with different letters are significantly different ($p<0.05$)

The proximate compositions of gelatins are given in Table 4.2.7. Generally, the skin gelatins of the three species showed high values for proteins and low values for moisture and fat, indicating efficient removal of water and fat from the skin. Grass carp gelatin contained significantly higher content of protein (91.54%) than the other two gelatins. Jongjajareonarak, *et. al.,* (2006) reported a protein content of 87.9% & 88.6% for freeze dried gelatin from the skin of big eye snapper and brown eye snapper respectively. Freeze-dried gelatin from the skin of adult Nile perch contained 88% protein when extracted at 50 ^{0} C (Muyonga, *et. al,* 2004a).

Moisture content in all the samples were below 10% which is less than the limit prescribed for edible gelatin i.e., 15% (GME, 2005). The moisture content of gelatin may be as high as 16 %, however, more normally it is about 10 % to 13 % because at 13 % moisture content the glass transition temperature of gelatin is about 64°C which allows particle size reduction to be a simple operation (McCormick, 1995). At 6 to 8 % moisture content gelatin is very hygroscopic and it becomes difficult to determine the physical attributes with accuracy (Cole, 2000).

The ash content in all the three samples were in the range of 1.10 - 1.18% , much less than the recommended maximum limit of 2.6%

(Jones.1977) and the limit set for edible gelatin(2%) (GME, 2005). The different mineral contents between the skins of the species might be associated with the varying ash contents obtained. However the nature of the ash is important. For example, 2 % $CaSO_4$ content in gelatin can impart excellent clarity in spite of the higher ash content .However, on dilution of the gelatin in a confectionery formulation, the ash can precipitate. Furthermore, ammonia is often used as a pH modifier in gelatin preparation and salts like NH_4Cl are not determinable by pyrolysis (Cole, 2000).

4.2.4.2 pH

The pH of the gelatins is given in Table 4.2.7. The pH varies between 4.05 and 4.42. Grass carp gelatin shows significantly higher values for pH ($p< 0.05$) than the other two gelatins. The values of pH for gelatin samples are outside the range prescribed for Type A Gelatin (pH 6.0 - 9.5) and Type B Gelatin (pH 4.7 – 5.6). This is because the pretreatment method employed during the extraction process involves both alkali and acid treatments. Functional properties of Gelatins viz., gel strength and melting point are dependent on pH. Choi and Regenstein (2000) observed that the gel strength of the fish and pork gelatins decreased markedly below pH 4 and slightly above pH 8. For the melting point also similar dependencies were observed in relation to pH. Crumper and Alexander (1954) observed that the rigidity of pork gelatin is maintained at pH range 4 – 10. Cole (2000) reported that for Type B gelatin, the viscosity is minimal and the gel strength is maximal at a pH of 5; hence from the manufacturers' point of view it is advantageous to manufacture gelatin at this pH. The pH reported for gelatin extracted from the skin of red and black tilapia was 3.05 and 3.91 respectively (Jamilah & Harvinder,2002).

4.2.4.3 Amino acid composition

The amino acid composition of the gelatins extracted from Rohu, Common carp and Grass carp skins are given in Table 4.2.8. All the samples had high content of imino acids (Proline +Hydroxyproline) in the range of 19.49 – 20.80 % of protein. Kasankala, *et. al.,* (2007) reported an imino acid

content of 19.47% for gelatin prepared from the skin of Grass carp. Piez and Gross (1996) reported Proline + Hydroxyproline content of 19.7% for carp fish. Imino acid content of Nile perch gelatin was ~ 21.5% irrespective of the source (Muyonga, *et. al.*,2004a). Grossman & Bergman (1992) reported~17% imino acid content for cod gelatin, and ~25% for Tilapia . Mammalian gelatins contain generally 30% imino acids (Poppe, 1992). High content of imino acids (Pro + Hyp) improves the rheological properties of gelatine as it is involved in formation of triple helical regions that immobilize water (Christopher,1993). Johnston-Banks, (1990) reported that the imino acids (proline and hydroxyproline) impart considerable rigidity to the collagen structure and that a relatively limited imino acid content should result in a less sterically hindered helix and may affect the dynamic properties of the gelatin. However, Gudmunsson & Hafsteinsson (1997) reported that the viscosity of the gel may be mainly due to the molecular weight distribution rather than the amino acid composition of the gelatin. Grass carp skin gelatin contained the lowest proline (9.2%) and highest hydroxyproline content (11.66%) among the three gelatins. Maximum gel strength was observed for Grass carp gelatin which shows that hydroxyproline is the major determinant of stability due to its hydrogen bonding ability through its hydroxyl group, although proline is also important (Burjandze, 1979; Ledward, 1986). Rohu, common crap and Grass carp gelatins contain approximately the same quantity imino acids as in the case of mammalian gelatins. These also had significant amounts of serine and threonine with free hydroxyl groups which can contribute to the gel strength by the formation of hydrogen bonds and helical structures.

The amino acid profile obtained was from an acid hydrolysate. A proportion of the acidic amino acids occur as the side chain amides of glutamine and asparagine in collagen (Ward & Courts, 1977) i.e., during acid hydrolysis of gelatin, some of the glutamine and asparagine will be converted to the acidic forms, i.e. glutamic acid and aspartic acid, respectively. Rohu, Common carp and Grass carp skin gelatins contain significant quantities of glutamic and aspartic acids which together constitute 13.19%, 13.08% and 14.85% respectively.

Glycine, the simplest amino acid, constitutes 21 -25% of the total amino acid residues in Rohu, Common carp and Grass carp skin gelatins. In mammalian gelatins glycine accounts for approximately one –third of the total amino acid residues. Glycine values of 29% and 32% were reported for gelatins from sin croaker and short fin scad respectively by Cheow,*et. al.*,(2007). The stability of the collagens and gelatins is also proportional to the glycine content, apart from total imino acid content (Lehninger, *et. al.,* 1993).

Significantly higher values for alanine was observed for Grass carp skin gelatin (8.3%) when compared to the other two samples under study. This amino acid, together with proline and hydroxyproline is found in the non-polar regions where sequences of the type Gly-Pro-Y predominate (Ledward, 1986). A higher content of this amino acid can be one of the reasons for higher viscoelastic properties of Grass carp skin gelatin than the other two gelatins. The same reason was attributed for the higher viscoelastic properties of commercial gelatin from Tilapia when compared to megrim gelatin (Sarabia, *et. al.,*2000).

The four amino acids viz., glycine, proline, hydroxyproline and alanine account for approximately two out of every three amino acid residues in mammalian collagen used in gelatin manufacturing .Fish collagens show a wider variation in composition. Their hydroxyproline and, to a lesser extent, proline contents are lower than that of mammalian collagens and this is compensated for by higher concentrations of serine and threonine (Balian & Bowes 1977). Glycine, proline, hydroxyproline and alanine account for 44-52% of the total amino acid residues in Rohu, Grass carp and Common carp skin gelatins and more than 60% of the total residues when combined with serine and threonine.

The amino acid composition of gelatins from the skin of Rohu, Common carp and Grass carp are similar to the gelatins derived from mammalian sources with respect to the imino acid and other important amino acids content. This could be the reason for the compatibility of these gels with mammalian gels in respect of physico-chemical properties.

Table 4.2.8 Amino Acid Composition of Gelatin from Carp Skin*

Amino acids	Amino acids g / 100g protein		
	Source of fish skin Gelatin		
	Rohu	Common carp	Grass carp
Aspartic acid	2.56 (0.27)	2.61 (0.25)	4.22 (0.30)
Threonine	4.41 (0.68)	4.19 (0.42)	2.09 (0.10)
Serine	4.69 (0.65)	4.34 (0.38)	2.77 (0.14)
Glutamic acid	10.63 (0.33)	10.47 (0.87)	10.63 (0.38)
Proline	11.59 (0.30)	11.72 (0.50)	9.20 (0.38)
Glycine	24.93 (1.60)	20.99 (0.94)	23.30 (1.34)
Alanine	1.16 (0.21)	3.54 (0.31)	8.30 (0.22)
Cysteine	ND	ND	ND
Valine	2.62 (0.82)	2.14 (0.19)	1.79 (0.14)
Methionine	2.43 (0.23)	3.94 (0.43)	1.28 (0.19)
Isoleucine	0.15 (0.04)	0.40 (0.05)	1.08 (0.12)
Leucine	3.21 (0.72)	1.40 (0.18)	1.89 (0.18)
Tyrosine	0.48 (0.13)	0.21 (0.03)	0.22 (0.04)
Phenylalanine	1.11 (0.15)	0.66 (0.06)	1.18(0.12)
Histidine	0.71 (0.38)	0.03 (0.01)	0.10 (0.03)
Lysine	2.83 (0.42)	4.20 (0.47)	2.16 (0.03)
Arginine	4.93 (0.36)	4.75 (0.20)	5.87 (0.13)
Hydroxyproline	7.90 (0.13)	7.78 (0.55)	11.66 (0.22)
Imino acids(Pro + Hyp)	19.49	19.50	20.86
Total	**86.34**	**83.37**	**87.74**

*Values in brackets are standard deviations of triplicate samples.

4.2.4.4. Molecular weight distribution

SDS PAGE Profile (Fig. 4.2.1) show the molecular weight distribution and protein patterns of fish skin gelatins from Common carp, Rohu, and Grass carp. The Common carp gelatin shows greater composition of α-chains with molecular weights in the range of 116 KDa to 97 KDa and sub α- units of molecular weight range of 24 to 66 kDa. The β-chains with molecular weights of 200 kDa are also present, but their bands are less intensive than the α-chain bands. The Grass carp skin gelatin has predominantly β-chains with molecular weights of 200 kDa and less

intensive α-chain bands with molecular weights in the range of 116 KDa to 97 KDa and sub α-units of molecular weights 55 to 66 kDa. The Rohu gelatin has predominantly α-chains with molecular weights in the range of 116 KDa to 97 KDa and a wide range of sub α-units of molecular weights 6.5 to 66 kDa. As in the case of Common carp skin gelatin, the β-chains with molecular weights of 200 kDa are also present, but their bands are less intense than the α-chain bands. The proportion of low molecular weight α-fraction peptides was higher in Common carp skin gelatin and Rohu skin gelatin than for Grass carp skin gelatin. Grass carp skin gelatin in this study had a higher proportion of the β-chain component than the other two gelatins. This indicates that collagen of Rohu and Common carp skins were more degraded during the extraction than Grass carp skin collagen. The extraction temperature plays a role in the degradation of the collagen as higher temperature extraction was found to produce more low molecular weight peptides (α-chains) and lower proportion of high molecular weight (β-chains) peptides (Muyonga, *et.al.,* 2004a). The extraction temperature for Grass carp skin was significantly lower than that of Common carp and Rohu skin, which may have caused a higher concentration of high molecular weight peptide fractions in Grass carp skin gelatin.

The formation of degradation fragments (low molecular weight α chains and sub α units) is associated with low viscosity, melting point, setting point and high setting time (Ledward, 1986; Normand, *et. al.*2000; Tavernier, 1989). Muyonga, *et. al.,* (2004a) reported a high positive correlation of bloom, viscosity and hardness to the β-chain peptides in Nile perch skin gelatin. According to Yau, *et.al,* (1979) the wide molecular weight distribution also negatively affects the functional properties of macromolecules like gelatin. The results in this study agrees to the observations above since Grass carp skin gelatin with higher concentration of β-chain peptides and lower amounts of low molecular weight α-chains and sub α-units showed better functional properties and high values for bloom, viscosity, melting point, setting point and a faster setting time than the other two fish skin gelatins.

The conversion of tropocollagen to gelatin involves the breaking of hydrogen bond, which stabilize the triple coil helix and transform it into the random coil configuration of gelatin. The hydrolyzed product depends on the cross links that remain between the peptide chains and reactive amino terminal and carboxyterminal groups that have been formed. Because the three chains are not identical, three basic types of new chains result after cleavage: the α-chain with one peptide chain, the β-chain with two peptide chains still connected and the gamma chain with three connected peptide chains: therefore a single gelatin sample has several molecular weights. The molecular weight distribution of gelatin determines its characteristics such as colloidal dispersion in water, viscosity, adhesiveness and gel strength (Ockerman & Hansen, 1999).

Eventhough the amino acid composition of the gelatin preparations were quite similar, differences among gelatin preparations arise from the type of chains formed during extraction. Gelatin molecules are subdivided into several molecular weight ranges corresponding to the most commonly occurring types. The more commonly found ranges correspond to the following categories:

(i) α-chains with molecular weight of 80 kDa to 125 kDa and sub α-units(Unit 1: 49 to 80 kDa, Unit 2: 35 to 49 kDa , Unit 3: 25 to 35kDa & Unit 4: 10 to 25 kDa).

(ii) β-chains with molecular weight of 125 kDa to 230 kDa.

(iii) Gamma chains with molecular weight of 230 kDa to 340 kDa.

It is apparent that the amount of these fractions, to some extent, reflected in the gel strength and viscosity. A higher content of gamma chains give a high setting gel while a sample rich in sub α units give a low setting gel. High molecular weight chains are the major determinants of viscosity and gel strength which correlates quite well with sum of α, β and high molecular weight components and is inversely proportional to the concentration of sub α particles (Philips & Williams, 2000).

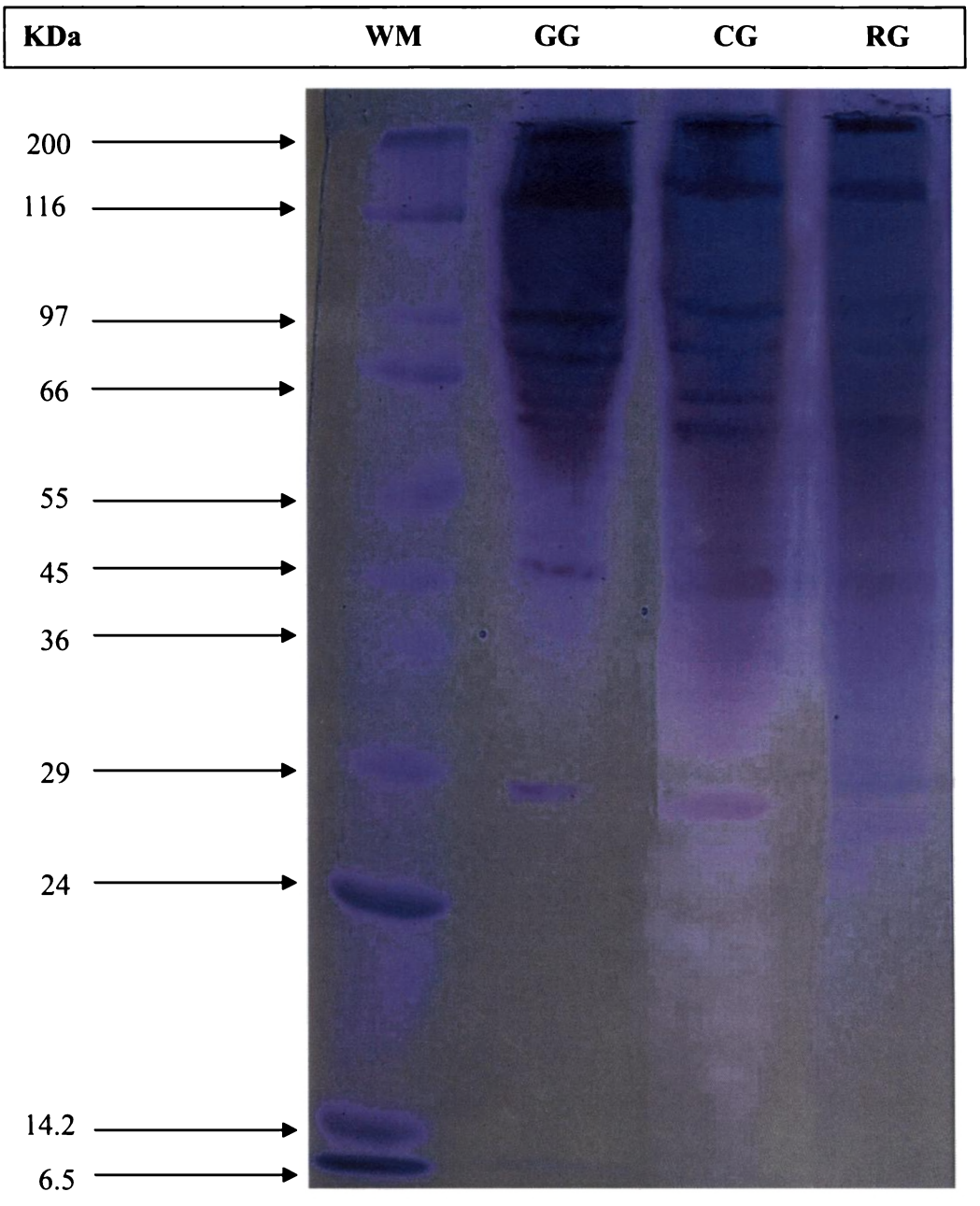


Fig. 4.2.1 Electrophoretic Profile of the Carp Skin Gelatins

WM –Wide Range Sigma Molecular marker, GG – Grass carp skin gelatin: CG – Common carp skin gelatin: RG - Rohu skin gelatin

4.2.4.5 FTIR Spectra of Carp Skin Gelatins

The frequencies at which major peaks occurred for acid soluble collagen and the different gelatins and collagens are summarized in Table 4.2.9 and the FTIR spectra is given as Figures 4.2.2 (a ,b & c). The spectra of carp skin gelatins are found to be dependent on the extraction temperature. Grass carp and Rohu gelatins with lower extraction temperatures at 40 and 49 ^{0} C respectively showed the low intensity amide A, I and II bands and the amide III band was not fully distinguished. These changes are indicative of greater disorder in gelatin and are associated with loss of triple helix state. This is consistent with changes expected as a result of denaturation of collagen to gelatin (Friess & Lee, 1996). The Common carp skin gelatin extracted at the higher temperatures, however, exhibited distinct amide III peaks. It seems therefore, that the extent of order in the high temperature-extracted gelatins may be higher than that in low temperature-extracted gelatins.

The Common carp skin gelatin extracted at higher temperature exhibited a much broader amide A than was observed for the low temperature extracted Rohu and Grass carp gelatins. The stable intermolecular crosslinks may not break during extraction of gelatin. Instead, solubilisation may be achieved by cleavage of peptide bonds. Hence, high temperature-extracted Common carp skin gelatin may contain a significant amount of intermolecular crosslinks. This can produce FTIR spectra showing a higher degree of molecular order. Paschalis *et.al.,* (2001) isolated stable crosslinks from bovine bone gelatin, supporting the assertion that intermolecular crosslinks may survive the process of gelatin extraction. Studies of FTIR spectra of skin and bone gelatins produced from Nile perch by sequential extraction process showed that the first gelatin extracts had diminished amide III bands while the last gelatin extracts showed distinct amide III bands and their amide I bands consisted of a higher percent area of a component around1690cm^{-1} (Muyonga *et.al.,*2004b). Aewsiri *et.al.,* (2008) reported that FTIR Spectra of both dorsal and ventral skin gelatin from cuttlefish displayed major bands at 3264 cm^{-1} (amide A), 1628 cm^{-1} (amide I) 1550 cm^{-1} (amide II) and 1240 cm^{-1} (amide III).

Fourier Transform Infrared (FTIR) Spectroscopy has been used to study changes in the secondary structure of gelatin. The spectral changes which are indicative of changes in collagen secondary structure have been shown to include changes in the amide A (Milch, 1964), amide I (1636–1661 cm^{-1}), amide II (1549–1558 cm^{-1}) (Renugopalakrishnan, *et. al.,* 1989) and the amide III (1200–1300 cm^{-1}) regions (Friess & Lee, 1996). Denaturation of collagen has been found to lead to reduction in the intensity of amide A, I, II and III peaks (Friess & Lee ,1996), narrowing of amide I band (Prystupa & Donald, 1996), increase in amide I component found around 1630 cm^{-1} and reduction in the intensity of amide I component, found around 1660 cm^{-1} (George & Veis, 1991; Payne & Veis, 1988; Renugopalakrishnan, *et. al.,* 1989). The amide I is the most useful peak for infrared analysis of the secondary 4 structure of protein including gelatin (Surewicz & Mantsch, 1988).

Table 4.2.9 FTIR Spectra Peak Position and Assignments for Carp Skin Gelatins

Region	Peak wave Number (cm^{-1})			Assignment	References
	Grass carp	Rohu	Common carp		
Amide A	-	-	3782.68	NH stretch, coupled with Hydrogen Bond	Sai and Babu (2001)
	3436.63	3419.00	3423.32		
	2880.84	2880.60	2917.14	CH_2 asymmetrical Stretch	Abe and Krimm (1972)
Amide I	-	1670.83	1688.42	C=O stretch/HB coupled with COO-	Jackson *et. al.*, (1995)
	1630.94	1626.18	-		
Amide II	1559.52	1557.46	1560.50	NH bend coupled with CN stretch	Jackson *et. al.*, (1995)
	1426.87	1445.64	1456.41	CH2 bend	Jackson *et. al.*, (1995)
	1343.93	1337.87	1331.67	CH2 wagging of proline	Jackson *et. al.*, (1995)
Amide III	1241.09	1238.79	1240.31	NH bend	Jackson *et. al.*, (1995)
	1168.22	1106.92		C-O stretch	Jackson *et. al.*, (1995)
	1115.79	-			
	-	-	1094.67	Skeletal stretch	Abe and Krimm (1972)
	-	-	1029.28		
	-	-	972.08		
	-	743.25	704.64		
	-	-	669.83		
	-	-	640.85		
	628.43	624.74			

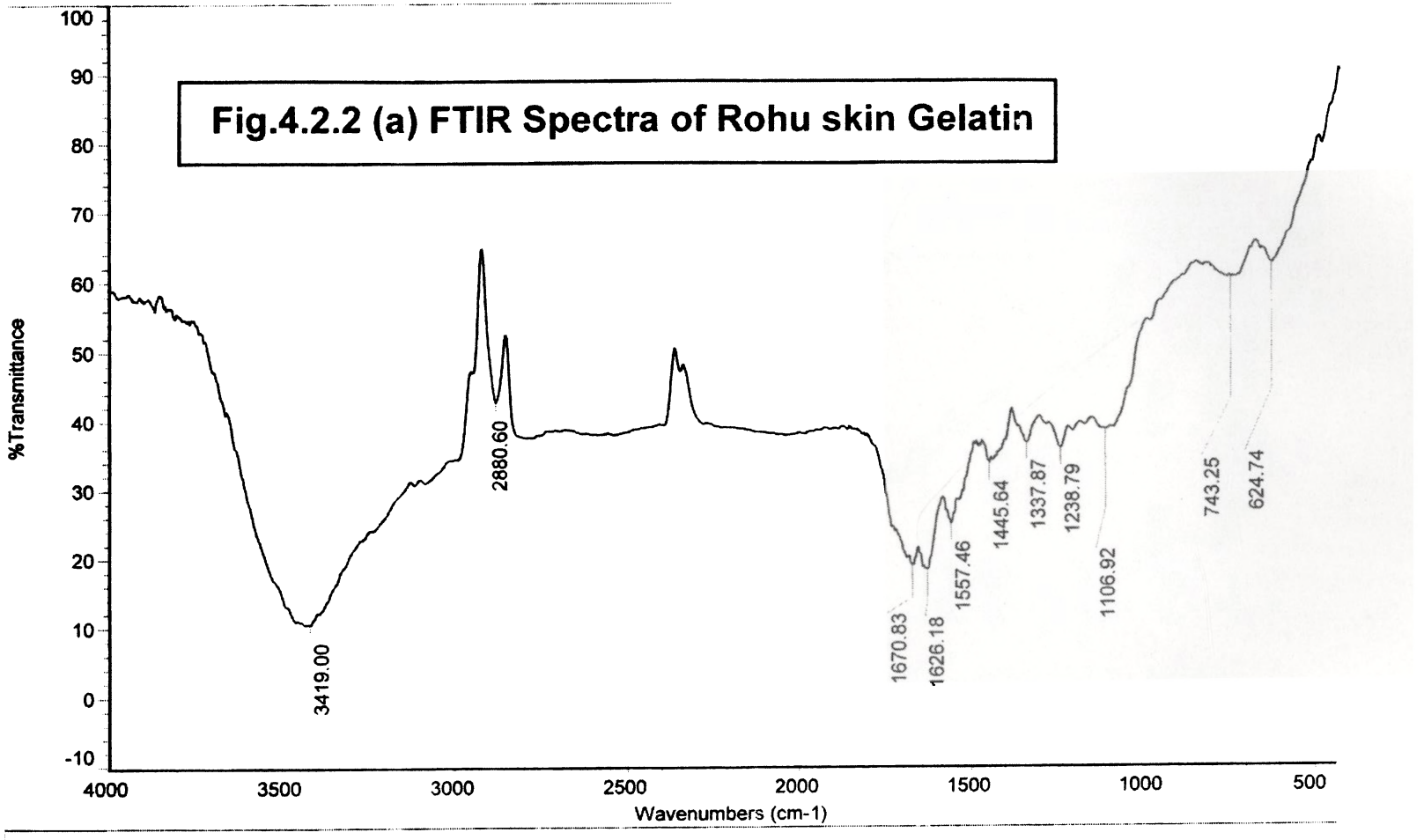


Fig.4.2.2 (a) FTIR Spectra of Rohu skin Gelatin

Date: Fri Jun 06 15:59:44 2008 (GMT-07:00)SAIFFT080602A-18(CIFT FP 18)

Scans: 32

Resolution: 4.000

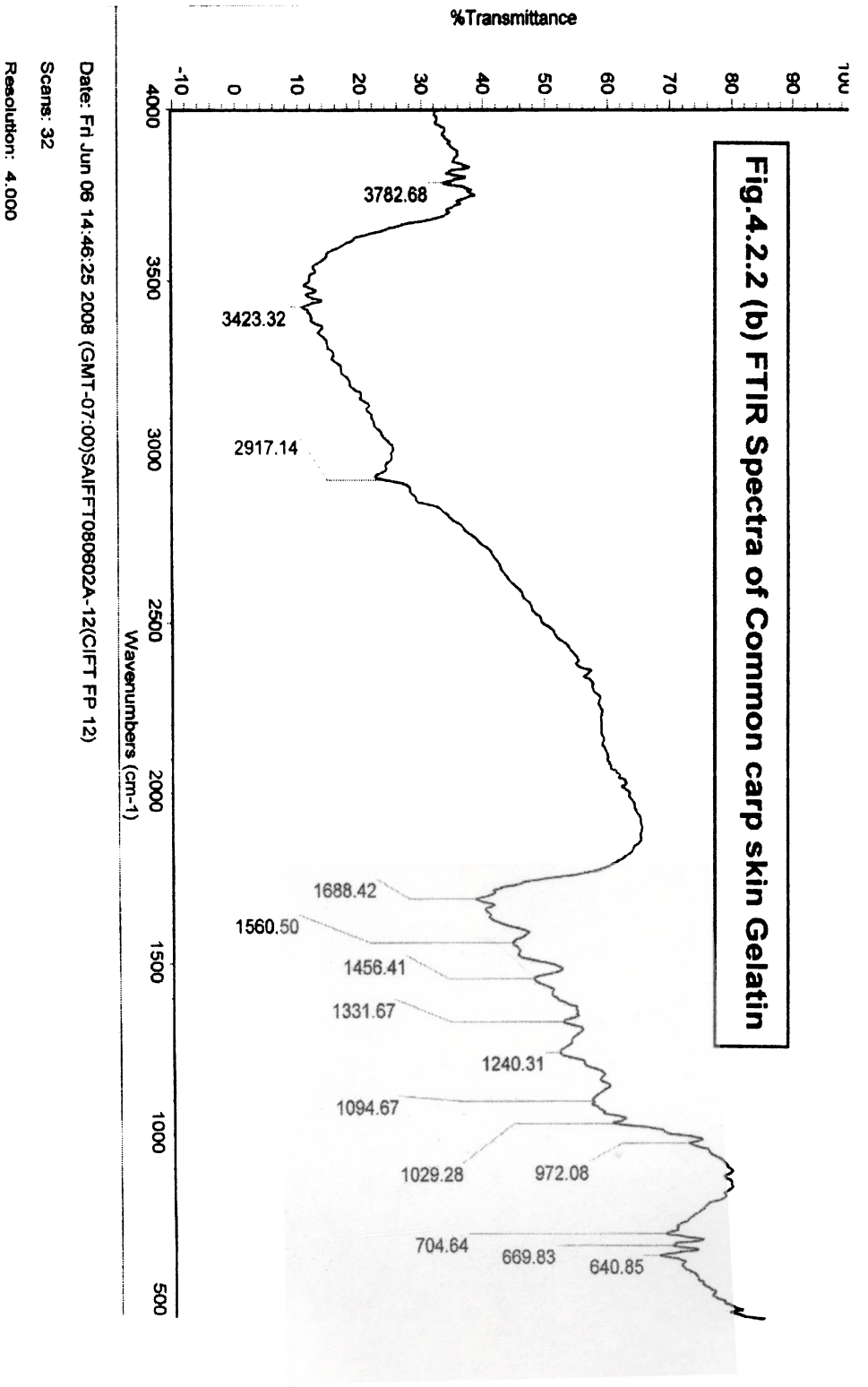


Fig.4.2.2 (b) FTIR Spectra of Common carp skin Gelatin

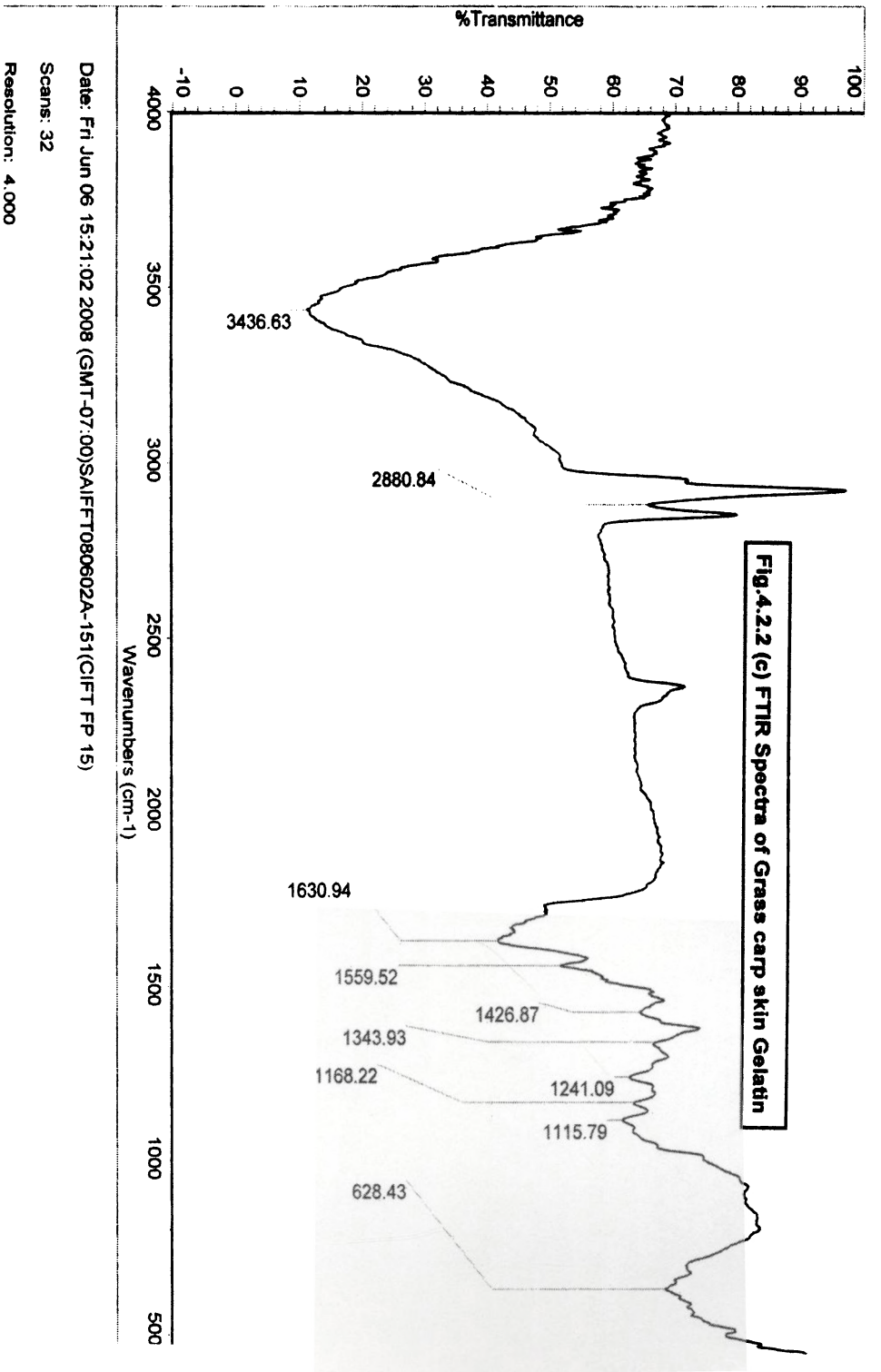


Fig.4.2.2 (c) FTIR Spectra of Grass carp skin Gelatin

4.2.5 Heavy Metal Content in the Carp Skin Gelatin

Determination of heavy metal content is a chemical quality requirement for edible gelatin (GME, 2005) with maximum allowable limits prescribed. Heavy metal contamination of gelatin can occur during the process of extraction from the process water. Fish skin gelatin may also likely to contain significant amounts of heavy metals if the subcutaneous fat is not properly removed from the raw material (fish skin) before the extraction since it is a depository of many heavy metals, particularly in fish caught from polluted waters. In this study the gelatin samples were analysed for arsenic, lead, copper, zinc, cadmium and chromium. Table 4.2.10 shows the quantum of heavy metal content in the carp skin gelatins with respect to the above elements. The heavy metal content in all the samples was negligible and well below the maximum allowable limits.

Table 4.2.10 Heavy Metal Content of Carp Skin Gelatin*

Heavy metals	Maximum Allowable Limit *	Source of fish skin Gelatin		
		Rohu	Common carp	Grass carp
Arsenic (ppm)	01	0.11	0.41	0.33
Lead (ppm)	05	0.88	0.67	0.91
Copper (ppm)	20	10.22	10.31	10.23
Zinc (ppm)	20	10.01	10.06	8. 34
Chromium (ppm)	10	1.34	1.87	1.90
Cadmium (ppm)	0.5	0.14	0.12	0.05

*Limits prescribed by European Commission Scientific Committee on Food SCF/CS/CNTM/MET/27-Opinion of the Scientific Committee on Food on specifications for gelatine in terms of consumer health (adopted on 27 February 2002).(http://europa.eu.)

4.2.6 Microbiological Quality of Carp Skin Gelatin

The results of the analysis for microbiological parameters are given in Table 4.2.11. The total plate counts were well below the allowable limit of 1000/g. Other bacterial groups were not detected in the samples. The gelatins from the skin of Rohu, Common carp and Grass carp are safe with respect to microbiological quality and conform to the standards of edible gelatin. Gelatin is extensively used in the microbiological media for solidifying the media. The ability of the bacteria to dissolve gelatin is used as a characteristic for the bacterial identification. Most countries have microbiological specifications for gelatin. Total mesophilic plate counts of 1000 are generally accepted with various countries limiting the presence of *Coliforms, E. coli, Salmonella, Clostridial spores, Staphylococci*, and sometimes even *Pseudomonas* (Cole, 2000).

Table 4.2.11 Bacteriological Quality of Fish Skin Gelatin

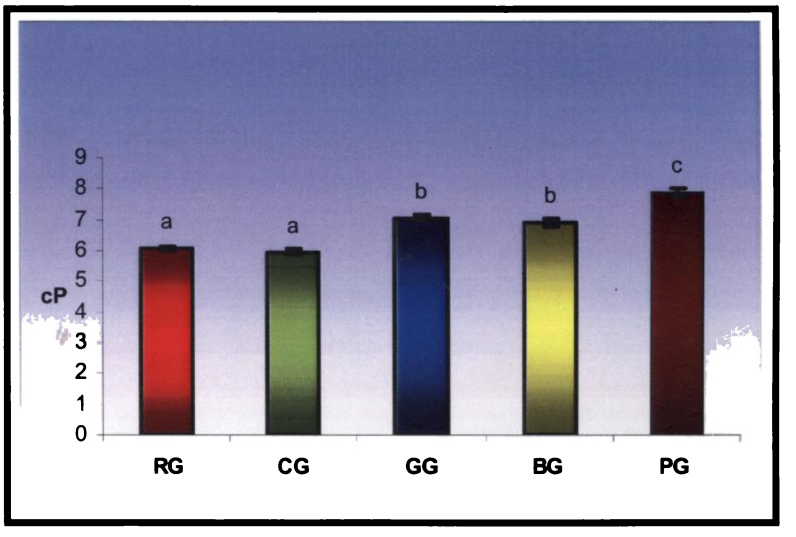
	Maximum Allowable Limit (GME,2005)	Source of fish skin Gelatin		
		Rohu	Common carp	Grass carp
Total aerobic count (30°C)	1.0×10^3/g	2.28×10^2 /g	2.52×10^2 /g	3.4×10^2 /g
Coliforms (30°C)	Negative/g	ND	ND	ND
Coliforms (44.5°C)	Negative/10g	ND	ND	ND
Sulphite-reducing anaerobic spores (37°C)	1.0×10^1 /g	ND	ND	ND
Clostridium perfringens	Negative/g	ND	ND	ND
Staphylococcus aureus	Negative/g	ND	ND	ND
Salmonella	Negative/25g	ND	ND	ND

4.3 Physical, Chemical and Functional Properties of Carp Skin Gelatin compared to Mammalian Gelatins.

4.3.1 Carp Skin Vs Mammalian Gelatin - Physical Properties

4.3.1.1. Viscosity

A comparison of the important physical properties of gelatins viz., viscosity, clarity, melting point, setting point, setting time and odour are given in Figures 4.3.1(a) to (f).Among the gelatin samples, maximum viscosity was noted for Porcine skin gelatin (7.89 cP) followed by Grass carp gelatin (7.07cP). Bovine skin gelatin and Grass carp skin gelatin had similar values for viscosity. Rohu skin gelatin and Common carp skin gelatin had significantly lower values of viscosity compared to the other three samples (Fig.4.3.1.a). Viscosity is partially controlled by molecular weight and molecular size distribution (Sperling, 1985). The viscosities of most of the commercial gelatins have been reported to be in the range of 2.0 to 7.0 cP for most gelatins and up to 13.0cP for specialized ones (Johnston-Banks, 1990).

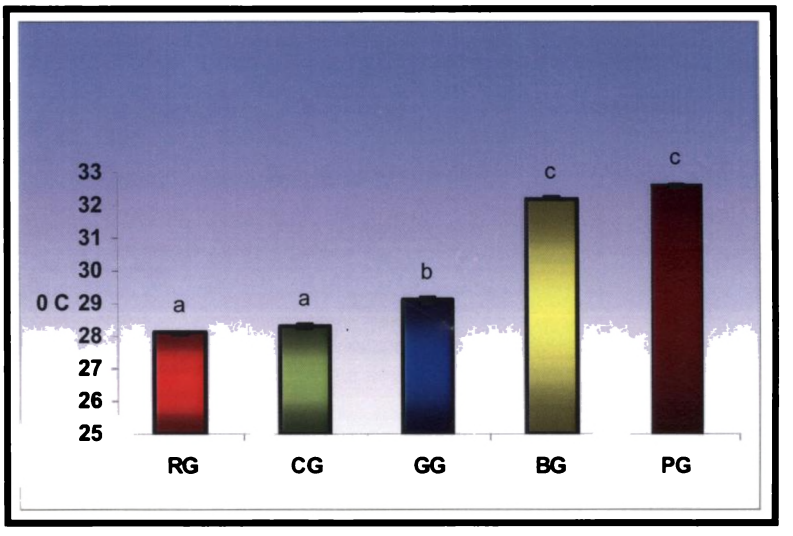


*PG = Porcine Skin Gelatin, Type A (300B); BG =Bovine Skin Gelatin, Type B (225B); RG = Rohu Skin Gelatin; CG = Common carp Skin Gelatin; GG = Grass carp Skin Gelatin

Fig. 4.3.1(a) Viscosity of Mammalian and Carp Skin Gelatin *

4.3.1.2. Melting point

The melting point of the gel samples are illustrated in Fig. 4.3.1(b). Mammalian gelatins showed significantly higher melting points (32.2 -32.6 ^{0}C) than carp skin gelatins. The melting temperature of gelatin has been found to correlate with the proportion of the imino acids proline and hydroxyproline (both with a 5-membered pyrrolidine ring) in the original collagen (Ledward,1986; Piez & Gross 1960; Veis,1964). Here it can be seen the imino acid content of Grass carp gelatin was maximum (20.80%) followed by Common carp (19.50%) and Rohu (19.49%) gelatins whereas the imino acid content of Bovine and Porcine skin gelatin is 22.9 and 23.7% respectively (Table 4.3.1). The melting point values correspond to the imino acid content in the samples. Gomez-Guillen *et. al.,* (2002) correlated the thermal stability of gelatin to the number and stability of Proline rich region in collagen or gelatin molecules, which are high in fresh warm water fish and mammalian species.

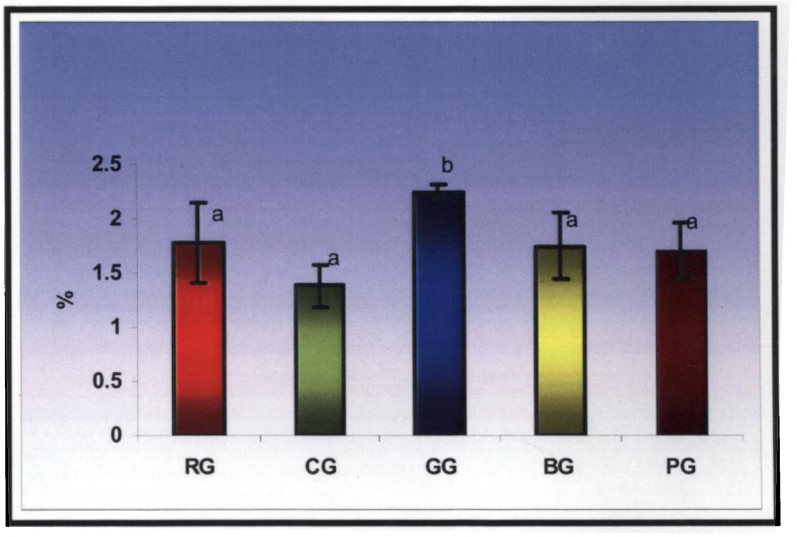


*PG = Porcine Skin Gelatin, Type A (300B); BG =Bovine Skin Gelatin, Type B (225B); RG = Rohu Skin Gelatin; CG = Common carp Skin Gelatin; GG = Grass carp Skin Gelatin

Fig. 4.3.1(b) Melting Point of Mammalian and Carp Skin Gelatin*

4.3.1.3. Clarity

Carp skin gelatins, with the exception of Common carp skin gelatin had better clarity than mammalian gelatins (Fig. 4.3.1c). Clarity was lowest for Common carp skin gelatin, which could be due to the higher extraction temperature employed. High temperature extraction can result in higher molecular weight aggregates which will increase the turbidity of the gel and affect the clarity (Montero *et. al.,* 2002). Clarity is important in commercial applications and this property is frequently assessed for determining the quality of gels.



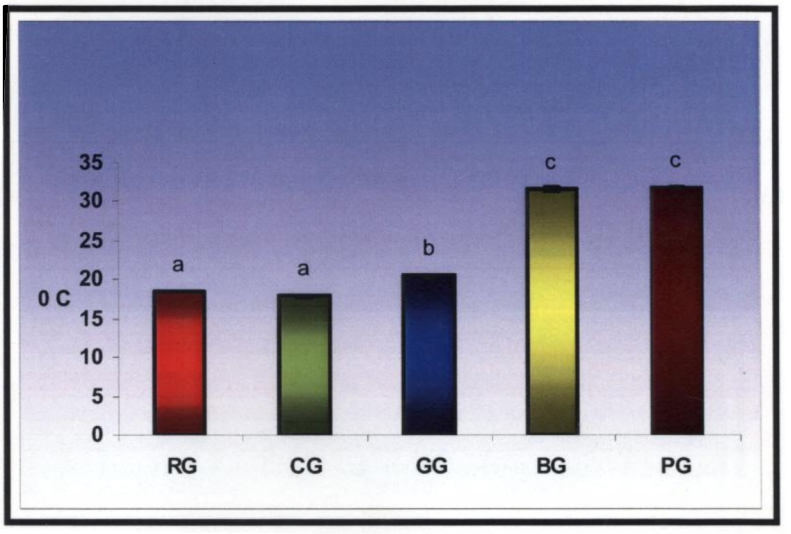
*PG = Porcine Skin Gelatin, Type A (300B); BG =Bovine Skin Gelatin, Type B (225B); RG = Rohu Skin Gelatin; CG = Common carp Skin Gelatin; GG = Grass carp Skin Gelatin.

Fig. 4.3.1(c) Clarity of Mammalian and Carp Skin Gelatin*

4.3.1.4. Setting Point and Setting Time

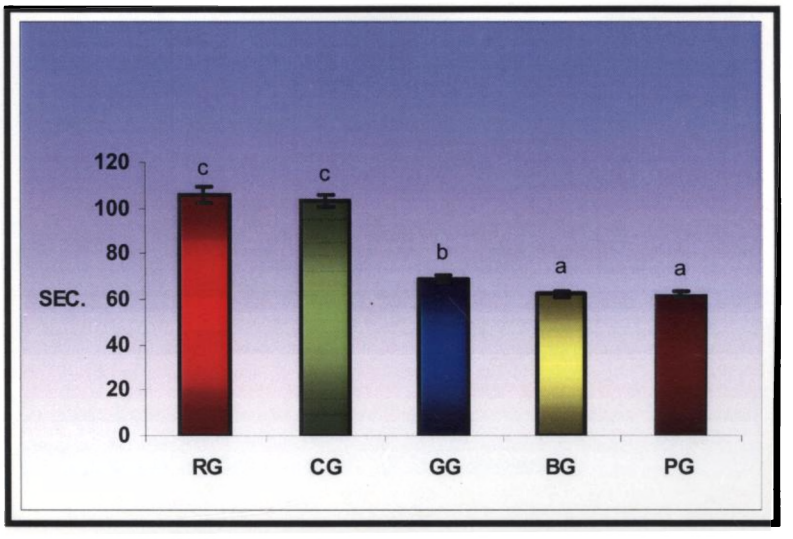
Setting point and setting time of the gels are given in Figures 4.3.1(d) & 4.3.1 (e).Mammalian gels have significantly higher setting temperatures (31.6 – 31.8 ^{0}C) than carp skin gelatins. Also, the gel setting time was significantly faster for mammalian gels. Grass carp skin gel had a setting time of 68.6 seconds which is comparable to mammalian gels. Bovine and porcine gelatins have considerably higher gelling and melting points than most fish gelatins, and the high gelling and melting points expand the range of gelatin application. Setting temperature of gelatin has also been found to correlate with the imino acid content which is typically ~24% for mammals and 16–18%

for most fish species (Choi & Regenstein, 2000; Gilsenan & Ross-Murphy, 2000a; Gudmundsson, 2002; Leuenberger, 1991)..



*PG = Porcine Skin Gelatin, Type A (300B); BG =Bovine Skin Gelatin, Type B (225B); RG = Rohu Skin Gelatin; CG = Common carp Skin Gelatin; GG = Grass carp Skin Gelatin.

Fig. 4.3.1(d) Setting Point of Mammalian and Carp Skin Gelatin*



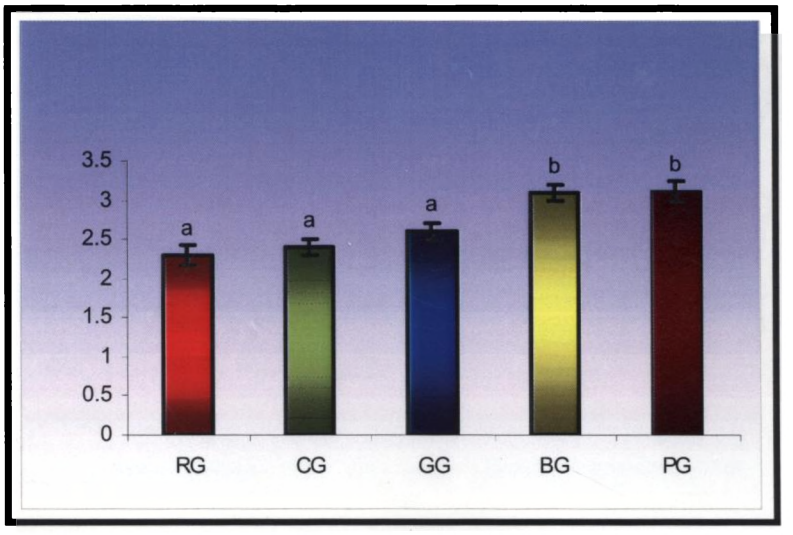
*PG = Porcine Skin Gelatin, Type A (300B); BG =Bovine Skin Gelatin, Type B (225B); RG = Rohu Skin Gelatin; CG = Common carp Skin Gelatin; GG = Grass carp Skin Gelatin.

Fig. 4.3.1(e) Setting time of Mammalian and Carp Skin Gelatin*

4.3.1.5. Sensory Evaluation

Odour score of the gels as is given in Fig.4.3.1 (f). The odour scores were significantly higher for bovine and porcine skin gelatins (3.1 – 3.12) than carp skin gelatins, indicating that they had a distinguishable odour and hence

can be considered as inferior to fish skin gelatins in organoleptic qualities. Choi & Regenstein (2000) observed that fish gelatins had less off odour and better aroma than pork gelatins on sensory evaluation. They noted that flavored fish gelatin dessert gel product had less undesirable off-flavors and off-odors, with more desirable release of flavor and aroma than the same product produced with pork gelatin possessing equal Bloom values, but a higher melting point.



*PG = Porcine Skin Gelatin, Type A (300B); BG =Bovine Skin Gelatin, Type B (225B); RG = Rohu Skin Gelatin; CG = Common carp Skin Gelatin; GG = Grass carp Skin Gelatin.

Fig. 4.3.1(f) Odour Score of Mammalian and Carp Skin Gelatin*

4.3.2 Carp Skin Vs Mammalian Gelatin – Amino Acid Composition

The amino acid composition of the carp skin and mammalian gelatins is given in Table 4.3.1. The main difference in the amino acid profile is the lower content of imino acid in carp skin gelatins than mammalian skin gelatins. The imino acid content in carp skin gelatin ranges from 19.49 -20.86% whereas for mammalian gelatin it is 22.91 – 23.7%. Overall, fish gelatins have lower concentrations of imino acids (proline and hydroxyproline) compared to mammalian gelatins, and warm-water fish gelatins (such as bigeye-tuna and tilapia) have a higher imino acid content than cold-water fish (such as cod, whiting and halibut) gelatins (Eastoe & Leach, 1977). The proline and hydroxyproline contents are approximately 30% for mammalian gelatins, 22–25% for warm-water fish gelatins (tilapia and Nile perch), and 17% for cold-water fish gelatin (cod) (Muyonga *et. al.,* 2004a). Fish gelatin gave gels with a considerably lower storage modulus, gelling (4–5 ^{0}C) and melting temperature (12–13 ^{0}C) compared to mammalian gelatin gels which could probably due to the lower content of proline and hydroxyproline in fish gelatin (Haug *et. al.*, 2004). Four amino acids viz., glycine, proline, hydroxyproline and alanine account for two out of every three amino acid residues in mammalian gelatins (Balian & Bowes 1977). In this study these four amino acids accounted for 63.51% and 62.59% of the total amino acid residues in Bovine and Porcine skin gelatins respectively. For carp skin gelatins the corresponding values were less than 50%, except in the case of Grass carp skin gelatin which had 52.46% of the above mentioned amino acids. The stability of the collagens and gelatins is also proportional to the glycine content, apart from total imino acid content (Lehninger, *et. al.,* 1993). In this study the glycine content in carp skin gelatins was in the range of 20.99 – 24.93%. Higher concentrations of serine and threonine are reported for fish skin gelatins than mammalian skin gelatins (Balian & Bowes 1977). In this study these two amino acids accounted for 9.1 % and 8.53% in Rohu and Common carp skin gelatins. The quantity of serine and threonine was lower (4.86 – 5.07%) in mammalian and Grass carp gelatins.

Table 4.3.1 Amino Acid Profile of Mammalian and Carp Skin Gelatin*

Amino acids	Amino acids g / 100g protein				
	RG	CG	GG	BG	PG
Aspartic acid	2.56	2.61	4.22	2.5	3.01
Threonine	4.41	4.19	2.09	2.11	2.06
Serine	4.69	4.34	2.77	2.95	3.01
Glutamic acid	10.63	10.47	10.63	7.23	10.32
Proline	11.59	11.72	9.20	11.89	12.44
Glycine	24.93	20.99	23.30	29.20	27.69
Alanine	1.16	3.54	8.30	11.4	11.2
Cysteine	ND	ND	ND	ND	ND
Valine	2.62	2.14	1.79	1.80	1.88
Methionine	2.43	3.94	1.28	1.01	1.43
Isoleucine	0.15	0.40)	1.08	1.11	0.98
Leucine	3.21	1.40	1.89	1.90	1.73
Tyrosine	0.48	0.21	0.22	0.11	0.08
Phenylalanine	1.11	0.66	1.18	1.60	1.20
Histidine	0.71	0.03	0.10	0.08	0.03
Lysine	2.83	4.20	2.16	4.01	3.29
Arginine	4.93	4.75	5.87	5.1	4.90
Hydroxyproline	7.90	7.78	11.66	11.02	11.26
Imino acids (Pro +Hyp)	19.49	19.50	20.86	22.91	23.70
Total	**86.34**	**83.37**	**87.74**	**95.01**	**96.51**

*PG = Porcine Skin Gelatin, Type A (300B); BG =Bovine Skin Gelatin, Type B (225B); RG = Rohu Skin Gelatin; CG = Common carp Skin Gelatin; GG = Grass carp Skin Gelatin.

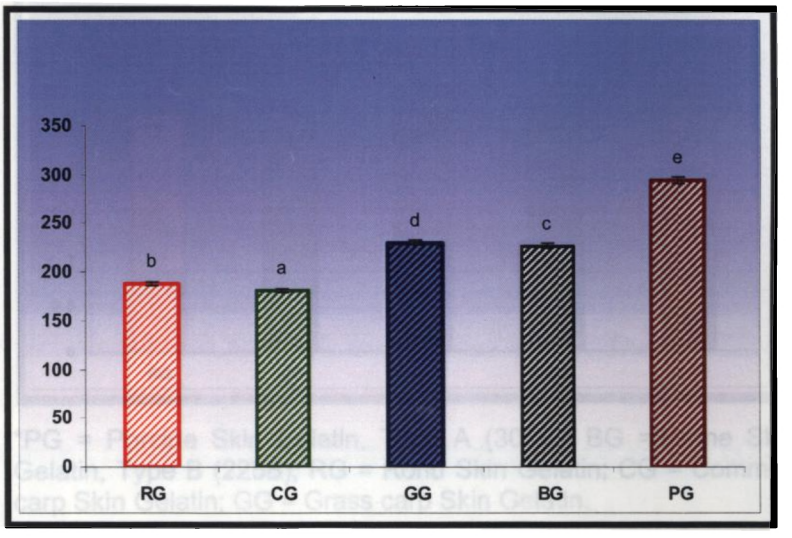
Lysine stabilizes gelatin structure by forming cross links between intermolecular chains (Cho *et. al.,* 2004). The percentage of lysine was 2.16 %, 2.83 % and 4.20 % for Grass carp, Rohu and Common carp skin gelatins respectively. The corresponding values for Bovine and Porcine skin gelatins were 4.01 % and 3.29%. The different ratios of amino acids in carp skin gelatin can be one of the reasons for the difference in functional properties of the former from mammalian gelatins. In this study, Grass carp skin gelatin was found to have an amino acid profile which showed similarity to that of the mammalian gelatin profile, particularly with respect to the content of hydroxyproline and glycine residues. This could be the reason for the similarities in functional properties of the former to that of mammalian gels.

4.3.3 Carp Skin Vs Mammalian Gelatin – Functional Properties

4.3.3.1. Gel Strength

The gel strength of carp skin and mammalian gelatins expressed as Bloom is given in Fig.4.3.2 (a). Grass carp gelatin had gel strength of 230.2 B which is comparable to the reported value for Bovine skin gelatin (227.2 B). The bloom values of Rohu and Common carp skin gelatins were 188.6 B and 181.3 B respectively which was significantly lower than mammalian gelatins. Fish gelatin typically has a Bloom value ranging from as low as zero to 270 B, compared to the high Bloom values for bovine or porcine gelatin, which have Bloom values of 200–240 B. However, a Bloom value as high as 426 B has been reported for yellowfin tuna skin (Cho, *et. al.,* 2005). Some species of warm water fish gelatins have been reported to exhibit relatively high Bloom values, close to that of high Bloom pork gelatin (Gudmundsson & Hafsteinsson, 1997). Such high gel strength characterizes only those gelatins extracted from the skins of warm-water fish such as tilapia (Grossman & Bergman, 1992; Jamilah, & Harvinder, 2002; Zhou, *et. al.,* 2006) and Grass carp (Kasankala *et. al.,* 2007). For example, Bloom values ranging from 128 B to 273 B have been reported for tilapia gelatin (Jamilah, & Harvinder, 2002; Zhou, *et.* al., 2006). On the other hand, cold-water fish gelatin solutions may remain in a liquid state under the conditions of the standard Bloom test at 10^0C (Norland, 1990). Typical Bloom values ranging from 70 to 110 B have been reported for cod, Alaska pollock, salmon, and hake .The wide range of

Bloom values found for the various gelatins arises from differences in proline and hydroxyproline content in collagens of different species, and is also associated with the temperature of the habitat of the animals.

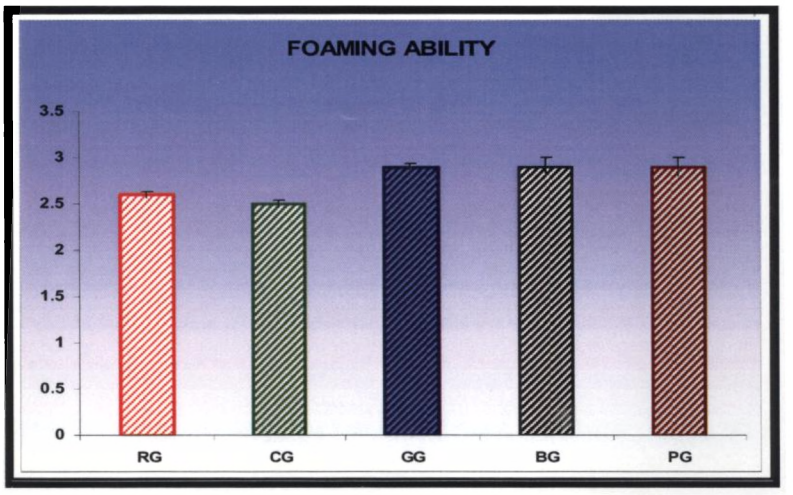


*PG = Porcine Skin Gelatin, Type A (300B); BG =Bovine Skin Gelatin, Type B (225B); RG = Rohu Skin Gelatin; CG = Common carp Skin Gelatin; GG = Grass carp Skin Gelatin.

Fig. 4.3.2(a) Bloom Value of Mammalian and Carp Skin Gelatin*

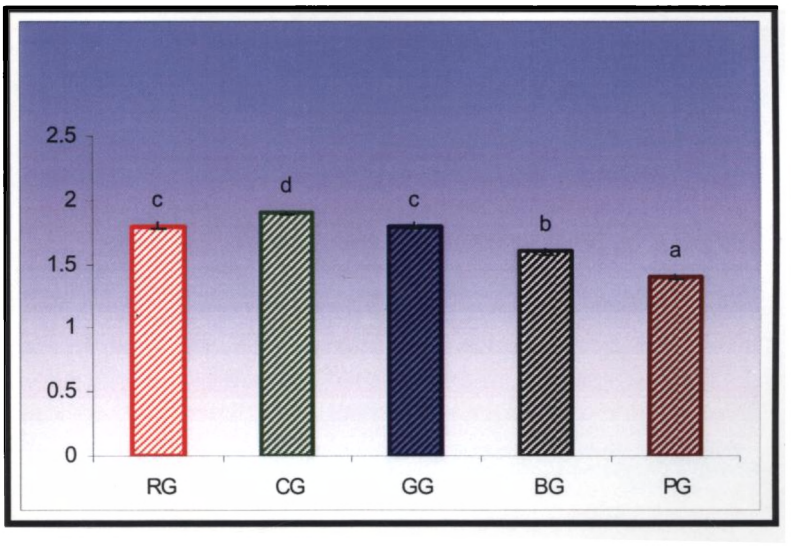
4.3.3.2. Foaming Ability and Foam Stability

The foaming ability and foam stability of carp skin and mammalian gelatins are given in Figures 4.3.2(b) & 4.3.2 (c). Grass carp skin and mammalian gelatins exhibited better foam formation abilities (2.9) than Rohu and Common carp skin gelatin. The hydrophobic areas on the peptide chain are responsible for giving gelatin its emulsifying and foaming properties (Cole, 2000; Galazka, *et.al.*, 1999). Foam stability was significantly higher for mammalian gelatins (1.4 – 1.6) than for carp skin gelatins (1.8 – 1.9). The reduced foam formation and stability may be due to aggregation of proteins which interfere with interactions between the protein and water needed for foam formation (Kinsella, 1977).



*PG = Porcine Skin Gelatin, Type A (300B); BG =Bovine Skin Gelatin, Type B (225B); RG = Rohu Skin Gelatin; CG = Common carp Skin Gelatin; GG = Grass carp Skin Gelatin.

Fig. 4.3.2(b) Foaming Ability of Mammalian and Carp Skin Gelatin*

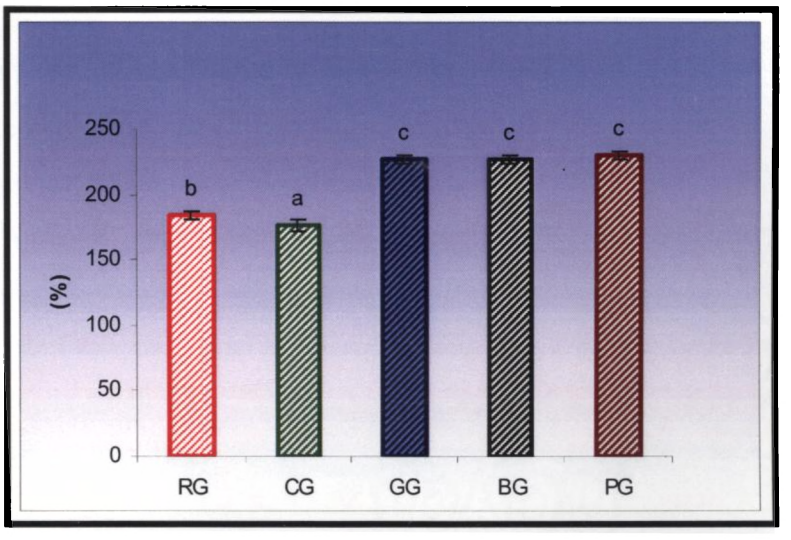


*PG = Porcine Skin Gelatin, Type A (300B); BG =Bovine Skin Gelatin, Type B (225B); RG = Rohu Skin Gelatin; CG = Common carp Skin Gelatin; GG = Grass carp Skin Gelatin.

Fig. 4.3.2(c) Foam Stability of Mammalian and Carp Skin Gelatin*

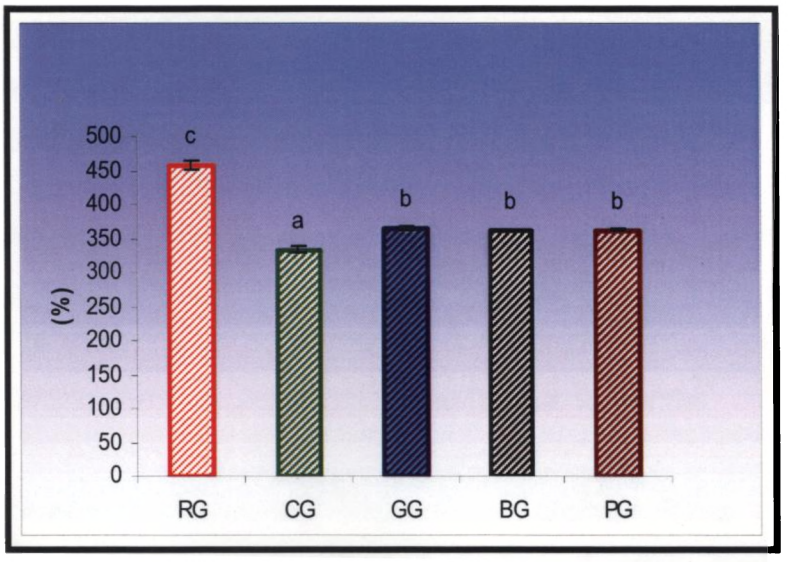
4.3.3.3 Water holding capacity and Fat binding capacity

Water holding and Fat binding capacities of carp skin and mammalian gelatins are given in Figures 4.3.2(d) & 4.3.2 (e). Rohu skin gelatin had the maximum fat binding capacity (457.3%) and Common carp skin gelatin had the minimum water-holding capacity (176%). No significant differences were observed in the water holding and fat binding capacities of Grass carp and mammalian skin gelatins. Water-holding and fat-binding capacities are functional properties that are closely related to texture by the interaction between components such as water, oil and other components. Fat binding capacity depends on the degree of exposure of the hydrophobic residues inside gelatin .Rohu skin gelatin had the highest percentage of hydrophobic residue tyrosine (Table 4.3.1) among the five gelatins which could explain its higher capacity for fat binding. Water-holding capacity is affected by the amount of hydrophilic amino acids like hydroxyproline. In this study highest water holding capacity (227 – 230%) was observed for Grass carp skin and mammalian gelatins since these had significantly higher percentage of hydroxyproline (Table 4.3.1).



*PG = Porcine Skin Gelatin, Type A (300B); BG =Bovine Skin Gelatin, Type B (225B); RG = Rohu Skin Gelatin; CG = Common carp Skin Gelatin; GG = Grass carp Skin Gelatin.

Fig. 4.3.2(d) Water Holding Capacity of Mammalian and Carp Skin Gelatin*



*PG = Porcine Skin Gelatin, Type A (300B); BG =Bovine Skin Gelatin, Type B (225B); RG = Rohu Skin Gelatin; CG = Common carp Skin Gelatin; GG = Grass carp Skin Gelatin.

Fig. 4.3.2(e) Fat binding Capacity of Mammalian and Carp Skin Gelatin*

Although porcine gelatin accounts for the highest levels of production, a significant amount of gelatin used in the food and pharmaceutical industries is also derived from cows. The BSE episode, as well as religious concerns, has led to intensive research, especially in Europe, to identify and develop alternatives to mammal-derived gelatin. Furthermore, strong competition exists among manufacturers for the procurement of pigskin or other mammalian sources, which has created increased demand and raised costs. To date, however, few alternatives are available, and as a result it has not been possible to eliminate gelatin (Karim & Bhat, 2009).

Within the past decade there has been intense interest in the market in gelatin derived from fish and poultry. Poultry skin and bones are expected to yield gelatin in the near future, but commercial production is currently limited by low yields. The skin obtained from poultry is also a coveted raw material for other food applications (Schrieber & Gareis, 2007). In this regard, fish gelatin has been highlighted as a better alternative to mammalian gelatins, particularly with qualities such as a lower melting point, resulting in faster dissolution in the mouth with no residual 'chewy' mouth feel. However, the production of fish gelatin is still in its infancy, contributing only about 1% of the annual world gelatin production (Arnesen & Gildberg, 2006).

4.4 Comparison of Physical, Mechanical and Barrier Properties of Edible Films Prepared from Rohu, Common carp and Grass carp gelatin with Mammalian Skin Gelatin Films.

Edible films were prepared from gelatins of Rohu, Common carp and Grass carp skins, Bovine skin gelatin of 225 bloom and Pork skin gelatin of 300 bloom to study the physical and barrier properties (Section 3.2.5). All the films were transparent, homogeneous and flexible to handle.

4.4.1 Physical and Mechanical Properties of Gelatin Films

The physical and mechanical properties of edible films are given in Table 4.4 .1. The average thickness of the films was in the range of 0.11-0.13mm. No significant deviations were observed between the films made from different sources. The thickness reported for wheat-gluten based edible films was in the range of 0.119 – 0.128mm (Gennadios, *et. al.,* 1993). Jongjareonrak, *et. al.,* (2006a) has reported a film thickness of 23.3 – 37.18 μm for edible films prepared from the skin gelatin of big eye snapper and brown stripe red snapper. In this study no significant difference was observed between film thicknesses among the different gelatins. This is important for the study of mechanical and barrier properties of the films since a comparison of these properties between the films will only be meaningful if these have similar thicknesses.

Tensile strength (TS) of different types of edible gelatin film are given in Table 4.4.1. Tensile strength is defined as the stress at which a material breaks or permanently deforms. Tensile strength was lowest for films from Common carp and Rohu skin gelatin (490 & 497 Kg/cm ^{2} respectively) and highest for film from Pork skin gelatin. Grass carp skin gelatin film showed higher tensile strength than film from Bovine hide gelatin. Significant differences ($p< 0.05$) were observed in the Tensile strengths of edible films made from Grass carp skin gelatin, Bovine hide gelatin and Pork skin gelatin. It is seen that the higher the gel strength of the gelatin, the better will be the tensile strength since among the five types of films, those made from Pork skin gelatin with bloom of 300 showed the maximum tensile strength.

Plasticizers can weaken the intermolecular forces between the chains of adjacent macromolecules, increasing the free volume and causing a reduction of glass transition temperature of the system (Jastrzebski, 1987). This causes reduction in puncture resistance and tensile strength of the film. Glycerol is the plasticizer used in the present study for the preparation of the films. Glycerol has relatively small molecule with hydrophilic characteristic which could be easily inserted between protein chains and establish hydrogen bonds with amide group and amino acid side chains of proteins (Gontard, *et.al.,*1993). When glycerol was incorporated in the gelatin film network, direct interactions and the proximity between protein chains were reduced. This can reduce the tensile strength at higher concentrations of glycerol as a plasticizer. In this study the glycerol concentration used for the preparation of films was at the rate of 20g/100g protein which can be considered as the optimum concentration with respect to mechanical properties of films. Similar observation was made by Sobral, *et.al,* (2002) while studying Nile tilapia protein films. The reduction of puncture force of myofibrillar protein film from Atlantic sardine was observed with the addition of glycerol as a plasticizer at concentrations between 0 and 40% of protein (Cuq, *et. al.,* 1997). Sobral, *et. al.,* (2001) also reported that puncture force of Bovine hide and Pig skin gelatin films decreased with the addition of sorbitol as a plasticizer at higher concentrations (45 and 55 g sorbitol / 100 g gelatin).

Elongation at Break (EAB) values varied significantly among the five films. EAB values exhibited the opposite trend to the TS of the film samples ie., the films with the highest TS had the lowest EAB and vice- versa (Table 4.4.1). EAB is the elongation recorded at the point of rupture of specimen, often expressed as a percentage of the original length. It corresponds to breaking or maximum load. Higher tensile strength indicates better mechanical strength. Similar observations were reported by Muyonga *et. al.,* (2004a) for films made from Nile perch skin and bone gelatin, commercial fish gelatin and bovine bone gelatin. Higher concentrations of plasticizers increase the moisture content of the film and can also contribute to the reduction of forces between the adjacent macromolecules which results in lower mechanical strength of the films (Sobral, *et.al.,* 1999). Glycerol as a plasticizer can form hydrogen bonds with proteins when introduced into the gelatin film

network. This will reduce the interaction between the proteins and increase the flexibility of the films which can result in higher EAB values. Many workers have reported decrease in TS and increase in EAB due to the increase in glycerol content in gelatin films (Lim, *et. al.*1999; Zhang, *et. al.,* 2007). In this study the same glycerol concentration was used for film preparation, hence the differences in TS and EAB could be possibly due to differences in gelatin from different sources, particularly in terms of amino acids and protein chain size (Muyonga, *et. al.,* 2004a; Paschoalik, *et. al.,* 2003).

Among the five types of films studied, the mechanical properties of Grass carp skin gelatin based film was superior to Rohu and Common carp skin gelatin films and comparable to films made from commercial bovine hide and pork skin gelatin.

Table 4. 4 .1 Physical & mechanical properties of gelatin based Edible Films *

	Thickness (mm)	Tensile Strength (TB) (kg/cm^2)	Elongation at Break (EAB) (%)
RG	0.11 ± 0.01	497 ± 7.31 [a]	57.16 ±1.24 [a]
CG	0.10 ± 0.01	490 ± 4.90 [a]	60.89 ±1.56 [b]
GG	0.12 ± 0.01	560 ± 6.66 [b]	27.00 ±0.72 [c]
BG	0.13 ± 0.02	527 ± 8.64 [c]	30.83 ±0.45 [d]
PG	0.11 ± 0.01	645 ± 6.15 [d]	24.98 ±0.18 [e]

*BG =Bovine Skin Gelatin (225B); PG = Porcine Skin Gelatin (300B); RG = Rohu Skin Gelatin; CG = Common carp Skin Gelatin; GG = Grass carp Skin Gelatin. All values were mean ± standard deviation of triplicate analyses. Different superscripts in the same column indicate significant differences ($P < 0.05$).

The colour of the films is given as L* a* b* values in Table 4.4.2. All the films_were transparent. The degree of transparency (L*) was significantly higher for films from Grass carp, bovine hide and pork skin gelatin. The a * values for the three above mentioned films showed negative values indicating a shift of colour towards green. The b* values were positive for all the films indicating the degree of yellowness. The yellowness was maximum for Common carp gelatin film which also showed the lowest L*value. This film was the least transparent sample among the five films. The colour of the films primarily depends on the colour of the gelatin which in turn depends on the

pretreatment method employed. Zhang, *et. al.,* (2007) reported that pretreatment with acidic solution with low pH prior to main extraction will yield a transparent gelatin whereas pretreatments at basic solutions with high pH prior to the main extraction resulted in dark coloured gelatin. In this study, gelatin extraction was carried by acid pretreatment at a pH level of around 4 prior to main extraction which confirms the above observation.

Table. 4. 4 .2 Colour of gelatin based Edible Films *

	L *	a*	b*
RG	94.44 ± 0.07 [a]	0.53 ± 0.01 [a]	3.27 ± 0.03 [a]
CG	93.49 ± 0.16 [b]	0.79 ± 0.03 [b]	4.20 ± 0.02 [b]
GG	97.79 ±0.16 [c]	-0.33 ±0.03 [c]	2.92 ± 0.05 [c]
BG	98.13 ± 0.05 [c]	-0.09 ± 0.02 [d]	2.74 ± 0.07 [d]
PG	98.10 ± 0.07 [c]	-0.11 ± 0.03 [d]	2.07 ± 0.05 [e]

*BG =Bovine Skin Gelatin (225B); PG = Porcine Skin Gelatin (300B); RG = Rohu Skin Gelatin; CG = Common carp Skin Gelatin; GG = Grass carp Skin Gelatin.All values were mean ± standard deviation of triplicate analyses. Different superscripts in the same column indicate significant differences ($P < 0.05$).

4.4.2 Barrier Properties of Gelatin Films

At the same level of plasticizer used, films produced from fish skin gelatin had significantly lower Water Vapour Permeability (WVP) than mammalian skin gelatin films (Table 4.4.3). Water Vapour Permeability is the rate of water vapour transmission per unit area per unit vapour pressure differential under test conditions. The low water vapour permeability of fish skin gelatin films is related to their high hydrophobicity due to the reduced amount of proline and hydroxyproline compared to the mammalian gelatins. Bustillos, *et. al.,* (2006) has reported that gelatin films prepared from coldwater fish skin gelatin had the lowest water vapour permeability followed by warm water fish skin gelatin films and mammalian gelatin films.

Increase in WVP with the increase in plasticizer concentration in edible films was reported by several workers (Buttler, *et. al.,* 1996; Cuq, *et. al.,* 1997; McHugh, *et. al.,* 1993; Aravnitoynnis, *et. al.,* 1998). The protein network becomes less dense and more permeable with the addition of plasticizer. Also, the hygroscopic character of the plasticizers increase the water content of the film and mobility of the molecules which result in increased permeation of the film. However, cross linking of films can improve the water vapour and oxygen barrier properties. This has been reported by Chiou, *et. al.,* (2008) in the case of fish gelatin films from Alaska Pollock and Alaska Pink salmon skin which showed better barrier properties when cross linked with glutaraldehyde.

The Gas Transmission Rate (GTR) which is measured as Oxygen Transmission Rate (OTR) of films from different sources is given Table 4.4.3. Lowest oxygen permeability was noticed for films based on Rohu, Common carp and Grass carp gelatins. Films based on bovine hide and pork skin gelatin had significantly higher values for OTR which indicate that fish skin gelatin based films have superior gas barrier property than mammalian skin gelatin films. Similar results were observed for gelatin films from the skin of cold water fishes Alaska Pollock and Alaska Pink salmon which showed superior water vapour and Oxygen barrier property than mammalian gelatins (Chiou, *et. al.*, 2008).

Gas Transmission Rate (GTR) is the rate at which a given gas will diffuse through a stated area of a specimen at standard pressure and temperature. In this study GTR is measured as the rate of diffusion of oxygen i.e., Oxygen Transmission Rate (OTR). Many edible films are highly sensible to moisture, but due to the high degree of hydrophilic properties, present an excellent barrier to oxygen and to some aromatic components. Normally, an increase in crystallinity, orientation, molecular mass or degree of cross-linking will result in a decrease in permeability (Miller & Krochta, 1997). Collagen and gelatin coatings have been used in meats and sausages to reduce gas permeability and / or water vapour permeability (Hood, 1987). Gelatin film is a good gas barrier although highly hydrophilic. At low or intermediate pH, protein based films have oxygen permeability values lower than the

polyethylenes, comparable to moderate oxygen barriers such as polyesters and approaching those of the best oxygen barriers like EVOH and PVDC. Gelatin films are potentially useful for fabrication of coatings and pouches for oxygen sensitive products due to their low oxygen permeability (Arvanitoyannis 2002).

The barrier properties of the five types of films show that films based on fish skin gelatins is superior to that of films from mammalian gelatin with respect to water vapour and gas permeation characteristics. Addition of plasticizer will have a negative impact on barrier properties as plasticizers are known to increase the water vapour permeability and gas permeability. Here same concentration of glycerol was used for all the films and hence its effect will be uniform for all the films.

Table 4. 4.3 Barrier Properties of gelatin based Edible Films*

	Water Vapour Permeability (WVP) (g/m^2/24hrs at 90±2% RH &37^0 C)	**Gas Transmission Rate (as OTR)** (cc/m^2/24hrs at 1 Atmos pressure difference)
RG	1.06± 0.03 ^{a}	0.39 ± 0.01 ^{a}
CG	1.10 ± 0.05 ^{a}	0.35 ± 0.02 ^{a}
GG	1.32 ± 0.02 ^{b}	0.40 ± 0.04 ^{a}
BG	1.49± 0.04 ^{c}	1.05 ± 0.06 ^{b}
PG	1.44 ± 0.03 ^{c}	1.03 ± 0.08 ^{b}

*BG =Bovine Skin Gelatin (225B); PG = Porcine Skin Gelatin (300B); RG = Rohu Skin Gelatin; CG = Common carp Skin Gelatin ; GG = Grass carp Skin Gelatin . All values were mean ± standard deviation of triplicate analyses. Different superscripts in the same column indicate significant differences ($P < 0.05$).

4.5. Comparison of Gel Strength, Melting Point, Odour and Texture Profile of Gelatin water Desserts.

4.5.1 Gel Strength, Melting Point and Odour of Gelatin Water Desserts

The method of preparation of Gelatin water desserts from the fish skin and mammalian gelatin sources is described in Section 3.2.4. The gelatin desserts prepared had the same gelatin concentration (3%w/w). Gelatin concentration lower than 3% resulted in desserts which are very soft and disintegrated immediately after formation, particularly in the case of fish skin gelatins. Gelatin concentration above 3% produced hard gels which is an undesirable feature for the desserts. Hence 3% concentration of gelatin was found to be the optimum concentration for the preparation of desserts. The gel strengths, melting point and the odour of the desserts are given in Table 4.5.1. The gel strengths of the gelatin desserts correspond to the gel strength of the gelatin used. The mammalian gelatins used are standard Sigma Grade Bovine and Pork skin gelatin with gel strength of 225 Bloom and 300 Bloom respectively. In the dessert formulations, the highest bloom was observed for Pork skin gelatin based dessert (67.33 B) , followed by Bovine skin(46.70 B), Grass carp (45.96 B), Rohu (30.51 B) and Common carp (28.71 B) skin gelatin desserts . Desserts from Common carp skin and Rohu skin gelatin had similar gel strength. No significant difference in gel strength observed in the case of desserts from Grass carp and Bovine skin gelatin desserts. Melting points of the fish skin gelatin water desserts showed lower values than the mammalian skin gelatins desserts. The lower melting point of the fish gelatin helps in better flavour release in dessert preparations.

The gel strength and melting point of the gel product is directly related to many factors viz., concentration of the gelatin in the sample, maturation time of the gel, maturation temperature and pH. Ferry (1948) has observed that the gel strength was almost squarely proportional to the concentration of the gelatin. Choi and Regenstein (2000) has reported that the rate of increase of melting point decreased with increasing concentration, while that of gel strength increased with gelatin concentration in fish and pork gelatins. In this experiment the same gelatin concentration (3%w/w) was used for all the gel water desserts, hence the influence of gelatin concentration on the melting

point and gel strength cannot be ascertained, however it has been found in the preliminary experiments that a gelatin concentration below 3% yielded desserts with poor gel stability and a concentration above 3% resulted in hard desserts which did not give the characteristic "melt –in – the mouth "feeling of soft gel desserts.

Gel strength of the gel decreases linearly with increasing maturation temperature and melting point, in contrast increased with increasing maturation temperature (Nijenhuis, 1981), The similar pattern has been observed for fish and mammalian gelatins. In this study, the maturation temperature was 20-24 hours (Ref.Section 3.2.4) which could be the reason for the significantly low gel strengths of the desserts. However an increase in melting points of the desserts with that of gelatin was not observed, in fact the melting points of the desserts were lower than that of corresponding gelatins. This can be explained only on the basis of the influence of other factors like pH.

The gel strength of the gelatins decreased markedly below pH 4 and slightly above pH 8 (Crumper & Alexander,1954, Choi & Regenstein, 2000). The gelatin water desserts prepared had a pH in the range of 3.6 to 3.8 which could also be the reason for low gel strength of the desserts. The melting point of the gelatin can have a marked drop below pH 4 which may be the reason for the low melting points of desserts.

Apart from the above mentioned physicochemical properties, the gel strength and melting point of the gelatin water dessert preparations can be influenced by the other ingredients used in the formulation of desserts. Water (87%), sugar (9%) salts (1%) and flavour mix are the important ingredients. The ingredients in the flavour mix control and regulate the pH. Water plays an important role in the gelation and gel setting and sugar can stabilize the hydrogen bonding in the gel. Salts, particularly sodium chloride can decrease the gel strength and melting point of gels. This is due to the fact that sodium chloride is capable of breaking both hydrophobic and hydrogen bonds, thus preventing the stabilization of the gel junction sites, either directly by preventing hydrogen bond formation and/or by modifying the structure of the liquid water in the vicinity of these sites (Finch, *et al.*, 1974).

Determination of the odour of the gelatin water desserts (Table 4.5.1) by sensory evaluation showed that desserts based on fish skin gelatin had a mild barely detectable odour while the odour of desserts made from mammalian gelatin was easily detectable, although not offensive. In all the dessert samples the flavour of the instant drink used in the formulation was predominating. This is consistent with the observation by Choi & Regenstein, (2000) that flavored fish gelatin dessert gel product has less undesirable off-flavour and off-odour than the same product made with pork gelatin.

Table 4.5.1 Gel Strength, Melting Point and Odour of Gelatin Water Desserts

	Gel Strength(Bloom)	Melting Point (^{0} C)	Odour
CGD	28.71 ±1.06 [a]	27.10 ± 0.08 [a]	Mild, only perceivable on careful assessment
RGD	30.51 ±1.24 [a]	27.21 ± 0.05 [b]	Mild, only perceivable on careful assessment
GGD	45.96 ±0.91 [b]	28.73 ± 0.11 [c]	Mild, only perceivable on careful assessment
BGD	46.70 ±1.59 [b]	30.41± 0.14 [d]	Mild, but easily perceivable.
PGD	67.63 ±1.13 [c]	31.30 ± 0.11 [e]	Mild, but easily perceivable.

BGD =Bovine Skin Gelatin Dessert; PGD = Porcine Skin Gelatin Dessert; RGD = Rohu Skin Gelatin Dessert; CGD = Common carp Skin Gelatin Dessert; GGD = Grass carp Skin Gelatin dessert.All values were mean ± standard deviation of triplicate analyses. Different superscripts in the same column indicate significant differences ($P < 0.05$).

4.5.2 Texture Profile Analysis of Gelatin Water Desserts

Texture Profile Analysis was carried out using a Lloyds Texture Analyzer (Lloyd Instruments, Model LRX Plus, U.K.) with 50% deformation. At 50% deformation, the measurements of TPA carried out was non destructive. From the TPA curve the mechanical textural parameters viz., hardness, cohesiveness, gumminess, springiness, chewiness and adhesive force were

calculated. The instrumental textural parameters of gelatin desserts studied are given in Table 4.5.2.

In gelatin water dessert samples maximum hardness was noticed for Pork skin gelatin dessert (1021.73gf), followed by Bovine skin gelatin dessert (769.35 gf) and fish skin gelatin desserts. Among fish skin gelatin desserts, minimum hardness was observed for Common carp skin gelatin dessert (231.29gf). The hardness of the desserts was significantly ($p< 0.05$) different among the samples. The hardness is dependent on the gel strength and the sample with the maximum gel strength (pork skin gelatin dessert) showed maximum hardness. This observation agrees to the report that in desserts prepared from Alaska Pollock, Tilapia and Pork skin gelatins, hardness correlated well with gel strength (Zhou and Regenstein 2007).

Cohesiveness is a measurement of the degree of difficulty in breaking down the gel's internal structure. A value of 1 indicates total elasticity and a value of 0 imply that the sample did not recover at all, indicating total loss of elasticity. In this study the desserts prepared from Grass carp skin gelatin and mammalian gelatin showed significantly high values (0.60 – 0.67) for cohesiveness than the other two fish skin gelatin desserts which indicate the high degree of elasticity of the desserts. Cohesiveness reported for desserts prepared from Alaska Pollock and Tilapia skin was 0.9 and 0.93 respectively, indicating a very high elastic gel (Zhou and Regenstein 2007).

Gumminess was found to be significantly higher for mammalian skin gelatin desserts (Table 4.5.2). It is a desirable attribute in marshmallow type of products where the product gives "a feel in the mouth" sensation while chewing.

In desserts, significantly higher values ($p< 0.05$) for springiness were observed for mammalian and Grass carp skin gelatins (6.21 – 7.17 mm). For soft desserts high springiness is not a desirable trait. High springiness results from the gel structure being broken into a few large pieces during the first TPA compression while low springiness results from the gel breaking into many small pieces (Lau *et. al.,* 2000).

Grass carp skin and mammalian skin gelatin desserts had significantly higher chewiness than Common carp and Rohu skin gelatin based desserts

(Table 4.5.2). This implies that soft textured desserts can be made from Common carp and Rohu skin gelatin. . Adhesive force was also found to be highest for mammalian and Grass carp skin gelatin based desserts.

Table 4.5.2 Instrumental Textural Parameters of Gelatin Water Desserts*

	Hardness (gf)	Cohesive-ness	Gumminess (gf)	Springiness (mm)	Chewiness (gf.mm)	Adhesive Force (gf)
CGD	231.2± 12.7[a]	0.38±0.05 [a]	87.6±13.9 [a]	4.0±0.14 [a]	350.6±0.03 [a]	10.0±0.55 [a]
RGD	296.9 ± 9.3 [b]	0.39±0.02 [a]	115.7±7.8 [b]	4.6± 0.14 [b]	538.0± 0.02 [b]	11.8±0.62 [b]
GGD	536.5 ± 14.8[c]	0.61±0.04 [b]	327.1±24.3 [c]	6.2± 0.26 [c]	2031.4±0.28 [c]	20.1±0.90 [c]
BGD	769.3 ± 17.1[d]	0.60±0.03 [b]	461.5±15.5 [d]	6.6± 0.32 [c]	3087.5±0.19 [d]	26.2±0.80 [d]
PGD	1021.7±21.7[e]	0.67±0.05 [b]	684.1± 9.9 [e]	7.1± 0.4 [cd]	4905.4±0.13 [e]	39.2±1.04 [e]

*BGD =Bovine Skin Gelatin Dessert; PGD = Porcine Skin Gelatin Dessert; RGD = Rohu Skin Gelatin Dessert; CGD = Common carp Skin Gelatin Dessert; GGD = Grass carp Skin Gelatin dessert.All values were mean ± standard deviation of triplicate analyses. Different superscripts in the same column indicate significant differences ($P < 0.05$).

The study indicated that Grass carp skin gelatin based desserts had comparable physical and mechanical properties with that of desserts prepared from mammalian skin gelatin. Grass carp skin and bovine skin gelatin based desserts had similar values for gel strength, cohesiveness and springiness which indicated the similarity of the gels. A notable observation was that the melting points of the gelatin desserts from fish skins were lower than mammalian skin gelatin desserts. This will help in better release of the flavour from the gels. The fishy odour was not prominent in fish gelatin based desserts. In mammalian gelatin based desserts, the characteristic odour was easily detectable. Hence the fish gelatin based desserts were rated higher in organoleptical evaluation. The information on the physical and texture properties of fish skin based gelatin desserts will be particularly useful in formulating kosher and halal gelatin desserts, and may also be helpful to add more textural variety to commercial gelatin desserts.

5.0 Summary and Conclusions....

The present study has evaluated the suitability of the skin of the freshwater fish as a raw material for the production of gelatin, optimized the process parameters for the gelatin extraction and studied the physico-chemical properties of gelatins & gel based products. The raw materials for the study were the skins of three cultured freshwater carps viz., Rohu (*Labeo rohita* – Hamilton Buchanan), Common carp (*Cyprinus carpio)* and Grass carp (*Ctenopharyngodon idella*). To summarize the work, the following conclusions are drawn from the study:

The gelatin extraction process from the skin of carps was optimized by adopting Response Surface Methodology which consisted of a preliminary screening and Response Surface Modelling to optimize the process parameters.

A two level fractional factorial screening design was employed to identify the critical independent variables that had significant influence on the gelatin extraction based on the responses for dependent variables viz., gel strength and yield. Alkali pretreatment concentration , Acid pretreatment concentration , Pretreatment time (min) and Extraction temperature (°C) were identified as critical independent variables that had influence on two responses studied in carp skin gelatin extraction.

The Response Surface Model was built on the basis of these factors as a four factor, five level Central Composite Design where the experimental values were compared with predicted values. A total of 31 experimental runs using the Central Composite Design were carried out to study the effect of the selected independent variables on the responses. The Analysis of Variance showed that the predicted models were statistically valid.

The optimization solutions for the extraction of gelatin from the skin of carps gave a composite desirability value above 0. 8 based on the set parameters. The responses predicted by the solutions are within the range of the experimental values obtained in the response surface model. Alkali

concentration was found to be the most important factor affecting the gel strength and yield in the gelatin extraction process from carp skin under a given set of optimization parameters.

Verification experiments were conducted under optimal conditions to compare predicted values and actual values of responses and similar results were obtained. Therefore, the estimated response surface model was adopted for optimization of gelatin processing from the skins of Rohu, Common carp and Grass carp.

Physical properties of the carp skin gelatins were studied.The gelatin yield was significantly lower in Grass carp than the other two sources.The maximum yield was observed for Rohu (12.9%) followed by Common carp (12%) and Grass carp (10.5%). High viscosity gelatin was obtained from Rohu, Common carp and Grass carp, which is suited for film forming applications. The viscosity value was in the range of 5.96 – 7.07 and was significantly higher for Grass carp gelatin followed by Rohu and Common carp gelatins.

Melting point of gelatin obtained from carps (28.13 -29.1 ^{0}C) was comparable to gelatin from mammalian sources. The comparatively high amount of imino acid content can be a contributory factor for the high melting point characteristics of gelatins from these species. The setting temperature observed for the gels from Rohu, Common carp and Grass carp skins were in the range of 17.9 ^{0} C – 20. 5^{0} C. Common carp had the lowest setting temperature and the highest was for Grass carp. Grass carp gel showed a significantly faster setting time of 68.6 seconds when compared to the other two gels. The setting and melting temperatures observed for gelatins from the skin of Rohu, Common carp and Grass carp are similar, if not better than many of the gelatins from animal sources and can possibly substitute the same in many applications without extensive modifications. This offers future scope for developing binary blends of these gelatins with animal gelatins that are completely compatible and commercially useful in many applications.

Gelatins from the skins carps were found to have a mild but easily perceivable odour, had a snowy white appearance and were light-textured. This could be a positive attribute, since it is easier to incorporate these gelatins into any food system without imparting any strong colour.

A study on the functional properties of the carp skin gelatins showed that the gels had medium gel strengths in the range of 181 – 230 B which are of commercial significance, considering the potential applications in food and edible film preparations. Gel strengths of carp skin gelatins were comparable to that of gelatins obtained from tropical fish species. Foam formation ability is an important functional property of gelatin which has significance in food applications viz., in the preparation of products like marshmallows. Foam formation ability of Common carp gelatin was 2.44, significantly lower than Rohu (2.51) and Grass carp (2.83) gelatins. Foam stability of Common carp was 1.90, significantly less than Rohu (1.86) and Grass carp (1.78) gelatins, demonstrating the lower stability of Common carp gelatin. Rohu skin gelatin had the highest fat-binding capacity (457.3%) and Common carp skin gelatin had the lowest water-holding capacity (176%). The high amount hydrophobic amino acid, tyrosine is probably responsible for the high fat binding capacity of Rohu skin gelatin. Grass carp gelatin had the maximum content of hydrophilic amino acid hydroxyproline among the carp gelatins and a correspondingly high value for water holding capacity.

Texture Profile Analysis of carp skin gelatins showed that hardness and cohesiveness values were maximum for Grass carp gelatin. Springiness index was significantly lower for Common carp gelatin than the other two gels. Grass carp gel showed significantly higher values for Gumminess, chewiness and fracture force than the other gels. Gelatins from Rohu, Common carp and Grass carp had very low adhesiveness, implying their chewability. The texture attributes of the gelatins of Rohu, Common carp and Grass carp gives an indication that these are useful in food applications for the preparation of the products like fruit gums where gelatin helps in thermo reversible gel formation, provide taste and color neutrality, gives easy pouring ability due to

low viscosity and excellent clarity. In addition gelatin gives unique texture and excellent mouth feeling, chewability and attractive appearance

Chemical analysis of carp skin gelatin indicated that the moisture content of the samples was below 10%, which is less than the limit prescribed for edible gelatin ie, 15% (GME, 2005). The ash content in gelatin samples were in the range of 1.10 - 1.18%, much less than the recommended maximum limit (2%) set for edible gelatin (GME, 2005). The pH varies between 4.05 – 4.42. Grass carp gelatin shows significantly higher values for pH. The values of pH for gelatin samples are outside the range prescribed for Type A Gelatin (pH 6.0 - 9.5) and Type B Gelatin (pH 4.7 – 5.6). This is because the pretreatment method employed during the extraction process involves both alkaline and acid treatments.

The amino acid composition of Gelatins from the skin of Rohu, Common carp and Grass carp was higher than gelatin from many other tropical and coldwater fish species with respect to the imino acid and other important amino acids content. This could be the reason for the compatibility of these gels with mammalian gels in respect of physico-chemical properties. Carp gelatin samples had high content of imino acids (Proline +Hydroxyproline) in the range of 19.49 – 19.86 % of protein. High content of imino acids improves the rheological properties of gelatin as it is involved in formation of triple helical regions that immobilize water. Imino acids also impart considerable rigidity to the gelatin structure. Grass carp skin gelatin contained the lowest proline (8.2%) and highest hydroxyproline content (11.66%) among the three gelatins. However, maximum gel strength was observed for Grass carp gelatin which shows that Hydroxyproline is the major determinant of stability due to its hydrogen bonding ability through its hydroxyl group, although proline is also important. Carp skin gelatin samples had significant amounts of serine and threonine (4.88 – 9.1%) with free hydroxyl groups which can contribute to the gel strength by the generation of hydrogen bonds and helical structures. Significantly higher values for alanine was observed for Grass carp skin gelatin (8.3%).A higher content of this amino acid can be one of the reason for higher viscoelastic properties Grass carp

skin gelatin than the other carp skin gelatins. The amino acid composition of Gelatins from the skin of Rohu, Common carp and Grass carp was higher than gelatin from many other fish species with respect to the imino acid and other important amino acids content.

Molecular weight distribution pattern of carp skin gelatins was studied which showed that Grass carp skin gelatin had predominantly β-chains with molecular weights of 200 kDa and less intensive α-chain bands with molecular weights in the range of 116 KDa and 97 KDa and sub α-units of molecular weights 55 to 66 kDa. Rohu gelatin had predominantly α-chains with molecular weights in the range of 116 KDa and 97 KDa and a wide range of sub α - units of molecular weights 6.5 to 66 kDa. In the case of Common carp skin gelatin, the β- chains with molecular weights of 200 kDa are also present, but their bands are less intensive than the α-chain bands. Grass carp skin gelatin with higher concentration of β-chain peptides and lower amounts of low molecular weight α - chains and sub α- units showed superior functional properties and high values for bloom, viscosity, melting point, setting point and a faster setting time than the other carp skin gelatins.

The FTIR spectra of carp skin gelatins were found to be dependent on the extraction temperature. Grass carp and Rohu gelatins with lower extraction temperatures at 40 and 49 ^{0} C respectively showed the low intensity amide A, I and II bands and the amide III band was not fully distinguished. These changes are indicative of greater disorder in gelatin and are associated with loss of triple helix state. This is consistent with changes expected as a result of denaturation of collagen to gelatin. The Common carp skin gelatin extracted at higher temperature exhibited a much broader amide A than was observed for the low temperature extracted Rohu and Grass carp gelatins. High temperature-extracted Common carp skin gelatin may contain a significant amount of intermolecular crosslinks. This can produce FTIR spectra showing a higher degree of molecular order.

Gelatins from the carp skins are safe with respect to heavy metals. The quantum of arsenic, lead, copper, zinc, cadmium and chromium in the samples were below the limits prescribed. Baceriological analysis showed that the microbiological quality and conforms to the standards of edible gelatin.The total plate counts were well below the allowable limit of 1000/g. Other bacterial groups viz., *Coliforms, E. coli, Salmonella, Clostridial spores, Staphylococci, & Pseudomonas* were not detected in the samples.

A comparative sudy of mammalian skin gelatins and carp skin gelatins showed that mammalian skin gelatins (bovine & porcine) showed significantly higher viscosity, melting & setting temperature and faster setting time when compared to carp skin gelatins. The odour scores were higher for mammalian gelatins, indicating that they had a distinguishable odour and hence can be considered as inferior to carp skin gelatins in organoleptic qualities.

Gel strengths of Grass carp and bovine skin gelatins were comparable. Foam formation ability was similar for mammalian and Grass carp skin gelatins and mammalian skin gelatins exhibited significantly better foam stability than carp skin gelatins. No significant differences were observed in the water holding and fat binding capacities of Grass carp and mammalian skin gelatins.

Studies on the physical and mechanical properties of mammalian and carp skin gelatin based films showed that the mechanical properties of Grass carp skin gelatin based film was superior to Rohu and Common carp skin gelatin films and comparable to films made from commercial bovine hide and pork skin gelatin. Carp skin gelatin films had significantly lower water vapour permeability than mammalian skin gelatin films. Low oxygen permeability was noticed for carp skin gelatin films than mammalian skin gelatins which indicate that carp skin gelatin based films have superior gas barrier property than mammalian skin gelatin films.

In the gel dessert formulations, the highest bloom was observed for pork skin gelatin based dessert, followed by bovine skin, Grass carp, Rohu

and Common carp skin gelatin desserts. Desserts from Common carp & Rohu skin gelatin had similar gel strength. No significant difference in gel strength observed in the case of desserts from Grass carp and Bovine skin gelatin desserts. Melting points of the fish skin gelatin water desserts showed lower values than the mammalian skin gelatins desserts. The lower melting point of the fish gelatin helps in better flavour release in dessert preparations. The fishy odour was not prominent in fish gelatin based desserts. In mammalian gelatin based desserts, the characteristic odour was easily detectable. Hence the fish gelatin based desserts were rated higher in organoleptic evaluation. Grass carp skin and bovine skin gelatin based desserts had similar values for gel strength, cohesiveness and springiness which indicated the compatibility in textural properties.

Future Outlook & research Needs

Increasing demand for fish gelatin may pave the way for further research and exploration of fish gelatin as an alternative for mammalian gelatin, as it fulfills the majority of consumer needs and complements the increasing global demand for gelatin. The current production of fish gelatin may not increase significantly in near future, as the availability of raw material, coupled with the relatively low yield will be limiting factors in fish gelatin production. However, though fish gelatin will be unable to completely replace mammalian gelatin, it might become a niche product offering unique and competitive properties to other biopolymers, as well as meeting the demand of global halal / kosher market.

Carp skin gelatin has superior physico-chemical and functional properties when compared to other gelatins of fish origin and hence assumes commercial significance. Utilization of carp skins for gelatin extraction can alleviate the problems of waste generation during commercial processing of carps, besides the production of an important biopolymer for food and industrial applications. The future emphasis on research in the utilization of freshwater fish skins for gelatin production should be on scaling up the extraction and production process and securing control of the extraction conditions during this process. As Good Manufacturing Practices (GMP) and HACCP (Hazard Analysis and Critical Control Point) are becoming

increasingly important in food manufacturing, future research has to be directed towards the development of low cost and high quality fish gelatins with minimal or no contaminants . Detailed investigations are to be carried out to standardize the purity of samples / raw material used to ensure uniformity. The use of physical (ultrasound and ionizing radiation), enzymatic, and natural (plant phenolics and genipin) crosslinking agents to enhance the gel strength and other functional properties of freshwater fish skin gelatin is an emerging area in gelatin research.

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