Production, purification, genetic characterization and application studies of tannase enzyme from marine fungus *Aspergillus awamori*

Thesis submitted to the
Cochin University of Science and Technology
under the Faculty of Science
in partial fulfillment of the requirements
for the degree of

Doctor of Philosophy
In
Biotechnology

by

Beena P.S.
Reg. No. 3174
Microbial Technology Laboratory
Department of Biotechnology
Cochin University of Science and Technology
Cochin – 682 022
Kerala, India

January 2010

DEPARTMENT OF BIOTECHNOLOGY
COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY
COCHIN-682 022, KERALA, INDIA
Ph: 91-484-2576267 Fax: 91-484-2577595

Email:saritagbhat@gmail.com

Dr. Sarita G. Bhat

Reader

4-01-2010

CERTIFICATE

This is to certify that the research work presented in this thesis entitled **"Production, purification, genetic characterization and application studies of tannase enzyme from marine fungus *Aspergillus awamori*"** is based on the original research work carried out by Mrs. Beena P.S. under the guidance and supervision of Dr. M. Chandrasekaran and my Co-guidance at the Department of Biotechnology, Cochin University of Science and Technology, in partial fulfillment of the requirements for the degree of Doctor of Philosophy, and that no part thereof has been presented for the award of any other degree, diploma, associateship or other similar titles or recognition.

.

SARITA G. BHAT

The dream begins with a teacher who believes in you, who tugs and pushes and leads you to the next plateau, sometimes poking you with a sharp stick called "truth." ~Dan Rather. It is beyond my expressions how much I am indebted to Dr. D. Girija, Assistant Professor, CPBMB, KAU for her encouragement and support extended towards me. She gave me a chance to explore, which gave me confidence and enthusiasm in the world of research through which I could reach here.

Words fail to express my heartfelt gratitude to Ms. Aswathy N, guest lecturer for her constant support, companionship and encouragement. I would like to thank Ms. Harisree and Ms. Mridula, guest lecturers for their help and support.

I express my deep sense of gratitude to all my MTL seniors, who played different roles in my life. My heartfelt thanks to Dr. Keerthi, who introduced me to MTL, Dr. Suresh PV, who was my M Sc dissertation Co-guide, Dr. Swapna, Dr. Sabu, Dr. Jayachandran, Dr. Mohanan, Dr. Dasan and Dr. Rajeev Kumar.

I extent my heartfelt thanks to Dr. Sreeja Chellappan, who made me to step back in to research, for her moral support, valuable and critical suggestions, continuous interest and constant encouragement throughout my work. I greatly acknowledge the extensive co-operation and spontaneous help received from Dr. Archana, Dr. Jasmin, , Dr. Madhu, Dr. Lailaja and Mr. Sijo.

"Gratitude is the fairest blossom which springs from the soul". My deepest sense of gratitude towards Soorej M Basheer and Jissa G Krishna may be failed in getting words but I am always indebted to them for their supportive hearts. Even though now they are away, along with our sir they were with me in completing each and every word of this thesis.

I feel I am very lucky to be a part of MTL and I would like to express my deep gratitude towards my colleagues Mrs. Roselin Alex, Mr. Sajan, Mr. Karthikeyan, Mr. Doles, Mrs. Manjula and Mr. Cikesh, who were with me during all my ups and downs.

My heartfelt gratitude fails to find words to Mr. Abraham for his moral support, effective discussions and timely help extended by him towards me. My sincere thanks to Mr. Manzur Ali, Mrs. Sapna, Pippy, Ms. Rekha and Mr. Ramesh for their support and inspiration during my research.

I am always indebted to MGL for their support and fun that they had with me. I am really thankful to Mr. Rahul, Ms. Jeena, Ms. Smitha, Mrs. Helvin, Mrs. Linda, Mr. Satheesh, Mr. Siju and Mrs. Alphonsa Vijaya.

DECLARATION

I hereby declare that the work presented in this thesis entitled **"Production, purification, genetic characterization and application studies of tannase enzyme from marine fungus *Aspergillus awamori*"** is based on the original research carried out by me at the Department of Biotechnology, Cochin University of Science and Technology, Cochin under the guidance of Dr. M. Chandrasekaran, Professor, Cochin University of Science and Technology and co-guidance of Dr. Sarita G. Bhat, Reader, Cochin University of Science and Technology and the thesis or no part thereof has been presented for the award of any degree, diploma, associateship or other similar titles or recognition.

Cochin 22, **Beena P.S**

4. 01. 2010.

Acknowledgement

At times our own light goes out and is rekindled by a spark from another person. Each of us has cause to think with deep gratitude of those who have lighted the flame within us.
-- Albert Schweitzer

Even though the words could not fulfill the gratitude towards all the supporters who contributed their efforts for this partial fulfillment, I would like to take this opportunity to share my heartfelt thanks to all without whose help and support this thesis would ever have been possible. The discussions, encouragement, suggestions and critiques made by them were the essence of this work.

I owe my heartfelt sincere gratitude to my supervising guide, Prof. (Dr.) M. Chandrasekaran who gave me an opportunity to pursue research which leads to a Ph.D., which was once a dream of mine. I am grateful to you Sir for your constant inspiration, effective discussions, encouragement and help given to me during the course of this study. His support was not only limited to my scientific issues his philosophical advices have influenced me a lot in day today life. His creativity, scientific aptitude and perseverance have made him a constant oasis of knowledge, which inspired and enriched me as a researcher and I am indebted to him more than he knows. The encouragement and help extended by him helped me to get DST sponsored Women Scientist scheme. I am privileged to get him as my guide and Scientist mentor of this study.

I express my sincere gratitude to Dr. Sarita G Bhat, Reader Department of Biotechnology, for providing me advices and tips in time that helped me a lot in completing the work in time. I am indebted to her for her valuable suggestions and support as a Co-guide and Doctoral Committee member.

I am very thankful to Prof. Dr. C.S. Paulose, Head, Department of Biotechnology for his constant support and help extended towards me. The effective functioning of my ongoing DST-WOS-A project including its financial dealings, are running smoothly because of you sir and I am very thankful to you.

I express my heartfelt gratitude to Prof. Dr. Elyas KK for all his critical comments, supportive comments, motivational talks and the helps he extended towards me during the course of this work. His experimental tips have helped me a lot for achieving the results and interpreting the results. Words could not express my indebtedness to you sir.

I also extend my appreciation to Dr. Padma Nambisan faculty member and Dr. E. Vijayan, Emeritus Professor for their kind support and co-operation.

The moral support and prayers extended by Ms. Jasmine Koshy during my work supported me a lot and I would like to express my deep sense gratitude towards her. I also extent my thanks to Ms. Aneeta, Mrs. Jikku jose, Ms. Habeeba and Mrs. Regi, Plant Biotechnology Laboratory, Department of Biotechnology for their support.

I would like to thank all the M Sc students who were here during the time of my work especially Ms. Sneha and Ms. Serene for their support. I also extend my appreciation to Dr. Binoy, Dr. Gireesh, Dr. Akash, Dr. Finla, Dr. Remya, Dr. Savitha, Dr. Riyas, Dr. Amee, Dr. Anu, Mrs. Sherine, Mrs. Anju, Ms. Pretty, Mr. Peeyush, Mr. Jes, Mr. Nandu, Mr. Korah, Mrs. Anita, Ms. Chindu, Mr. Jaya Narayanan and Mr. Smijin and all other friends at Neuroscience Laboratory, Department of Biotechnology, for their help, support and co-operation.

I am also grateful to all staff members of the Department of Biotechnology, Planning and Academic sections of Cochin University of Science and Technology for their help and co-operation.

The financial support from SERC, DST, Ministry of Science and Technology, Government of India in the form of Women Scientist Scheme (WOS-A) (WOS-A/LS-130/2006) is greatly acknowledged. I would like to express my gratitude to Dr. HB. Singh, Officer-in- Charge, DST-WOS-A project and all the evaluation committee members for their valuable suggestions.

Words could not extent my deep sense of gratitude to all my family members especially my daughter Aardhra who spent several late nights in the lab with me and adjusted a lot with her mother's research, and my husband who tolerated and supported me throughout my study. I would like to thank my father, mother and sisters for their constant inspiration, motivation and prayers from my childhood. Their sustained encouragement and effort made me to reach here. I would like to thank my in laws and particularly valiyamma for her motivating words.

A number of friends, colleagues and well wishers were involved in this work directly or indirectly. I thank one and all for their generous help, extended to me has made this research work a reality. I hereby extend my sincere gratitude and appreciation to all of them who made this thesis possible.

Above all I am grateful to The Creator of all "GOD" for giving me all the blessings and strength for making this effort a success.

Beena P.S.

“The mediocre teacher tells.
The good teacher explains. The superior teacher
demonstrates. The great teacher inspires.”

(William Arthur)

Dedicated to all those who inspired me

ABBREVIATIONS

%	-	Percentage
ºC	-	Degree Celsius
A	-	Adenine
Aº	-	Angstrom
A_{260}	-	Absorbance at 260 nm
A_{280}	-	Absorbance at 280nm
ANOVA	-	Analysis of variance
APS	-	Ammonium per sulphate
BLAST	-	Basic Local Alignment Search Tool
bp	-	Base pairs
BSA	-	Bovine serum albumin
C	-	Cytosine
cDNA	-	Complementary Deoxyribonucleic acid
cfu	-	Colony forming units
Conc.	-	Concentration
DNA	-	Deoxy ribo nucleic acid
dNTP	-	Deoxy nucleotide tri phosphate
dsDNA	-	double stranded DNA
DW	-	Distilled water
E.coli	-	*Escherichia coli*
EC	-	Enzyme Classification
EDTA	-	Ethylene diamine tetra acetic acid
g	-	grams
G	-	Guanine

GC	-	Gas Chromatography
h	-	Hours
kbp	-	kilo base pairs
kDa	-	kilo Dalton
Km	-	Substrate concentration at which the reaction velocity is half maximum
kV	-	kilo Volt
LB	-	Luria Bertani
M	-	Molar
mg	-	milligram
ml	-	milliliter
mm	-	millimeter
mM	-	millimolar
min	-	minute
MSSF	-	Modified Solid State Fermentation
nm	-	Nanometer
OD	-	Optical Density
ORF	-	Open Reading Frame
PAGE	-	Poly acrylamide gel electrophoresis
PB	-	Plackett –Burman
PCR	-	Polymerase Chain Reaction
rpm	-	Revolutions per minute
RSM	-	Response Surface Methodology
RT	-	Room temperature
SLF	-	Slurry State Fermentation
SMC	-	Submerged culture
SSC	-	Solid state culture
SDS	-	Sodium deodecyl sulphate
SmF	-	Submerged Fermentation
sp.	-	Species
SSF	-	Solid State Fermentation
T	-	Thymine
TAE	-	Tris acetic acid EDTA
Taq	-	*Thermus aquaticus*
TE	-	Tris EDTA
TEMED	-	N-N-N’-N’- tetramethyl ethylene diamine
TLC	-	Thin Layer Chromatography
U/mg	-	Units/milligram
U/ml	-	Units/milliliter
UV	-	Ultra violet
v/v	-	volume/volume
V	-	volts
V_{max}	-	maximal velocity
µg	-	microgram
µl	-	microliter
µM	-	micromole

CONTENTS

LIST OF TABLES

LIST OF FIGURES

enzymes for specific applications will be a future trend with continuously improving tools and understanding of structure-function relationships and increased search for enzymes from exotic environments. New technical tools to use enzymes as crystalline catalysts, ability to recycle cofactors, and engineering enzymes to function in various solvents with multiple activities are important technological developments, which will steadily create new applications.

Among the source of enzymes *i.e*., animals, plants and microbes, enzymes from microorganisms have become the choice for industrial production. Microbial enzymes are more useful than enzymes derived from plants or animals because of the availability of great variety of catalytic activities, the possible high yields, ease of genetic manipulation, regular supply due to absence of seasonal fluctuations and rapid growth of microorganisms on inexpensive media. The technological application of enzymes under demanding industrial conditions makes the currently known arsenal of enzymes insufficient and the search for new microbial sources continued.

In fact only 2 % of the world's microorganisms have been tested as enzyme sources (Wiseman, 1985) although microorganism from diverse and exotic environments and extremophiles are considered as an important source of enzymes, and their specific properties are expected to result in novel process applications (Govardhan and Margolin, 1995). Within the microbial diversity, there is always a chance of finding microorganisms producing novel enzymes with better properties that are suitable for commercial exploitation. The multitude of physico chemically diverse habitats has challenged nature to develop equally numerous molecular adaptations in the microbial world. Microbial diversity is a major resource for biotechnological products or processes.

Chapter 1

INTRODUCTION

The use of enzyme mediated processes can be traced to ancient civilization. Enzymes are the focal points of biotechnological process since they are involved in all aspects of biochemical conversions. They are used as cost effective eco-friendly substitutes for chemical processing in several industries. Today enzymes have become major market next to pharmaceuticals. The enzyme industry feed a vast range of industries, domestic and environmental applications. Industrial enzyme market is growing steadily. Enzymes have become one of the major commodities today and their manufacture and applications in recent past has emerged as one of the major industry and the global market for industrial enzymes is estimated as $2 billion in 2004 and is expected to rise at an average annual growth rate (AAGR) of 3.3 % to $2.4 billions in 2009. Indian market for enzymes was estimated to be more than Rs 320 crores in the year 2004-05. Detergents (37 %), textiles (12 %), starch (11 %), baking (8 %) and animal feed (6 %) are the main industries, which use about 75 % of industrially produced enzymes. Today nearly 4000 enzymes are known and of these about 200 are in commercial use. Until the 1960's, the total sale of enzymes were only a few million dollars annually, but the market has since grown spectacularly (Godfrey and West, 1996; Wilke, 1999). The world enzyme demand is satisfied by 12 major producers and 400 minor suppliers. Around 60 % of total world supply of industrial enzyme is produced in Europe.

More than 75 % of industrial enzymes are hydrolases. Protein-degrading enzymes constitute about 40 % of all the enzyme sales. More than fifty commercial industrial enzymes are available and their number is increasing steadily. Tailoring

Gallic acid (3,4,5-trihydroxy benzoic acid), an organic substance occurring in many plants either as a free molecule or as part of tannic acid molecule, is a useful product with wide applications. It is utilized in the production of trimethoxy benzaldehyde, which is used in ink industry, dye industry and most importantly pharmaceutical industry. In pharmaceutical industry 3, 4, 5 trimethoxy benzaldehyde is converted to trimethoprim, a broad spectrum antibiotic. A combination of trimethoprim and sulphonamide is effective against many otherwise resistant species of bacteria. Though technological advances have introduced a number of antibiotics in markets, trimethoprim is still very significant. In combination with sulphonamides, it is highly effective against many drug resistant species of bacteria. Of the total requirement of gallic acid of 8000 tons per year, 75 % of it is used in production of trimethoprim. Gallic acid can also be used as a raw material for manufacturing an intermediate for anti oxidants, preservatives like propyl gallate. It is very important to have an economical indigenous technology for its commercial production.

Conventionally gallic acid is produced by acid hydrolysis of tannic acid but it has cost, yield and low purity disadvantages. Alternatively, gallic acid can be produced by the microbial hydrolysis of tannic acid by tannase (tannin-acyl hydrolase), an inducible enzyme, secreted by microorganisms. Mainly *Aspergilli* have been used for hydrolysis of tannic acid to yield gallic acid (Mondal *et al*., 2001; Seth and Chand, 2000) among bacteria *Klebsiella pneumoniae* and *Corynebacterium* sp. have been reported to produce gallic acid from crude extract of tara gallotannin (Deschamps and Lebeault, 1984).

In spite of the commercial importance of gallic acid not many reports are available in the literature on the development of a process for gallic acid production at fermenter level (Pouratt *et al*., 1987). The main hindrance in the development of a successful bioconversion process is the sensitivity of the

Tannase or tannin acyl hydrolase (E.C.3.1.1.20) catalyzes the hydrolysis of ester bond and depside bond present in hydrolysable tannins to form glucose and gallic acid. Industrial bioconversion of tannic acid is generally accomplished by enzyme tannase for the production of gallic acid (3, 4, 5-tri hydroxy benzoic acid), which is mostly used in pharmaceutical industry for production of anti-bacterial drug trimethoprim (Bajpai and Patil, 1996). It is also used as an important substrate for synthesis of propyl gallate in food industry as an anti oxidant (Lekha and Lonsane, 1997). Besides these applications tannase is widely used in the manufacture of instant tea and acorn wine. Tannase has also been applied for cleavage of poly phenolics such as dehydrodimer crosslinks present in the cell wall of plants, which is necessary for plant cell wall digestibility (Garcia-Conesa *et al.*, 2001).

Tannase has potential application in the clarification of beer and fruit juices, manufacture of coffee flavored soft drinks, and improvement in flavor of grape wine and as an analytical probe for determining the structure of naturally occurring gallic acid esters (Seth and Chand, 2000). In the case of the wines, it is important to state that the main tannins present are catechins and epi-catechins, which can get a complex with galacto-catechins and others galloyl-derivated. Fifty percent of the colour of the wines is due to the presence of the tannins; however, if these compounds are oxidized to quinones by contact with the air it could form an undesirable turbidity, presenting severe quality problems. The use of the tannase can be a solution to these problems. Chemical treatment of wine to remove unfavoured phenolics can be altered with tannase to hydrolyze chlorogenic acid to caffeic acid and quinic acid which influence the taste of wine favorably. In the manufacture of beer, the tannase could be used since the tannins are present in low quantities. When the proteins of the beer are in considerably high quantities an undesirable turbidity is presented by the reaction with these tannins. This problem could be resolved with the employment of the tannase.

Tannase can be potentially used for the degradation of tannins present in the effluents of tanneries, which contains high amounts of tannins, mainly polyphenols, which are dangerous pollutants, for this reason the use of the tannase represents a cheap treatment and removal of these compounds (Van de Lagemaat and Pyle, 2001). Its production at industrial level is carried out using microbes employing submerged culture (SmC), where the activity is expressed mainly of intracellular form, implying additional costs in its production (Lekha and Lonsane, 1994). Tannase is recently commercialized by Biocon (India), Kikkoman (Japan), ASA Special enzyme GmbH (Germany) and JFC GmbH (Germany) with different catalytic units depending of the product presentation (Aguilar *et al*., 2007).

Although tannase is present in the plants, animals and microorganisms, it is mainly produced by the microorganisms (Ayed and Hamdi, 2002; Belmares *et al*., 2004). Tannase is produced as a membrane bound or intracellular enzyme. Fungal tannases have a better activity in degrading hydrolysable tannins, whereas yeast tannases degrade tannic acid better and has a lower affinity for naturally occurring tannins (Deschamps *et al.*, 1983). On the other end of the spectrum, bacterial tannase can degrade and hydrolyse natural tannin and tannic acid very efficiently (Deschamps *et al.*, 1983, Lewis and Starkey, 1969). Of all the microorganisms that are known to produce tannase, *Aspergillus* sp. is being used commercially as the most efficient producer of this enzyme. The ability of *Aspergillus niger* to grow on tannic acid medium may be linked to its saprophytic lifestyle. Plants contain 5-20 % tannin by weight. *Aspergillus* sp. capable of growing on tannic acid medium containing it as sole carbon source might definitely produce tannase for its survival. According to Van Diepeningen *et al*., (2004), black *Aspergillus* sp. can utilize more tannic acid than non black *Aspergillus* sp. Although tannase production by *Aspergillus* can occur in the absence of tannic acid, *A. niger* tolerates tannic acid concentrations as high as 20 % without having a deleterious effect on both growth and enzyme production (Van Diepeningen *et al*.,

2004; Cruz-Hernandez *et al*., 2006). Studies on tannase production by *Aspergillus* have been carried out in submerged and solid-state cultures.

Tannins are defined as naturally occurring water-soluble polyphenols of varying molecular weight depending on the bonds possessed with proteins and polysaccharides (cellulose and pectin). Tannins are widespread in the plant kingdom, and are found in the leaves, fruits, bark and wood. They occur in many edible fruits and vegetables and are often considered nutritionally undesirable because they form complexes with protein, starch and digestive enzymes and cause a reduction in nutritional value of food (Chung *et al*., 1998). The usual complexation of proteins with tannic acid and naturally occurring tannins to form water insoluble complexes inactivate the enzymes (Haworth *et al*., 1985). Tannins have since been shown to be the natural substrate for the tannase enzyme, the enzyme also attacks gallic acid methyl esters, but it possesses high specificity towards the acyl moiety of the substrate.

Hydrolysable tannins are composed of esters of gallic acid (gallotannins) or ellagic acid (ellagitannins) with a sugar core, which is usually glucose (Bhat *et al*., 1998). They can occur in wood, bark, leaves, fruits and galls (Mueller-Harvey, 2001). Major commercial hydrolysable tannin sources are Chinese gall (*Rhus semialata*), sumac (*Rhus coriaria*), Turkish gall (*Quercus infectoria*), tara (*Caesalpinia spinosa*), myrobalan nuts (*Terminalia chebula*) and chestnut (*Castanea sativa*) (Bhat *et al*., 1998). Hydrolysable tannins are readily hydrolyzed chemically by acidification or biologically by tannase. Various groups have reported the gallic acid production from myrobalan (Mukherjee and Banerjee, 2004), tara (Pourrat *et al*., 1985), sumac (Pourrat *et al*., 1987), gall nuts (Regerat *et al*., 1989), Chinese tannins (Kar *et al*., 1999), teri pod (*Caesalpinia digyna*) (Kar *et al*., 1999; Mukherjee and Banerjee, 2004) and sake cake (Kawakubo *et al*., 1993).

OBJECTIVES OF THE PRESENT STUDY

Marine fungi remain totally unexplored as a source of industrial enzyme and prospective applications. Further tannase production by a marine organism has so far not been established. The primary objective of this study included the evaluation of the potential of *Aspergillus awamori* isolated from sea water as part of an earlier study and available in the culture collection of the Microbial technology laboratory for tannase production through different fermentation methods, optimization of bioprocess variables by statistical methods, purification and characterization of the enzyme, genetic study, and assessment of its potential applications.

SPECIFIC OBJECTIVES OF THE STUDY INCLUDE THE FOLLOWING:

1. Selection of natural substrates for induction of tannase and gallic acid production by *Aspergillus awamori.*
2. Bioprocess development for tannase enzyme and gallic acid production by *A. awamori* under slurry-state fermentation, submerged fermentation and solid state fermentation.
3. Purification and characterization of tannase.
4. Isolation of gene encoding tannase and its characterization.
5. Application studies.

microorganisms to tannic acid concentration and the oxidation of the unused tannic acid. This limits the use of high tannic acid concentration during bioconversion process resulting in low productivity.

Two critical factors, production costs and insufficient knowledge of the basic characteristics, physicochemical properties, catalytic characteristics, regulation mechanisms and potential uses, limit the use of tannase at the industrial level. The industrial applications of tannase have not been fully exploited because of its high cost, although there are a large number of reports on the production of tannase by submerged fermentation and solid state fermentation. Vegetable residues such as coffee wastes, grape wastes, cashew wastes, wheat bran, rice bran etc supplemented with tannic acid were used as substrates for tannase production employing SSF. However, several studies have reported interesting advantages of tannase production by solid state culture (SSC) compared with that produced by Submerged culture (Belmares *et al*., 2004).

The traditional one-at-a-time optimization strategy is simple and useful in screening procedures, and the individual effects of medium components can be seen on a graph with no need to revert to more sophisticated statistical analyses. Unfortunately, this simple method frequently fails to locate the optimal response region, because the joint effects of factors on the response are not taken into account. It has been reported that the complexities and uncertainties associated with large-scale fermentations usually arise from a lack of knowledge of the sophisticated interactions amongst the various factors acting during fermentation (Egmond and Vlieg, 2000). Statistical experimental designs provide an efficient approach to optimization. The Box Behnken design is especially suitable in accounting for the interactions and identifying the more significant components in a medium formula. A combination of factors generating a certain optimal response can be identified from the use of a factorial design and response surface

methodology. Response surface methodology (RSM) is a powerful technique for testing multiple process variables, because fewer experimental trials are needed as compared to studying one variable at a time. Also, significant interactions between the variables can be identified and quantified by this technique. An important aspect of the commercialization of tannase is the development of an efficient and low-cost production system. In this setting, one of the main drawbacks is the expensive, time-consuming and low yield process of producing tannase.

The optimization studies done by varying one parameter while keeping the others at constant level do not reflect the interaction effects among these variables employed and this kind of optimization studies do not depict the net effect of the various factors on the enzyme activity. In order to overcome this major problem, optimization studies are done using response surface methodology (RSM) which is a mathematical and statistical technique widely used to determine the effects of several variables and to optimize different biotechnological processes (He and Tan, 2006). RSM has been extensively applied to optimize culture medium and other process parameters for the production of tannase (Battestin and Macedo, 2007a).

The marine environment which encompasses about 71% of Earth's surface is potentially a vast resource for useful enzymes. Microbes live in various habitats in the marine environment, including neuston, plankton, nekton, seston and epibiotic, endo biotic, pelagic and benthic environments. These habitats harbor a diverse range of microbes including archaebacteria, cyanobacteria, actinomycetes, yeasts, filamentous fungi, microalgae, algae or protozoa. Almost all these microorganisms are potential sources of useful enzymes. The ecological role of these microorganisms is in the mineralization and recycling of complex organic matter through degradative pathways and thus also contributes to the secondary production in the sea. Bacteria and fungi secrete different enzymes such as protease, amylase, lipase, chitinase, cellulose, ligninase, pectinase, xylanase,

nucleases (DNAases, RNAses, restriction enzymes etc) based on their habitat and ecological functions. Marine microorganisms are underutilized and untapped resources in terms of industrial enzymes. They are recognized to return several industrial enzymes provided due efforts are made (Chandrasekaran, 1997; Chandrasekaran and Rajeev kumar, 2002). Thus there is enormous scope for investigations exploring the probabilities of deriving new products of economic importance from potential marine organism.

Aspergillus sp. is one of the most important sources of tannase for industrial application. A large number of other microbes are also exploited for tannase production till this time. Irrespective of the availability of few microbes there is still demand for new and better strains as source of tannase in industry. Further there is absolute lack of knowledge on the performance of marine fungi as source of industrial enzyme and prospective applications. Hence, in this study efforts were made to develop a suitable bioprocess for large scale production of tannase using a marine *Aspergillus awamori* isolated from sea water and evaluate the scope for their varied applications, besides isolating the gene encoding tannase and its characterization. Several natural substrates were also tried as source of tannin and as an inducer for tannase production and in turn gallic acid production. The notable source tried was *Garcinia gummi gutta* leaves, which has wide medicinal properties. *Garcinia gummi-gutta* (syn. *G. cambogia, G. quaesita*), commonly known as Gambooge, Brindle berry, Brindall berry or Malabar tamarind, Kodumpulli (Kerala) Goraka (Sri Lanka) is a subtropical species of *Garcinia* native to Indonesia. Gambooge is grown for its fruit in Southeast Asia, and west and central Africa. Although submerged and solid state fermentation processes were employed by earlier investigators an effort was made in the present study to explore the prospects of slurry state fermentation along with other conventional fermentation techniques for tannase and gallic acid production by this marine fungus.

products can be detected in the hydrolysate of 1,2,3,4,6,-pentagalloyl glucose and that gallic acid of methyl.m.digallate is liberated first (Fig.2.4) Teighem accidentally discovered this unique enzyme in 1867 (Teighem, 1867) and reported the formation of gallic acid when two fungal species were exposed to an aqueous solution of tannins. The fungal species were later identified as *Penicillium glaucum* and *Aspergillus niger* (Lekha and Lonsane, 1997). Unfortunately, although biotechnologically useful, tannases do not satisfy all requirements for optimal versatility in industrial processes. They exhibit rather limited substrate spectra and are relatively expensive to purify, because they are secreted only at low levels by their microbial producers. There is therefore an ongoing search for new sources of tannases with more desirable properties for commercial applications.

Chapter 2

REVIEW OF LITERATURE

2.1 Tannin

The word tannin is very old and reflects a traditional technology. "Tanning" (waterproofing and preserving) was the word used to describe the process of transforming animal hides into leather by using plant extracts from different plant parts of different plant species. Plant parts containing tannins include bark, wood, fruit, fruit pods, leaves, roots, and plant galls. Tannins are phenolic compounds that precipitate proteins. Tannins are the fourth most abundant plant constituent after cellulose, hemicelluloses and lignin (Swain, 1965). They are composed of a very diverse group of oligomers and polymers. Tannins can complex with proteins, starch, cellulose and minerals.

Tannins are naturally occurring water soluble polyphenols with molecular weight ranging from 0.3-5 kDa. They are classified into three groups, hydrolysable tannins which consist of polyhydric alcohol esterified with gallic acid, condensed tannins formed from monomeric flavan-3-ol and recalcitrant to hydrolysis and catechin gallates which occupy an intermediate position sharing the properties of hydrolysable and condensed tannins (Bhat *et al*., 1998). Tannins are in fact antimicrobial agents, and most of the microorganisms cannot tolerate its polyphenolic nature. Only a few microorganisms can degrade tannic acid and utilize it as nutrient (Lekha and Lonsane, 1997).

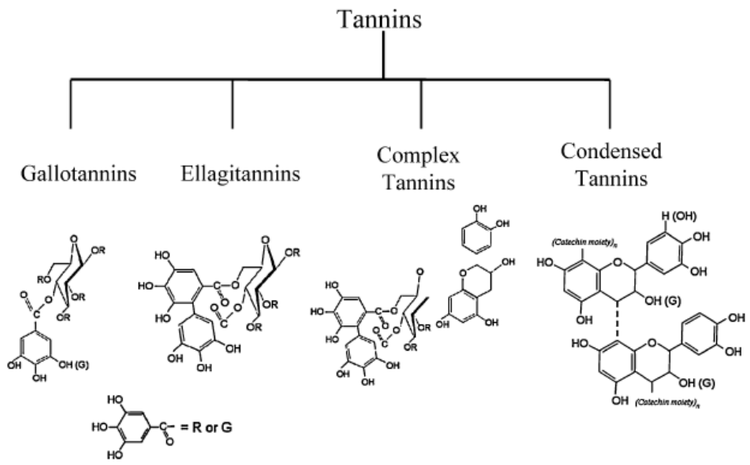


Fig.2.1 Major groups of tannins

According to Augilar *et al*., (2007) tannins are divided into four major groups: gallotannins, ellagitannins, condensed tannins, and complex tannins (Fig.2.1). Gallotannins are characterized by the presence of several molecules of organic acids, such as gallic, digallic, and chebulic acids, esterified to a molecule of glucose. On the other hand, ellagitannins have building blocks of ellagic acid units linked to glucosides. To maintain its binding capacity, gallotannins, and ellagitannins must have more than two acidic unit constituents esterified to the glucose core. Gallotannins can easily be hydrolysed under mild acid or alkaline conditions, either in hot water or enzymatically (Lopez-Rios, 1984). Ellagitannins are more stable than gallotannins. Condensed tannins or proanthocyanidins are complex compounds made of flavonoid building blocks (from 2 over 50) that are not considered to be easily hydrolyzable (Ramirez-Coronel *et al*., 2004). Complex

tannins can be generated through reactions between gallic or ellagic acids with catechins and glucosides.

Tannins are considered to be the plant's secondary metabolic products because they play no direct role in the plants metabolism. After lignin, tannins are the second most abundant group of plant phenolics. The large amount of phenolic hydroxyl groups allows the tannins to form complexes with proteins and to a lesser extent with other macromolecules like cellulose and pectin (Mueller-Harvey *et al.*, 1987). Hydrolysable tannins are composed of esters of gallic acid (gallotannins) or ellagic acid (ellagitannins) with a sugar core which is usually glucose (Bhat *et al*., 1998). They can occur in wood, bark, leaves, fruits and galls. Major commercial hydrolysable tannin sources are Chinese gall (*Rhus semialata*), sumac (*Rhus coriaria*), Turkish gall (*Quercus infectoria*), tara (*Caesalpinia spinosa*), myrobalan nuts (*Terminalia chebula*) and chestnut (*Castanea sativa*) (Bhat *et al*., 1998). Hydrolysable tannins are readily hydrolyzed chemically by acidification or biologically by tannase.

2.2 Tannase

Tannin Acyl Hydrolase (E.C. 3.1.1.20) is commonly referred to as tannase which catalyzes the hydrolysis of ester bond and depside bond present in hydrolysable tannins to form glucose and gallic acid (Fig.2.2). Tannase catalyzes the breakdown of hydrolysable tannins such as tannic acid, methyl gallate, ethyl gallate, n-propylgallate, and isoamyl gallate (Fig.2.3). It is well known that tannase hydrolyses the ester bonds of tannic acid, although tannic acid is known to denature proteins. Tannins have been shown to be the natural substrate for the tannase enzyme which also attacks gallic acid methyl esters. But it possesses high specificity towards the acyl moiety of the substrate. Tannase hydrolyses tannic acid completely to gallic acid and glucose through 2, 3, 4, and 6,-tetragalloyl glucose and two kinds of monogalloyl glucose. This is supported by the facts that the same

	Sabu *et al*., (2006)
Lactobacillus animalis	Nishitani *et al*., (2004)
Lactobacillus murinus	Nishitani *et al*., (2004)
Enterococcus faecalis	Goel *et al*., (2005)
Weissella paramesenteroides	Kostinek *et al*., (2007)
Leuconostoc fallax	Kostinek *et al*., (2007)
Leuconostoc mesenteroides	Kostinek *et al*., (2007)
Pediococcus acidilactici	Nishitani *et al*., (2004)
Pediococcus pentosaceus	Nishitani *et al*., (2004)
Citrobacter freundii	Belmares *et al*., (2004)
Selenomonas ruminantium	Belmares *et al*.,(2004)

Yeasts

Candida sp.	Aoki *et al*., (1976)
Saccharomyces cerevisiae	Zhong *et al*., (2004)
Mycotorula japonica	Belmares *et al*., (2004)
Pichia sp.	Deschamps *et al*., (1983)
Debaryomyces hansenii	Deschamps *et al*., (1983)

Fungi

Aspergillus niger	Bradoo *et al*., (1996); Rana and Bhat(2005); Cruz-Hernandez *e t al*., (2006); Trevino- Cueto *et al*., (2007); Murugan *et al*., (2007)
Aspergillus japonicus	Bradoo *et al*., (1997)
Aspergillus gallonyces	Belmares *et al*., (2004)
Aspergillus awamori	Bradoo *et al*., 1996); Mahapatra *et al*., (2005)
Aspergillus fumigates	Batra and Saxena, (2005)
Aspergillus versicolor	Batra and Saxena, (2005)
Aspergillus flavus	Yamada *et al*., (1968); Batra and Saxena, (2005)
Aspergillus caespitosum	Batra and Saxena, (2005)
Aspergillus oryzae	Bradoo *et al*., (1996)
Aspergillus aculeatus	Banerjee *et al*., (2001)
Aspergillus aureus	Bajpai and Patil, (1997)
Aspergillus fischeri	Bajpai and Patil, (1997)
Aspergillus rugulosus	Bradoo *et al*., (1996)
Aspergillus terreus	Bajpai and Patil, (1997)

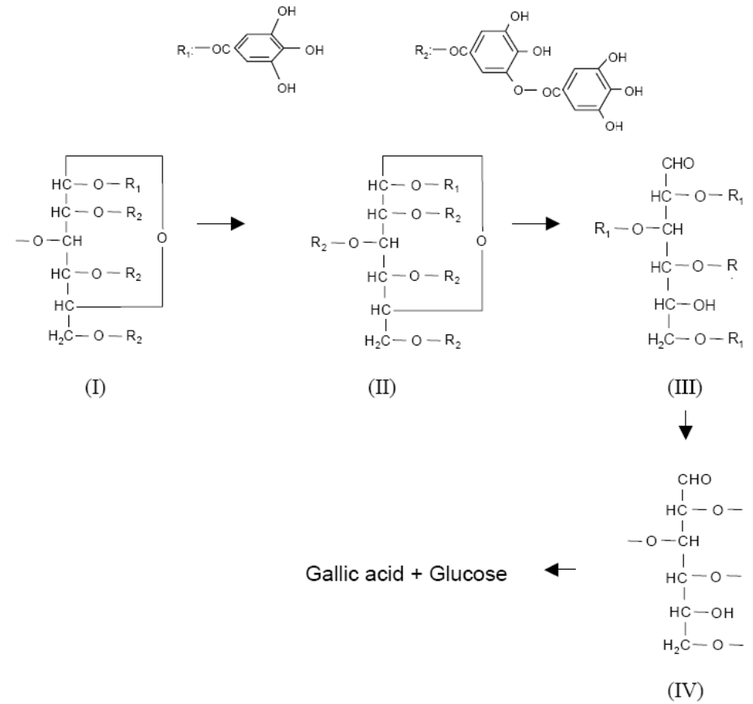


Fig. 2.2 Hydrolytic pathway of tannic acid by tannase.

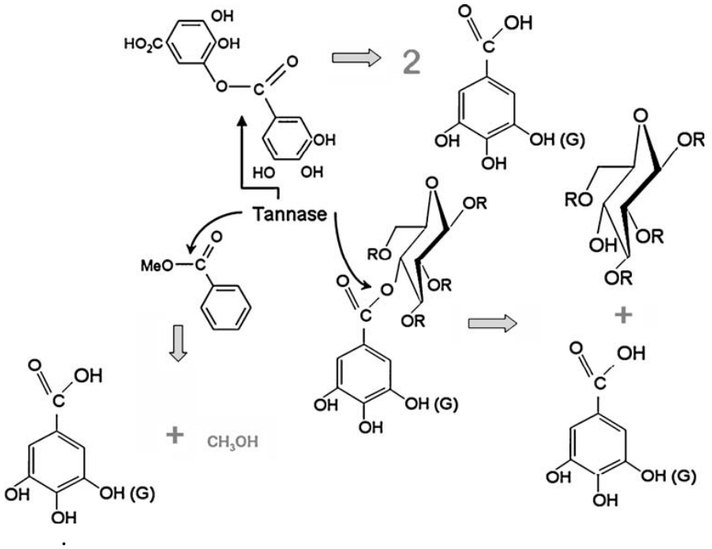


Fig.2.3 Mechanism of action

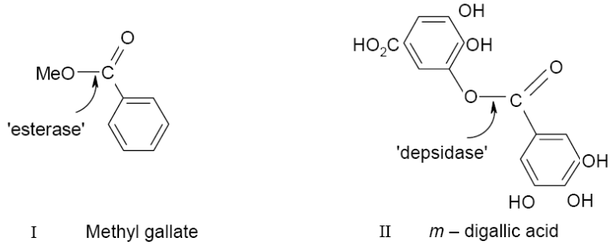


Fig.2.4 Esterase and depsidase activities of tannase

2.3 Microbial Sources of tannase

Although tannase is present in plants, animals, and microorganisms, it is mainly produced by the latter. Tannase is produced by bacteria, yeasts, and fungi (Table 2.1). The production and applications of tannase have been extensively studied; and investigations related to strain isolation and improvement, process development, and application of tannases has resulted in a great number of scientific publications and patents. According to Yamada *et al*., (1968) the enzyme was mainly found intracellularly although the culture broth also contained the enzyme. It was also evident from the growth studies that tannase enzyme was an inducible enzyme (Gupta *et al*., 1997; Jean *et al*., 1981 and Mattiason and Kaul, 1994). To utilize glucose, the organism may synthesize more tannase by which ester and depside bonds are hydrolyzed and glucose is available for the organism (Mohapatra *et al*., 2009). Table 2.2 presents some of the published patents regarding tannase production and application.

Table 2.1 Microbial sources of tannase

Microorganism	Reference
Bacteria	
Achromobacter sp.	Lewis and Starkey, (1969)
Bacillus pumilus	Deschamps *et al*., (1983)
Bacillus polymyxa	Deschamps *et al*., (1983)
Corynebacterium sp.	Deschamps *et al*., (1983)
Bacillus cereus	Mondal *et al*., (2001b)
Klebisella planticola	Deschamps *et al*., (1983)
Klebisella pneumoniae	Deschamps *et al*., (1983)
Pseudomonas solanaceanum	Deschamps *et al*., (1983)
Streptococcus bovis	Belmares *et al*., (2004)
Streptococcus gallolyticus	Sasaki *et al*., (2005)
Lactobacillus plantarum	Ayed and Hamdi, (2002); Kostinek *et al*., (2007)
Lactobacillus paraplantarum	Nishitani and Osawa, (2003); Nishitani *et al*., (2004)
Lactobacillus pentosus	Nishitani *et al*., (2004); Kostinek *et al*., (2007)
Lactobacillus acidophilus	Nishitani *et al*., (2004);

by many researchers in its original and modified forms. This method was based on decrease in the absorbance of the substrate tannic acid at 310nm. Haslam and Tanner, (1970) developed a spectrophotometric method which used p-nitrophenyl esters of gallic acid as substrate. Hagerman and Butler, (1976) developed a protein precipitation method for quantitative determination of tannins. Deschamps *et al*., (1983) studied tannase activity by measuring the gallic acid produced at 260 nm after precipitating the residual tannic acid using BSA solution. Inoue and Hagerman, (1988) described a method for determination of gallotannins which involves the formation of a chromogen between gallic acid obtained by the acid hydrolysis of gallotannins and rhodanine. Sharma *et al*., (2000) modified later their method using methyl gallate as a substrate for tannase based on the formation of chromogen between liberated gallic acid and rhodanine. Mondal *et al*., (2001a) developed a modified Hagerman and Butler method to measure the residual tannic acid, which in turn measures the tannase enzyme activity. Some investigators used gas chromatography (Jean *et al*., 1981) and high performance liquid chromatography (Barthomeuf *et al*., 1994; Niehaus and Gross, 1997) for measuring the gallic acid produced.

2.6 *Aspergillus* sp. as major source of tannase

Aspergillus is a genus under the Kingdom Fungi, Phylum Ascomycota, Class Eurotiomycetes, Order Eurotiales, Family Trichocomaceae. *Aspergillus* was first catalogued in 1729 by the Italian priest and biologist Pietro Antonio Micheli. Species of *Aspergillus* are highly aerobic and are found in almost all oxygen-rich environments, where they grow as molds on the surface of substrates, as a result of the high oxygen tension. They are common contaminants of starchy foods (such as bread and potatoes), and grow in or on many plants and trees. In addition to growth on carbon sources, many species of *Aspergillus* demonstrate oligotrophy where they are capable of growing in nutrient-depleted environments, or environments in which there is a complete lack of key nutrients.

Aspergillus foetidus	Banerjee *et al*., (2005)
Penicillium notatum	Ganga *et al*., (1977)
Penicillium islandicum	Ganga *et al*., (1977)
Penicillium chrysogenum	Bradoo *et al*., (1996)
Penicillium digitatum	Bradoo *et al*.,(1996)
Penicillium acrellanum	Bradoo *et al*., (1996)
Penicillium carylophilum	Bradoo *et al*., (1996)
Penicillium citrinum	Bradoo *et al*., (1996)
Penicillium charlessi	Bradoo *et al*., (1996); Batra and Saxena (2005)
Penicillium variable	Batra and Saxena, (2005)
Penicillium glaucum	Lekha and Lonsane, (1997)
Penicillium crustosum	Batra and Saxena, (2005)
Penicillium restrictum	Batra and Saxena, (2005)
Penicillium glabrum	Van de Lagemaat and Pyle, (2005)
Trichoderma viride	Bradoo *et al*., (1996)
Trichoderma hamatum	Bradoo *et al*., (1996)
Trichoderma harzianum	Bradoo *et al*., (1996)
Fusarium solani	Bradoo *et al*., (1996)
Fusarium oxysporium	Bradoo *et al*., (1996)
Mucor sp.	Belmares *et al*., (2004)
Paecilomyces variotii	Mahendran *et al*., (2005); Battestin and Macedo (2007a)
Rhizopus oryzae	Hadi *et al*., (1994); Purohit *et al*., (2006)
Cryphonectria parasitica	Farias *et al*.,(1994)
Heliocostylum sp.	Bradoo *et al*.,(1996)
Cunnighamella sp.	Bradoo *et al*., (1996)
Syncephalastrum racemosum	Bradoo *et al*., (1996)
Neurospora crassa	Bradoo *et al*., (1996)

Table 2.2 Published patents regarding tannase production and application

Year	Title	Patent no.
1974	Conversion of green tea and natural tea leaves using tannase	USP3812266
1975	Production of tannase by *Aspergillus*	JP7225786
1975	Tea soluble in cold water	UKP1280135
1976	Extraction of tea in coldwater	GP2610533
1976	Enzymatic solubilization of tea cream	USP3959497
1985	Gallic acid ester(s) preparation	EP-137601
1985	Enzymatic treatment of black tea leaf	EP135222
1987	Preparation of tannase	JP62272973
1987	Manufacturing of tannase with *Aspergillus*	JP62272973
1988	Production of tannase by *Aspergillus oryzae*	JP63304981
1988	Elaboration of tannase by fermentation	JP63304981
1989	Preparation of spray-concrete coating in mining shaft	SUP1514947
1989	Antioxidant catechin and gallic acid preparation	JP01268683
1989	Tannase production by culture of *Aspergillus tamarii*	EP-339011
1989	New *Aspergillus niger* B1 strain	EP307071
1989	Tannase production process by *Aspergillus* and its application to obtain gallic acid	EP339011
1992	Tannase preparation method	JP4360684
1995	Enzymatic clarification of tea extracts	USP5445836
1997	DNA fragment containing a tannases gene, a recombinant plasmid, a process for producing tannases, and a promoter	USP5665584

2000	Tea concentrate prepared by enzymatic extraction and containing xanthan gum that is stable at ambient temperature	USP6024991
2000	Producing theaflavin	USP6113965
2004	Compositions based on vanilloid catechin synergies	USP6759064
2006	Diagnostic agent and test method for colon cancer using tannases as index	USP7090997
2006	Isolation of a dimmer di-gallate a potent endothelium-dependent vasorelaxing compound	USP7132446
2007	Diagnostic agent and test method for colon cancer using tannase as index	USP7090997

2.4 Screening and selection of potential strains

Qualitative screening for tannase producers are carried out on tannic acid agar plates (TAA) containing 1 % tannic acid and 3 % agar and recording the clear zones formed due to hydrolysis of tannic acid around the fungal colony (Bradoo *et al*., 1997). Deceptive halo formation by tannase defective bacteria on tannic acid plate media due to catalase production was also reported (Nishitani and Osawa, 2005). Hence, a quantitative method adopted for the screening of tannase producers which was mainly based on tannase assay in the enzyme production medium.

2.5 Tannase Assay

Tannase catalyzes the hydrolysis of ester and depside linkages in hydrolysable tannins like tannic acid (Fig.2.4). The product of hydrolysis is glucose and gallic acid. Numerous methods are standardized and available in literature for measuring the hydrolytic activity. The oldest method of tannase assay was based on the titration of gallic acid released by the action of enzyme on tannic acid, but determination of end point was not successful (Nishira, 1961). Madhava Krishna *et al*., (1960) reported another method of estimation of glucose liberated by incubation with enzyme for 24 h which was unfit for routine assays of the enzyme. Iibuchi *et al*., (1967) described a photometric method that has been used

2.7.1 Submerged fermentation

Submerged liquid fermentations are traditionally used in the United States for the production of microbe derived enzymes. Submerged fermentation involves submersion of the microorganism in an aqueous solution containing all the nutrients needed for growth. Submerged fermentation with tannic acid as the substrate has been widely reported for tannase production. Some studies concerning optimum production and regulatory aspects of tannase by moulds have been carried out under submerged culture systems (Bradoo *et al*., 1997; Bajpai and Patil, 1997).

Key environmental parameters that play important role in tannase synthesis by *A. awamori* were identified and optimized by response surface methodology (Seth and Chand, 2000). Tannic acid concentration, agitation speed, and pH during the fermentation were identified as important process parameters effecting cell growth and enzyme synthesis by *A. awamori*. These parameters were optimized in a laboratory bioreactor by response surface methodology using Box and Behnken factorial design to determine the optimum conditions for enzyme production and gallic acid accumulation. Under optimum process conditions for enzyme synthesis, the fermentation run lasted 60 h with an initial tannic acid concentration of 35.0 g /l, yielding biomass concentration of 7.13 g /1 containing 771 IU of intracellular tannase per gram dry cell weight and 19 g /1 of gallic acid. However, maximum gallic acid accumulation (40.3 g /l) was obtained in 24 h with an initial substrate concentration of 45 g /l.

Tannases are induced by tannic acid or by some of its derivatives but the regulatory mechanism of its production remains uncertain. Huang *et al*., (2005) used tannase as a model system to view experimentally the differences in enzyme regulation mechanism in both culture systems. Induction and repression patterns of tannase production by *A. niger* Aa-20 in solid-state (SSC) and submerged culture

Earlier investigations on tannase production indicated very clearly that almost all species of *Aspergillus* are capable of synthesizing tannase on induction. *Aspergillus* sp. capable of growing on tannic acid medium containing it as sole carbon source might definitely produce tannase for its survival. Van Diepeningen *et al*., (2004) tested 34 black *Aspergillus* strains from culture collections and 46 wild isolates from air, soil surfaces and other substrates individually on different concentrations of tannic acid. They belonged to subgroups of *A. niger, A. aculeatus, A. ellipticus, A. heteromorphus, A. tubigenesis, A. braseliensis, A. carbonarius and A. japonicus.* All could grow on medium containing 20 % tannic acid. Some of the strains were tested on higher amounts and growth on 80 % tannic acid polymers was not exceptional. Although tannase production by *Aspergillus* can occur in the absence of tannic acid, black *Aspergillus* sp. could utilize more tannic acid than non black *Aspergillus* sp. Among the various species, *Aspergillus niger, A. flavus* and *A. oryzae* were found to be the best tannase producers using tannic acid as a sole source of carbon.

Out of 60 *Aspergilli* screened for tannase production, 25 were found to be tannase producers (Batra and Saxena, 2005). Tannase-producing ability of 35 *Aspergilli* and 25 *Penicillii* were examined both qualitatively on tannic acid agar plates and quantitatively in broth. Twenty-five *Aspergilli* and 20 *Penicillii* produced tannase in culture broth. Potent tannase-producing *Aspergilli* included *Aspergillus fumigatus* (8.3 IU/ml), *Aspergillus versicolor* (7.0 IU/ml), *Aspergillus flavus* (4.95 IU/ml) and *Aspergillus caespitosum* (4.47 IU/ml) and among *Penicillii*, species of *Penicillium charlesii* (4.82 IU/ml), *Penicillium variable* (4.70 IU/ml), *Penicillium crustosum* (4.7 IU/ml) and *Penicillium restrictum* (4.47 IU/ml) are known as tannase producers. The crude tannase from these fungi showed pH optima of 5.0, except in *A. caespitosum*, *P. crustosum* and *P. variable*, which had pH optima of 6.0. Optimum tannase activity was at 60ºC in most of the potent producers, except in *A. caespitosum, P. charlesii*, *P. crustosum* and *P. restrictum*,

which showed temperature optimum of 40ºC. Among the selected *Aspergilli* and *Penicillii*, tannase from *A. versicolor* and *P. restrictum* were stable over a broad pH range of 3.0 - 8.0 for 24 h. The tannase from *A. versicolor* is heat stable. The various species of *Aspergilli* that produce tannase is presented in the Table 2.3

Table 2.3 The various species of *Aspergilli* that produce tannase (Batra and Saxena, 2005).

***Aspergillus* sp. screened**	**Tannase activity (IU/ml)**	***Aspergillus* sp. screened**	**Tannase activity (IU/ml)**
Aspergillus columaris	0.10	*A. flavipes*	1.33
A. striatus	0.10	*A. flavus*	4.95
A. alliaceu	0.35	*A. fumigatus*	8.30
A. terreus	0.47	*A. janus*	0.05
A. amstellodemi	0.70	*A. japonicus*	2.47
A. versicolor	7.05	*A. nidulans*	0.82
A. awamori	4.11	*A. niger*	1.65
A. caespitoum	4.47	*A. oryzae*	0.10
A. carneus	0.47	*A. parasiticu*	4.12
A. fischeri	0.27	*A. penicilliformis*	0.94

The ability of *A. niger* to grow on tannic acid medium may be linked to its saprophytic lifestyle. *A. niger* tolerates tannic acid concentrations as high as 20 % without having a deleterious effect on both growth and enzyme production (Van Diepeningen *et al*., 2004; Cruz-Hernández *et al*., 2006). Lekha *et al*., (1993), isolated a potential tannase producer *A. niger* PKL104 from soil. Sharma *et al*., (1999), isolated *A. niger* from cattle feed with tannin rich material and purified tannase. *A. niger* GH1 was isolated from leaves of *Larrea tridentate* cov., a native plant of Mexican semiarid region as a best tannin degrading strain and is now a part of fungal collection Food Research Department UAdeC (Cruz-Hernandez *et al*., 2005). *A. niger* HA 37 was isolated from oil mill waste water, a substrate containing an important amount of hydrolysable tannins acting as inducer for tannase production (Aissam *et al*., 2002), Later this organism was used for

production of tannase from oil mill waste water (Aissam *et al*., 2005). Sabu *et al*., (2005a) purified the extracellularly produced tannase by the fungus *A. niger* ATCC16620.

Seth and Chand, (2000), reported biosynthesis of tannase and hydrolysis of tannin to gallic acid by *A. awamori* and optimization of process parameters. Mondal *et al*., (2001a) carried out a quantitative survey on microbial population producing tannase and reported *A. accalearus* DBFG as the best tannase producer. Bardoo *et al*., (1996) screened 55 fungi belonging to various groups for extracellular tannase production and reported *Aspergillus* species as the best producer. Several fungal strains isolated from different locations including garbages, forests and orchards were screened for tannase production and recorded *A. ruber* as the best strain with maximum enzyme yield.

2.7 Tannase production

Studies on tannase production by *Aspergillus* have been carried out under submerged, solid-state and modified solid state fermentation conditions. Depending on the strain and the culture conditions, the enzyme was induced and expressed with different levels of activity, showing different production patterns. Phenolic compounds such as gallic acid, pyrogallol, methyl gallate, and tannic acid induced tannase synthesis (Bajpai and Patil, 1997). However, the induction mechanism has not been clearly demonstrated, and there is some controversy about the role of some of the hydrolyzable tannin constituents as related to the synthesis of tannase (Deschamps *et al*., 1983; Aguilar *et al*., 2001a).

The concept of the tannase production process is to use coffee pulp or coffee pulp juice as a tannin-rich substrate and achieve direct breakdown of the hydrolysable tannins present. Earlier studies suggested that SSF is advantageous over conventional submerged fermentation for the productive yield of tannase which is an inducible enzyme using coffee wastes The developed process could potentially be used with other tannin-rich agricultural residues such as cassava, carob bean, wine-grape and tea waste (Lekha and Lonsane 1997).

Aguilar *et al*., (1999) reported productivities of 6.667 UE/Lh and 1.275 UE/Lh for tannase under SSC and SmC, respectively, and the maximum tannase activity expressed intracellularly was also 18 times more in SSC than in SmC, while the extracellular activity was 2.5 times higher in SSC than in SmC. The interaction effects of five variable parameters on gallic acid yield by the application of Evolutionary Operation (EVOP) factorial design technique were presented. Aguilar *et al*., (2001b) compared tannase production in SmC and SSC found that an initial glucose concentration of 25 mg/ml catabolically repressed the enzyme synthesis due to induction ratio (I.R.), which was lower than basal activity. While, in SSC the I.R. decreased significantly with an initial glucose amount of 200 mg/ml, however it was higher than basal activity. These results showed that SmC is highly sensitive to catabolic repression in comparison with SSC system. The differences in induction and repression patterns were associated with substrate diffusion aspects in both culture systems.

Enzyme production by *A. ruber* was studied under solid state fermentation using different tannin rich agro-wastes, as the substrate. They included ber leaves (*Zyzyphus mauritiana*), jamun leaves (*Syzygium cumini*), amla leaves (*Phyllanthus emblica*) and jawar leaves (*Sorghum vulgaris*). Jamun leaves were found to be the best substrate for enzyme production under solid-state fermentation (SSF) during which the maximum production of tannase was recorded at 30.1ºC after 96 h of

(SmC) were established. Tannic acid and glucose were used as carbon sources. Induction and repression ratios were obtained with different concentrations of tannic acid and glucose, respectively. Using valonia tannin (an ellagitannin) as the substrate, factors influencing the yield of ellagic acid and biosynthesis of valonia tannin hydrolase by *Aspergillus* SHL 6 were investigated. Valonia tannin concentration, pH, temperature, carbon, and nitrogen sources during the fermentation were identified as important hydrolysis parameters. The yield of ellagic acid and the hydrolase activity of valonia tannin both had maximum values (within the range of the tests) when 100 ml of culture medium containing 5.0 g/l of valonia tannin in a 250 ml conical beaker was incubated at 28º C and initial pH from 4.5 to 5.0 using a rotary shaker at 120 rpm for 72 h (Huang *et al*., 2005).

Production of tannase by *A. niger* HA37 on a synthetic culture medium containing tannic acid at different concentrations has been studied. Maximal enzyme activity increased according to the initial concentration of tannic acid. Tannase production by *A. niger* HA37 on four fold diluted olive mill waste waters (OMWW) as substrate, was between 0.37 and 0.65 EU/ ml. Enzyme production on the diluted OMWW remained globally stable during more than 30 h. Growth of *A. niger* HA37 on OMWW was correlated with about 70 % degradation of phenolic compounds present in the waste. This strain has therefore the capacity to degrade complex wastewaters which cause environmental damage to aquatic streams (Aissam *et al*., 2005). Maximum tannase production occurred in the culture broth containing 1-2 % (w/v) tannic acid and 0.05–0.1 % (w/v) glucose. The pH, incubation period, temperature and glucose concentration optima of tannase production was found at 5.5, 36 h, 35°C and 0.5 % respectively (Lokeswari and Jaya raju, 2007).

The optimization studies done by varying one parameter while keeping the others at constant level do not reflect the interaction effects among these variables

employed and this kind of optimization studies do not depict the net effect of the various factors on the enzyme activity. In order to overcome this major problem, optimization studies are done using response surface methodology (RSM), which is a mathematical and statistical technique widely used to determine the effects of several variables and to optimize different biotechnological processes (He and Tan, 2006). Response Surface methodology (RSM) has been extensively applied to optimize culture medium and other process parameters for the production of lipase (He and Tan, 2006; Liu *et al*., 2006), tannase (Battestin and Macedo, 2007), α-amylase (Uma Maheswar Rao and Satyanarayana, 2007), β-cyclodextrin glucanotransferase (Ibrahim *et al*., 2005), dextran dextrinase (Naessens *et al*., 2004) and chitinase (Nawani and Kapadnis, 2005).

2.7.2 Solid state fermentation

In addition to submerged liquid fermentation, an ancient fermentation technology known as solid-substrate fermentation is also used to produce enzymes. Solid-substrate fermentations are generally characterized by growth of microorganisms on water-insoluble substrates in the presence of varying amounts of free water (Mitchell and Lonsane, 1992). This process is also referred to as solid state fermentation (SSF). Solid-state fermentation can be defined as a fermentation that takes place on a solid or semisolid substrate or in a nutritionally inert support. The origin of SSF can be traced back to bread-making in ancient Egypt. Solid state fermentations include a number of well-known microbial processes such as soil growth, composting, silage production, wood rotting and mushroom cultivation. In addition, many familiar western foods, such as mold-ripened cheese, bread and sausage, and many oriental foods including miso, tempeh and soy sauce, are produced using SSF. Beverages derived from SSF processes include ontjom in Indonesia, shao-hsing wine and kaoliang (sorghum) liquor in China and sake in Japan (Mudgett, 1986).

Microbial attachment to the solids, whether inert or degradable, and the low water conditions make SSF rather different from the more common submerged fermentation. From the point of view of process intensity a key question is to what extent the advantage of operating in a concentrated environment is offset, if at all, by changes in the process kinetics (Van de Lagemaat and Pyle, 2001). If the process intensity and stability can be assured, SSF could be the system of choice for selected “added-value” applications such as the production of fermented foods, enzymes and other extracellular metabolites like organic acids and flavor compounds. Applications of SSF to other than purely profit-driven objectives, such as environmental control, include the production of compost and animal feed from solid waste.

In 1896, Takamine produced a digestive enzyme, Takadiastatse, by SSF employing *A. niger* on wheat bran (Takamine, 1914). This led to the application of SSF in other food and beverage industries. The most profitable applications of SSF are in the Oriental and African countries where SSF processes have been perfected over long periods. SSF processes are recognized by investigators to be suitable for the production of enzymes by filamentous fungi since they reproduce the natural living conditions of such fungi (Rodrıguez-Couto and Sanroman, 2005).

Several studies have reported interesting advantages of tannase production by solid state culture (SSC) over SmC (Barthomeuf *et al*., 1994; Chaterjee, *et al*., 1996; Lekha and Lonsane, 1997; Ramirez-Coronel *et al*., 1999; Aguilar *et al*., 1999; Aguilar *et al*., 2001a, b; Aguilar *et al*., 2002; Van de Lagemaat and Pyle, 2001). Attractive advantages indicated in the literature included the high production titers (up to 5.5 times more than in SmC), the extracellular nature of the enzymes and the stability to wide pH and temperature ranges (Lekha and Lonsane, 1994).

reported to produce gallic acid from crude extract of tara gallotannin (Deschamps, and Lebeault, 1984).

Despite the immense commercial importance of gallic acid a little work has been done on development of a process for gallic acid production at fermenter level (Pouratt *et al*., 1987). The main hindrance in the development of a successful bioconversion process is the sensitivity of the microorganisms to tannic acid concentration and the oxidation of the unused tannic acid. This limits the use of high tannic acid concentration during bioconversion process resulting in low productivity. Therefore, the authors have developed a strategy to overcome the above mentioned problems and achieve high percent conversion during bioconversion. A protocol for gallic acid production in a 5 liter fermenter at 40 g/l of tannic acid concentration extracted from *Quercus infectoria* gall nuts using *Aspergillus fischeri* MTCC 150 has been reported, (Bajpai and Patil, 2008).

Terpod (*Caesalpinia dignia*) cover powder containing tannin was used as the substrate for microbial conversion to gallic acid employing a new method, Modified Solid State Fermentation (MSSF), by Kar *et al*., (1999). The purpose of using such a system was to improve the yield of gallic acid by *Rhizopus oryzae*, a filamentous fungus. They reported that MSSF supported best results because some of the disadvantage of SSF could be easily overcome by MSSF method of cultivation. A comparison of different types of fermentation conditions optimized by Kar *et al*., (1999) is presented in Table 2.5.

incubation. Tap water was found to be the best moistening agent, with pH 5.5 in the ratio of 1:2 (w/v) with substrate. Addition of carbon and nitrogen sources to the medium did not increase tannase production (Kumar *et al.*, 2007).

Tannase production by *A. niger* Aa-20 was studied in submerged (SmF) and solid-state (SSF) fermentation systems with different tannic acid and glucose concentrations. Tannase activity and productivity were at least 2.5 times higher in SSF than in SmF. Addition of high tannic acid concentrations increased total tannase activity in SSF, while in SmF it was decreased. Tolerance to high concentrations of tannic acid by *A. niger* Aa-20 was lower in SmF than in SSF. In SSF an increase in tannic acid enhanced the expression of tannase activity. The addition of glucose (20 g /l) resulted in strong catabolite repression in SmF system. The tannase: biomass yield in SSF was at least 2 times higher than in SmF. The results presented the capacity of SSF to minimize catabolite repression. The role of gallic acid in tannase regulation was also studied (Aguilar, 2000; 2001b).

Palm kernel cake (PKC), the residue obtained after extraction of palm oil from oil palm seeds and tamarind seed powder (TSP) obtained after removing the fruit pulp from tamarind fruit pod were tested for the production of tannase under solid-state fermentation (SSF) using *A. niger* ATCC 16620. The fungal strain was grown on the substrates without any pretreatment. In PKC medium, a maximum enzyme yield of 13.03 IU/g dry substrate (gds) was obtained when SSF was carried out at 30°C, 53.5 % initial substrate moisture, 33×10^9 spores/5 g substrate inoculum size and 5 % tannic acid as additional carbon source after 96 h of fermentation (Sabu *et al*., 2005a). Extra and intracellular tannase production by *A. niger* GH1 has been evaluated using submerged (SmF) and solid-state fermentation (SSF) at different temperatures (30, 40 and 50°C). Effects of initial substrate (tannic acid) concentration, incubation time and temperature on tannase production in SSF have been studied. *A. niger* GH1 produced the highest tannase level

(2291 U/L) in SSF at 30°C during the first 20 h of culture at tannic acid concentration of 50 g/l, and under these conditions enzyme production was entirely extracellular *(*Cruz-Hernandez *et al*., 2005*)*. Tannase production under SSF by *A. niger* Aa-20 using gobernadora powder as the sole carbon source and as an inducer of tannin-degrading enzymes was evaluated. Tannase production reached values of 1040 Ul/1 at 43 h of culture. During the first 48 h of culture, the concentration of gallic acid accumulation was 0.33 g/l. It was observed that *Gobernadora* is a potential source of gallic acid and tannase production by solid state culture (Trevino-Cueto *et al*., 2006). Materials used as supports of SSF for tannase production and tannin-rich materials used as enzyme inducer in SSC and SmF are presented in Table 2.4.

Powdered fruits of *Terminalia chebula* and powdered pod cover of *Caesalpinia digyna* was used in the process and the different process parameters for maximum production of tannase and gallic acid by co-culture method were optimized through media engineering (Banerjee *et al*., 2005).

Table 2.4 Materials used as supports of SSC for tannase production and tannin-rich materials used as enzyme inducer in SSC and SmC

Traditional supports	Reference
Sugarcane bagasse	Lekha and Lonsane (1994); Garcia-Pena *et al*., (1999)
Wheat bran	Chaterjee *et al*., (1996); Sabu *et al*., (2005b)
Polyurethane foam	Ramirez-Coronel *et al*., (1999); Aguilar *et al*., (2001b); Van de Lagemaat and Pyle (2001, 2005)
Tamarind seed powder	Sabu *et al*., (2005a)
Palm kernel cake	Sabu *et al*., (2005b)
Chestnut bark	Deschamps *et al*., (1983)
Tara (*Caesalpinia spinosa*) tannins	Pourrat *et al*., (1985)
Gall nuts (*Quercus infectoria*)	Barthomeuf *et al*., (1994)
Rhus coriaria leaves	Barthomeuf *et al*., (1994)
Fruits of *Terminalia chebula*	Banerjee *et al*., (2005)
Pod cover of *Caesalpinia digyna*	Banerjee *et al*., (2005)
Quercus aegylops tannins	Shi *et al*., (2005)
Jawar leaves (*Sorghum vulgaris*)	Kumar *et al*.,(2007)
Amla leaves (*Phyllanthus emblica*)	Kumar *et al*.,(2007)
Jamun leaves (*Syzygium cumini*)	Kumar *et al*.,(2007)
Ber leaves (*Zyzyphus mauritiana*)	Kumar *et al*., (2007)
Creosote bush leaves	Trevino-Cueto *et al*., (2007)

2.7.2.1 Gallic acid Production

Conventionally gallic acid is produced by acid hydrolysis of tannic acid but it has cost, yield and low purity disadvantages. Alternatively, gallic acid can be produced by the microbial hydrolysis of tannic acid by tannase (tannin-acyl hydrolase), an inducible enzyme, secreted by microorganisms. Microbial production of tannase, especially from fungi, is well documented, however, the reports on tannic acid hydrolysis is limited. Mainly *Aspergilli* have been used for hydrolysis of tannic acid to yield gallic acid (Mondal *et al*., 2001; Seth and Chand, 2000), among bacteria *Klebsiella pneumoniae* and *Corynebacterium* sp. have been

exchange chromatography. A purification fold of 19.5 with 13.5 % yield was obtained (Chhokar *et al*., 2009). Precipitation of tannase using polymers 1-90 % such as polyethylene glycol, poly vinyl alcohol and dextran have been reported (Naidu *et al*., 2008). Ultra filtration membranes were also used in concentrating the enzyme recently (Sharma *et al*., 2007; Marco *et al*., 2009).

The second step involved in most of the cases was ion exchange chromatography (Raj kumar and Nandy, 1983; Lekha and Lonsane, 1994; Mahapatra *et al*., 2005; Battestin and Macedo, 2007b). Tannase is said to be an acidic protein (Adachi *et al*., 1968) and hence anion exchanger was used in all the cases reported. The last step employed in tannase purification was gel filtration chromatography. Since tannase is a high molecular weight protein sephadex G-200 was used by most of the workers (Raj kumar and Nandy 1983; Lekha and Lonsane, 1994; Sharma *et al*., 2007) An extracellular tannase produced by solid-state cultures of *A. niger* was purified to homogeneity from the cell-free culture broth by preparative isoelectric focusing and by fast protein liquid chromatography (FPLC) using anion-exchange and gel-filtration chromatography (Ramirez-Coronel *et al*., 2003). Sephadex G-100, and G-50 (Ramirez-Coronel *et al*., 2003) and sephacryl S-300 gelfiltration (Marco *et al*., 2009) were also used to separate a tannase from *A. niger* GH1.

Internal sequences were obtained from each of the gel-purified and trypsin digested tannase forms. The peptide sequences obtained from both forms were identical to sequences within a β-glucosidase from *A. kawachii*. The purified tannase was tested for β-glucosidase activity and was shown to hydrolyze cellobiose efficiently. However, no β-glucosidase activity was detected when the enzyme was assayed in the presence of tannic acid (Ramirez-Coronel *et al*., 2003).

Table 2.5 Comparison of different types of fermentation conditions optimized for gallic acid production by *Rhizopus oryzae* (Kar *et al*., 1999***).***

Parameters	**SSF**	**SMF**	**MSSF**
Incubation period	72h	48h	72h
Substrate concentration	2%	10%	0 20%
pH	4.5	5	4.5
Temperature	32ºC	37ºC	32ºC
Fermentation condition	Stationary	Agitation	Stationary
Tannase	18.87 U	23.86U	32.76U
Gallic acid yield	30.48%	27.5%	90.9%

Depending on the strain and the culture conditions, the enzyme can be constitutive or inducible, showing different production patterns. Phenolic compounds such as gallic acid, pyrogallol, methyl gallate and tannic acid induced tannase synthesis (Bajpai and Patil, 1997). However, the induction mechanism has not been demonstrated and there is some controversy about the role of some of the hydrolysable tannin constituents on the synthesis of tannase (Deschamps *et al*., 1983). For instance, gallic acid, one of the structural constituents of some hydrolysable tannins, such as tannic acid, has been reported as an inducer of tannase synthesis under submerged fermentation, while it represses tannase synthesis under solid state fermentation. Nevertheless, independent of the involved mechanism, it has been well accepted that due to the complex composition of the hydrolysable tannins, some of their hydrolysis products induces tannase synthesis (Aguilar *et al*., 2002).

Catabolic repression of tannase in solid-state culture of *Aspergillus niger* Aa-20 on polyurethane foam was investigated considering the diffusion of a repressor molecule as the main responsible factor. The fundamental hypothesis considers that if repressor molecule uptake rate is higher than repressor molecule diffusion rate on solid matrix, the catabolic repression phenomenon will be

minimized. Results showed that addition of low glucose concentrations enhanced the extracellular and intracellular tannase activities (from 5.8 to 6.6 and from 0.96 to 1.76 IU). Besides the glucose uptake rate was higher (1.23 x 10^{-5} cm^2/seg) than glucose diffusion rate on solid matrix (2.8 x 10^{-6} cm^2/seg). On the contrary result at high glucose concentrations showed that extracellular tannase activity decreased from 6.6 to 1.87 IU (Cerda Montalvo *et al*., 2005).

2.7.3 Slurry state fermentation

The slurry state fermentation, recently reported by De Gregorio *et al.*, (2002), may be considered as an intermediate technology between solid-state fermentation and submerged fermentation. The slurry-state fermentation of pectinse by *A .niger* P-6021 on *Citrus changshan-huyou* peel residue, coupled with wheat bran, was found to be a feasible process (Zhong and Cen, 2005). A comparative study on detection of different pectinolytic activities of a novel strain of *Streptomyces lydicus* was performed in the three different modes of fermentation, SSF, SmF and SLF (Jacob and Prema, 2006). Single cell protein (SCP) and crude pectinolytic enzymes production from citrus pulps is reported. SCP and enzymes were produced by slurry-state flask cultivation of *Aspergillus niger* and *Trichoderma viride* on pulps from lemon juice clarification (De Gregorio *et al*., 2002). In fact till this time no reports are available on tannase production by slurry state fermentation by any fungus.

2.8 Purification and characterization of tannase

Several strategies can be used for tannase concentration or purification and immobilization after extraction from the biomass (solid state fermentation) or from the culture medium (submerged fermentation).

To increase the specific activity of the enzymatic preparation, tannase should be concentrated. For that, classical methods such as salt or solvent

precipitation, ultra filtration followed by ion exchange or size exclusion chromatography, as well as solvent extraction are used (Lekha and Lonsane, 1994). On the other hand, tannase extraction strongly depends on the fermentation system used. As tannase is mostly extracellular when produced by SSC, it can be easily extracted with water or a buffer. Two to three volumes of the reagent is well mixed with the fermented mass and pressed to obtain the enzymatic extract.

Tannase location during its production by SmF depends on the cultivation time (Rajkumar and Nandy, 1983). Though it is mainly intracellular at the beginning of the culture, it is later secreted to the culture medium. However, up to 80 % of tannase remained bound to the mycelium when the maximum overall tannase titer is attained. Bound tannase can be extracted after cell-wall hydrolysis with digestive enzymes such as chitinase. The cells can also be mechanically disrupted to recover the bound tannase.

Tannase has been purified from a variety of fungi like *A. flavus* (Yamada *et al.*, 1968), *A. oryzae* (Iibuchi *et al.*, 1968), *Candida* sp.(Aoki *et al*., 1976), *Penicillium chrysogenum* (Rajkumar and Nandy, 1983) and *A. niger* (Barthomeuf *et al*., 1994). Based on extracellular and intracellular nature of enzyme production, culture filtrate as such or mycelia extract after sonication (Yamada *et al.*, 1968) were used as the crude enzyme.

As an initial step of purification, ammonium sulphate precipitation (Yamada *et al.*, 1968; Sabu *et al*., 2005a; Naidu *et al*., 2008) or acetone precipitation were attempted (Beverini and Metche, 1990; Lekha and Lonsane, 1994) which resulted in an initial concentration as well as purification. Aoki *et al*., (1976) and Lekha and Lonsane, (1994) reported the failure of ammonium sulphate to precipitate tannase because of the very low yield. Tannase from *A. awamori* MTCC 9299 was purified using ammonium sulfate precipitation followed by ion-

When tannic acid is used as substrate, K_m values of 11.25and 0.048 mM were obtained with *Aspergillus* and *Penicillium*, respectively (Yamada *et al*., 1968; Adachi *et al*., 1971; Ibuchi *et al*., 1972; Aoki *et al*., 1976; Chae and Yu, 1983). The substrate specificity of the tannase was determined by high performance liquid chromatography (HPLC) analysis of tannin substrates and products. The enzyme was able to remove gallic acid from both condensed and hydrolysable tannins.

Tannase requires the presence of metal ions to express its full catalytic activity; in this sense, it is important to know the kind of ions and their concentrations in achieving maximal reaction efficiency. The effect of metal ions on tannase activity was studied (Kar *et al*., 2003). One mM Mg^{+2} or Hg^{+} activated tannase activity. Ba^{+2}, $Ca^{+2,}$ Zn^{+2}, Hg^{+2}, and Ag^{+} inhibited tannase activity at 1.0 mM concentration, and Fe^{+3} and Fe^{+2} completely inhibited tannase activity. Ag^{+}, Ba^{+2}, and Hg^{+2} competitively inhibited tannase activity (Mukherjee and Banerjee, 2005). Among the anions studied 1 mM Br^{-}or $(S_2O_3)^{-2}$ enhanced tannase activity. Tween 40 and Tween 80 enhanced tannase activity whereas Tween 60 inhibited tannase activity. Palmitic acid and oleic acid enhanced tannase activity, whereas stearic acid inhibited tannase activity (Kar *et al*., 2003). Sodium lauryl sulfate and triton X-100 inhibited tannase activity. Urea stimulated tannase activity at a concentration of 1.5 M. Among the chelators, 1mM EDTA or 1,10-o-phenanthrolein inhibited tannase activity dimethyl sulphoxide and β-mercaptoethanol inhibited tannase activity at 1 mM concentration whereas soybean extract inhibited tannase activity at concentrations varying from 0.05 to 1.0 % (w/v). Among the nitrogen sources selected ammonium ferrous sulfate, ammonium sulfate, ammonium nitrate and ammonium chloride enhanced tannase activity at 0.1 % (w/v) concentration (Kar *et al*., 2003; Mukherjee and Banerjee, 2005). The tannase from *A. niger* was reported to be inactivated by β-mercaptoethanol (Aguilar and Gutierrez-Sanchez, 2001). No inhibition by EDTA was observed in the case of the tannase from *A. flavus* (Yamada *et al*., 1968). The addition of metal

The molecular weight of tannase was shown to vary from 186 kDa to 300 kDa. Tannase is stable for several months at 30°C. The activity of tannase increased with temperature up to a threshold at 47.5ºC, and successive fell beyond this value enlightened the occurrence of reversible biocatalyst inactivation. The experimental results confirmed that the deactivation process of mycelium-bound tannase follows first-order kinetics pattern, and the mycelium-bound enzyme showed improved stability in organic solvent. In consideration of both the activity and stability of tannase, the optimum reaction temperature for tannase-catalyzed esterification should be 40ºC (Farias *et al*., 1994). SDS-PAGE analysis, as well as gel localization studies of purified tannase, indicated the presence of two enzyme forms. Tannases of *Aspergillus* strains have a molecular weight around 150-350 kDa. Their activity and stability pH are 5-6.0 and 3.5-8.0, respectively, while optima temperatures from 35 to 40°C have been reported (Table 2.6).

Purification of a commercial tannase from *A. oryzae* by affinity chromatography on Con A-Ultrogel resulted in the separation of two fractions (tannase I and tannase II) (Beverini and Metche, 1990). Tannase from *A. oryzae* was shown to consist of two subunits of 30 and 33 kDa. (Hatamoto *et al*., 1996) and the study concluded that native tannase consisted out of four pairs of the two subunits, forming a hetero octamer with a molecular mass of about 300 kDa. Tannase from *Candida* sp.K.1 also consisted two subunits of 120 kDa each that could be separated after treatment with SDS and β mercaptoethanol (Aoki *et al.*, 1976). Tannase produced under SSF, was observed to be present in monomeric and dimeric forms with molecular masses of 90 and 180 kDa (Ramirez-Coronel *et al*., 2003). The tannase had an isoelectric point of 3.8, a temperature optimum of 60-70°C, and a pH optimum of 6.0.

An *A. falvus* with molecular weight 192 kDa had 25.4 % carbohydrate content (Yamada *et al*., 1968; Adachi *et al*., 1971). *A. niger* with a molecular

weight of 186 kDa was reported to have 43 % carbohydrate content (Barthomeuf *et al*., 1994; Parthasarathy and Bose, 1976). Whereas *A. oryzae* tannase with 300 kDa molecular weight had 22.7 % carbohydrate content (Hatamoto *et al.*, 1996; Abdel-Naby *et al*., 1999).

All fungal tannases reported are glycoproteins, primarily consisting of neutral sugars like mannose, galactose and hexosamines (Aoki *et al*., 1976; Piater, 1999). Circular dichroism analysis of purified fractions of tannase indicated that the β-sheet structure in tannase was predominant, indicating its globular nature (Mahapatra *et al*., 2005). The polypeptide moiety for tannase was shown to be very small varying between organisms, for example the tannase from *A. flavus* consisted of 12.5% nitrogen in contrast to tannase from *Candida* sp. K-1 consisting of 38% glycoprotein. The biological significance of such high carbohydrate content is yet unknown, however it is strongly suggested that the carbohydrate moiety protects the carboxyl groups of the protein peptide bonds against hydrogen bond formation due to the large amount of phenolic hydroxyl groups present in the substrate for tannase (Lekha and Lonsane, 1994).

Tannase from *A. awamori* MTCC 9299 recorded 30°C and pH of 5.5 as optimum for enzyme activity. The effects of metals and organic solvents on the activity of tannase were also studied. Metal ions Mg^{+2}, Mn^{+2}, Ca^{+2}, Na^{+}, and K^{+} stimulated the tannase activity, while Cu^{+2}, Fe^{+3}, and Co^{+2} acted as inhibitors of the enzyme. The addition of organic solvents like acetic acid, isoamylalcohol, chloroform, isopropyl alcohol, and ethanol completely inhibited the enzyme activity. However, butanol and benzene increased the enzyme activity (Chhokar *et al*., 2009).

Table 2.6 Temperature optimum and temperature stability of a few other reported tannases.

Group	Organism	Optimum temperature	Temperature stability	Reference
Fungal tannase	*A. flavus*	50 - 60°C	≤ 70°C	Yamada *et al*., 1968, Pourrat *et al*., 1982.
"	*A. oryzae*	30-40°C	55° C	Yamada *et al*., 1968, Pourrat *et al*., 1982.
"	*A. niger*	35°C	≤ 50°C	Haslam and Tanner, 1970
"	*P. chrysogenum*	30-40°C	45°C	Rajakumar and Nandy, 1983
"	*C. parasitica*	30°C	25 - 40°C	Farias, *et al*., 1992, Iibuchi *et al*.,1968.
Plant tannase	Penduculate oak	35 ,40ºC	≤ 50°C	Niehaus and Gross, 1997.
Yeast tannase	*Candida* sp. K-1	50ºC	≤ 50ºC	Aoki *et al*., 1976.

Good stability was maintained even if the enzyme was incubated for 24 h at a pH of 5.0 (Madhavakrishna and Bose, 1962). The optimum pH for tannase isolated from *A. niger* was shown to be between 5.0 and 6.0, with instability occurring at above pH 6.0 (Iibuchi *et al*., 1968). Barthomeuf *et al*., (1994) confirmed that the tannase from *A. niger* contained both esterase and depsidase activity with the esterase and tannase activities peaking at a pH of 5.0. The stability was also good over a wide pH range between a pH of 3.5 and 8.0. The fungal tannase from *A. flavus* has also been characterized extensively and the authors showed that the enzyme could be preserved at a pH range of 5.0 and 5.5. A rapid decrease in activity occurred outside this pH range. An interesting observation was that on surface cultures the mycelial tannase activity peaked at a pH of 3.7 but in culture media the tannase activity was active between a pH of 4-7, here the activity increased with an increase in pH (Pourrat *et al*., 1982). Not many pI values has been reported for tannase except for *A. oryzae* tannase which had a pI value of near to 4.0 (Iibuchi *et al*., 1968).

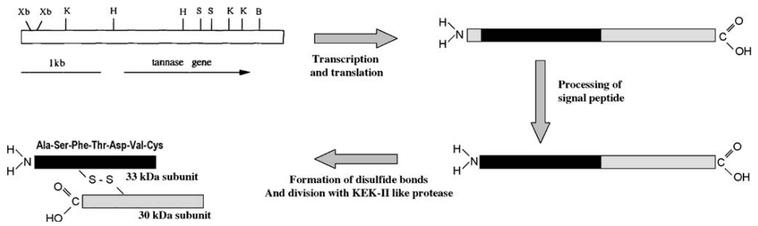


Fig 2. 5 Descriptive scheme of the proposed post-translational modification of tannase precursor (Hatamoto *et al*., 1996)

The tannase-encoding *Arxula adeninivorans* gene *ATAN1* was isolated from genomic DNA by PCR, using as primers oligonucleotide sequences derived from peptides obtained after tryptic digestion of the purified tannase protein. The gene harbours an ORF of 1764 bp, encoding a 587-amino acid protein, preceded by an N-terminal secretion sequence comprising 28 residues. The deduced amino acid sequence was similar to those of tannases from *Aspergillus oryzae* (50 % identity), *A. niger* (48 %) and putative tannases from *A. fumigatus* (52 %) and *A. nidulans* (50%). The sequence contains the consensus pentapeptide motif (–Gly–X–Ser–X–Gly–), which forms part of the catalytic centre of serine hydrolases. Expression of *ATAN1* is regulated by the carbon source. Supplementation with tannic acid or gallic acid leads to induction of *ATAN1*, and accumulation of the native tannase enzyme in the medium. The enzymes recovered from both wild-type and recombinant strains were essentially indistinguishable. A molecular mass of 320 kDa was determined, indicating that the native, glycosylated tannase consists of four identical subunits. The enzyme had a temperature optimum at 35-40^{o}C and a pH optimum at 6.0. The enzyme was able to remove gallic acid from both condensed and hydrolysable tannins. The wild type strain LS3 secreted amounts of tannase equivalent to 100 U/l under inducing conditions, while the transformant strain, which over expressed the *ATAN1* gene from the strong, constitutively active

ions like Zn^{2+}, Mn^{2+}, Cu^{2+}, Ca^{2+}, $Mg^{2+}$and Fe^{2+} inhibited the enzyme activity. Only K^{+} ions enhanced tannase activity, and an activity of 4.31 U/ml was reported here. Enzyme activity was maximal after 15-20 min of incubation time, with an activity of 3.9 U/ml. *K*m was found to be 1.03 mM and V_{max} 4.25 mmol/min. Since the enzyme is active over a wide range of pH and temperature, it could find potential use in the food-processing industry (Sabu *et al*., 2005a). Tannase from *A. awamori* nakazawa exhibited optimum activity at 35°C and at a pH of 5.0. Urea concentrations higher than 3M were inhibitory. Increasing concentrations of sodium lauryl sulfate (SLS) also led to decrease in activity. Two percent SLS was inhibitory. Increasing concentrations of ethylenediamine tetraacetic acid (EDTA) had an inhibitory effect on tannase (Mahapatra *et al*., 2005).

A kinetic and thermodynamic study was performed on the esterification of propyl gallate from gallic acid and 1-propanol by mycelium-bound tannase from *A. niger* in organic solvent. A kinetic model of esterification by mycelium-bound tannase was developed based on the Ping–Pong Bi–Bi kinetic mechanism, considering not only the effect by substrates and products, but also tannase denaturation. A reasonable quality of fit was observed by fitting experimental rate data to the kinetic mode with an average correlation coefficient of 0.977. Further when neglected the inactivation of tannase, the kinetic model fitted the corresponding experimental data poorly (Yu and Li, 2006).

The only two K*m* values reported in literature were other enzymes from *A. flavus* and *P. chrysogenum.* The data available for the two species were for the different substrates, and not for the natural substrate on which the organism grows. Tannase from *P. chrysogenum* had a *Km* value of 0.48 X 10^{-4} M for tannic acid as substrate (Rajakumar and Nandy, 1983). In the case of *A. flavus* the *Km* values were 0.5 X 10^{-4} M for tannic acid as substrate, and 1.4 X 10^{-4} M for glucose.1.gallate (Yamada *et al.*, 1968).

2.9 Genetic characterisation

Hatamoto *et al*., (1996) cloned the *Aspergillus oryzae* tannase gene (Fig.2.5) using three oligo deoxyribonucleotide (oligo) probes synthesized according to the tannase N-terminal and an internal amino acid (aa) sequence. The nucleotide (nt) sequence of the tannase gene was determined and compared with that of a tannase DNA complementary to RNA (cDNA) by means of reverse transcriptase PCR. The results indicated that there was no intron in the tannase gene and that it coded for 588 aa with a molecular weight of about 64 000 Da. The low tannase producing strain *A. oryzae* AO1 was transformed with the plasmid pT1 which contained the tannase gene, and tannase activities of the transformants increased in proportion to the number of copies. Tannase consisted of two kinds of subunits, linked by a disulfide bond(s) with molecular weights of about 30kDa and 33 kDa, respectively. Purified these subunits and determined their N-terminal aa sequences. The large and small subunits of tannase were encoded by the first and second halves, respectively. Judging from the above results, the tannase gene product is translated as a single polypeptide that is cleaved by post-translational modification into two tannase subunits linked by a disulfide bond(s) and hence concluded that native tannase consisted of four pairs of the two subunits, forming a hetero-octamer with a molecular weight of about 300 kDa. A tannase from *A. oryzae* has been cloned and expressed in *Pichia pastoris*. The catalytic activity of the recombinant enzyme was assayed. A secretory form of enzyme was made with the aid of *Saccharomyces cerevisiae* factor, and a simple purification protocol yielded tannase in pure form. The productivity of secreted tannase achieved 7000 IU/l by fed-batch culture. Recombinant tannase had a molecular mass of 90 kDa, which consisted of two kinds of subunits linked by a disulfide bond(s) (Zhong *et al*., 2004). A comparison between the cDNA sequence for tannase from *A. oryzae* with the genomic DNA sequence for tannase from *A. niger* revealed that no introns were present in the genes. The tannase genes from *A. oryzae* and *A. niger* proved to be very similar. The tannase gene from *A. niger* was 1740 bp in size in

contrast to the tannase gene from *A. oryzae* being 1767 bp in size. DNA sequence alignment studies between the tannase gene sequences revealed that there was a 76.76 % homology between the two genes on nucleotide level. Amino acid alignments revealed that the ORF for tannase from *A. niger* showed a 71.5 % identity and a similarity of 10.19 % to the amino acid sequence for tannase from *A. oryzae*. During post translational modification the peptide sequence for tannase from *A. oryzae* is cleaved into two subunits by a KEX-II like protease at position 315 and 316, liberating two peptide subunits of 30 kDa and 33 kDa in size. The KEX-II like protease site is identifiable in the protein sequence of *A. niger* at positions 307 and 308 where the KEX-II protease cleaves the carboxyl side of dibasic residues (Lys-Arg). Therefore it was hypothesized that the tannase from *A. niger* also undergoes KEX-II like protease cleavage liberating two subunits of 33 700 Da and 30 400 Da. The tannase gene from *A. oryzae* was PCR amplified and cloned into an expression vector, pRS426 containing a PCR amplified *PDC1* glucose induced promoter (Hohmann, 1991). Low amounts of catalytically active tannase was expressed by *S. cerevisiae* and it was hypothesized that the recombinant tannase was hyper glycosylated by the expression host, resulting in secreted recombinant proteins that may be inactive or different from the natural proteins (Ballou, 1982). To confirm this hypothesis further investigation is however needed. To characterize the expressed recombinant tannase, further investigation is needed, with possibly using an *Aspergillus* tannase mutant strain as expression host (Albertse, 2002). Recently, Cerda-Gomez *et al*., (2006) reported the use of conserved tannase gene sequences from five different *Aspergillus* species to design a set of primers (Tan1, Tan2), which later were used to amplify by polymerase chain reaction (PCR), a DNA segment of 435bp from four different *Aspergillus* strains.

2.10.1.5 Cell wall digestation

Tannase may contribute to plant cell wall degradation by cleaving some of the cross-links existing between cell wall polymers (Garcia-Conesa *et al.*, 2001). Due to the shortage and high cost of the enzyme, the use of tannase in large-scale applications is limited at present. It is hoped therefore that the economic benefits of tannase production can help improve the overall viability of the process.

2.10.1.6 Effluent treatment

Tannery effluents contain high amounts of tannins, mainly polyphenols, which are dangerous pollutants and cause serious environmental problems (Van de Lagemaat and Pyle, 2001). Tannase can be potentially used for the degradation of tannins present in the effluents of tanneries offering a cheap treatment and removal of these compounds.

2.10.2 Environmental

Tannins are resistant to microbial attack and known to inhibit the growth of some microorganisms. The antimicrobial effect of tannin slows down the rate of biodegradation of soil organic matter (Scalbert, 1991). Polyphenolic compounds on tannin substrate structure form complex with extra cellular and intracellular enzymes from biodegradative organisms. The complexation leads to inhibition of biodegradative enzymes which leads to loss in microbial growth and increase in bioconversion time taken for decomposition of soil organic matter. Tannase could decrease the bioconversion time for decomposition of soil organic matter (Albertse, 2002).

Tannase is also used in the manufacture of sensitive analytical probes for determining the structure of naturally occurring gallic acid esters (Haslam and Tanner 1970). Moreover, it is incorporated into the manufacture of high grade leather (Barthomeuf *et al*., 1994) and is used to clean up the hard and acidic industrial effluent containing tannin materials (Banerjee, 2005).

A. adeninivorans TEF1 promoter, produced levels of up to 400 U/l when grown in glucose medium in shake flasks (Boer *et al*., 2009).

2.10 Applications of Tannase

Tannase is widely used in manufacture of instant tea and acorn wine. Tannase has potential application in the clarification of beer and fruit juices, manufacture of coffee flavored soft drinks, and improvement in flavor of grape wine, and as an analytical probe for determining the structure of naturally occurring gallic acid esters (Seth and Chand, 2000). Tannase has also been applied for cleavage of poly phenolics such as dehydrodimer crosslinks present in the cell wall of plants, which is necessary for plant cell wall digestibility (Garcia-Conesa *et al*., 2001). The industrial applications of tannase have not been fully exploited because of its high cost, although there are a large number of reports on the production of tannase by submerged fermentation, most of these do not involve the identification of critical parameters for enzyme biosynthesis and their optimization. Gallic acid is also used as an important substrate for synthesis of propyl gallate in food industry as a preservative and as an anti oxidant (Lekha and Lonsane, 1997). Industrial bioconversion of tannic acid is generally accomplished by the enzyme tannase for the production of gallic acid.

2.10.1 Industrial:

2.10.1.1 Pharmaceutical industry

Gallic acid (3, 4, 5-trihydroxy benzoic acid), which is synthesized chemically is used in pharmaceutical industry for production of anti bacterial drug trimethoprim (Bajpai and Patil, 1996; Lekha and Lonsane, 1997) used in combination with sulphonamide (Hadi *et al*., 1994; Kar *et al*., 1999; Kar and Banerjee, 2000). Gallic acid has been synthesized chemically, but this chemical synthesis has been known to be very expensive and not always very selective. Gallic acid is one of the

products liberated upon hydrolysis of tannic acid with tannase (Iibuchi *et al*., 1972). It is used as a synthetic intermediate for the production of pyrogallols and gallic acid esters. Propyl gallate which is very expensive is used as an anti oxidant in fats and oils, in foods, cosmetics, hair products, adhesives and lubricant industries (Gaathon *et al*., 1989; Haadi *et al*., 1994; Yamada and Tannaka, 1972). Tannase enzyme is beneficial in facilitating the breakdown of extrinsic stain and is useful in hydrolysis of tannins which is known to discolour the tooth surface (Laurence Du-Thumm, *et al*., 2005).

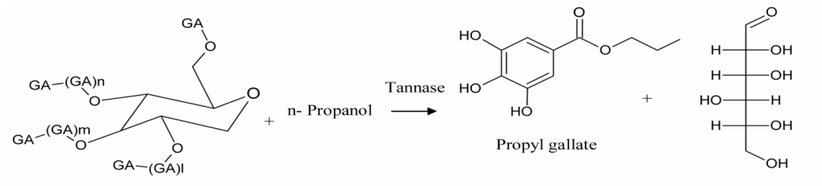


Fig.2.6 Transesterification of tannic acid to propyl gallate in presence of n-propanol

2.10.1.2 Beer and wine production

Tannase could hydrolyze wort phenolics which complex with other chemicals in beer mixture and result in haze formation (Giovanelli, 1989). Chemical treatment of wine to remove unfavoured phenolics can be changed with tannase to hydrolyse chlorogenic acid to caffeic acid and quinic acid which influences the taste of wine favorably (Chae and Yu,1983). In the case of wines, it is important to state that the main tannins present are catechins and epi-catechins, which can get a complex with galacto-catechins and others galloyl-derivated. The amount of catechin in white wines is around of 10 to 50 mg/l, while in other wines it can reach 800 mg/l (Ribereau-Gayon, 1973). Fifty percent of the colour of the wines is due to the presence of the tannins; however, if these

compounds are oxidized to quinines by contact with the air it could form an undesirable turbidity, presenting severe quality problems. When the proteins of the beer are in considerably high quantities an undesirable turbidity is presented by accomplishing with these tannins. The use of tannase can be a solution to these problems. In the manufacture of beer, the tannase could be used since the tannins are present in low quantities. Even fruit juices are treated with lactase and tannase to stabilize and clarify the product.

2.10.1.3 Cold tea products

The haze formation in tea is due to coacervation of tea flavanoids, consisting mainly of epicatechin, epicatechin gallate, epigallocatechin and epigallocatechin gallate. Tea polyphenols form hydrogen bonds with caffeine which leads to cream formation. Consumers would prefer clear products, so the compounds forming haze should be removed to get a product free of turbidity and chemicals used as clarifiers. Tannase has catalytic activity to remove gallic acid moieties from tannins and the polyphenols from tea extracts which results in coldwater soluble products. The treatment of tea with tannase enhances the natural levels of epicatechin and gallic acid which in turn favours the formation of epicatechin flavic acid which is responsible for bright reddish colour of tea with good cold water solubility and colour (Albertse, 2002).

2.10.1.4 Animal feed

The anti nutritional effects of tannins are well known (Singleton and Kratzer, 1969). They are present in a variety of plant materials which are used as feed (Bate-Smith and Rasper, 1969). The use of a number of enzymes in animal feed is gaining importance (Berry and Paterson, 1990). The use of tannase in the pretreatment of tannin containing feed may prove beneficial in the removal of these undesirable compounds and also improves digestibility. Tannase reduces the antinutritional effects of tannins in animal feed. Garcia-Conesa *et al*., 2001 reported the hydrolysis of diethyl diferulates by tannase from *Aspergillus oryzae* for animal feed improvement.

centrifuged at 4ºC and 10,000 rpm for 15 min, and the supernatant was treated as crude enzyme for all assays. This was the general procedure followed for all the studies, unless otherwise mentioned.

3.4. Analytical methods

3.4.1 Tannase assay

The tannase activity was estimated based on a modified method of Sharma *et al*., *(*2000) based on the formation of chromogen between gallic acid (released by the action of tannase on methyl gallate) and rhodanine (2-thio-4-ketothiazolidine). A standard curve was prepared using gallic acid.

i. The enzyme reaction mixture was prepared by the addition of 0.01M methyl gallate in 50 µl of 0.05 M citrate buffer, and 10µl of crude enzyme. Substrate blanks were maintained with buffer without substrate.
ii. The reaction mixture was incubated at 30ºC for 5 min.
iii. 60 µl of 0.667 % methanolic rhodanine was added again incubated at same conditions.
iv. 40 µl of 0.5 M Potassium hydroxide was added and then incubated again for 5 min.
v. Diluted 5 times with distilled water.
vi. The pink colour developed was read at 520 nm using UV-visible spectrophotometer (Shimadzu-160A). A set of blanks and controls were maintained.

One unit of tannase activity is defined as the amount of enzyme required to liberate 1µM of gallic acid /min under defined conditions. Enzyme activity was expressed as U/ml.

Chapter 3

MATERIALS AND METHODS

3.1 Microorganism

Aspergillus awamori BTMFW 032 isolated from sea water of Cochin (Soorej, 2008), available as stock culture at Microbial Technology Lab, Department of Biotechnology, Cochin University of Science and Technology was used as the source for production of tannase enzyme. *Aspergillus oryzae* (ATCC9362) a commercial strain known for tannase production, and *Aspergillus awamori* (ATCC44733) a terrestrial strain (both obtained from NCIM, Pune, India) were also included in this study.

All the three fungal cultures were maintained on Czapek Dox minimal media agar slants supplemented with 1 % tannic acid as the sole carbon source. *Aspergillus awamori* BTMFW 032 was maintained in media prepared with 50 % aged sea water. The fungal strains were sub cultured periodically, grown at 30ºC for 10 days and stored at 4ºC. Stock cultures were maintained in sterile paraffin oil at room temperature.

3.2 Medium

Czapek Dox minimal medium with the composition given below was used throughout the study for all the three strains. Sea water was also used as cultivation medium for the enzyme production by *A. awamori* BTMFW 032.

Czapek Dox minimal medium

Solution A

Sodium nitrate	-	40 g
Potassium chloride	-	10 g
Magnesium sulphate	-	10 g
Ferrous sulphate	-	0.2 g

Dissolved in 1Litre distilled water and stored at 4ºC.

Solution B

Di potassium hydrogen phosphate	-	20.0 g

Dissolved in 1Litre distilled water and stored at 4 ºC.

One liter of medium was prepared by the addition of

Stock solution A	-	50 ml
Stock solution B	-	50 ml
Distilled water	-	400 ml
Aged sea water	-	500 ml
Agar	-	20 g

* In the case of *Aspergillus oryzae* (ATCC9362) and *Aspergillus awamori* (ATCC44733) Distilled water was used instead of aged sea water for preparation of medium and cultivation of the strains.

To a liter add 1ml of both '**a**' and '**b**'.

a.	Zinc sulphate	-	1 % (w/v)
b.	Copper sulphate	-	0.5 % (w/v)

3.3 Culture conditions

The fungus was first reconfirmed for production of tannase on tannic acid agar plates containing 1 % tannic acid as sole source of carbon and 2 % agar. The

plates were spot inoculated and incubated at RT (28±2°C) for 48 h. Further cultivation of the fungus in liquid medium was carried out as described below.

3.3.1 Culture conditions in liquid media

Gallic acid is a product of conversion of tannic acid by tannase enzyme, an inducible enzyme produced by *Aspergillus* sp. Hence the strains were maintained on Czapek Dox minimal agar slants with 1 % tannic acid as the sole carbon source. Further cultivation of *A. awamori* and *A. oryzae* were done using pre induced inoculum.

3.3.2 Inoculum preparation

A spore suspension was prepared by addition of 5 ml of saline to 10 days old agar slope culture, scrapped well to get the spores released into the solution. The spore suspension thus prepared was then transferred to a sterile 100 ml conical flask, dispersed at 5000 rpm for 30 min at room temperature and used as inoculum. Appropriate dilutions were made with sterile saline such that the final concentration of the spores was $7.6x10^7$cfu/ml.

3.3.3 Inoculation and incubation

Cultivation of fungus was carried out in 250 ml Erlenmeyer flasks using the Czapek Dox minimal medium supplemented with filter sterilized tannic acid at 1 % concentration as sole source of carbon unless otherwise mentioned, and inoculated with prepared inoculum (1 % v/v), incubated for 72 h at room temperature (28±2ºC) and at 100 rpm in a rotary shaker. Samples were withdrawn at regular intervals of 24 h and assayed for tannase activity.

3.3.4 Recovery of enzyme

After incubation for the desired period the fungal mycelia were removed by filtration through Whatman No.1 filter paper and the supernatant was

production of the enzyme. The natural substrates tried were basically tannin containing agro residues which included fruits, different leaves, like ground mango leaves, *Garcinia cambogia* leaves, psidium leaves, coconut fiber and pith, grape waste, cashew apple, *Averrhoa bilimbi* fruits and leaves, tea powder, tamarind and dry tamarind pods, and those in combination with tannic acid. Their concentrations were variably fixed as inducer at 1 % concentration (w/v). Minimal medium and seawater with tannic acid as sole carbon source were maintained as control.

3.7 Development of an efficient bioprocess for the production of tannase enzyme and gallic acid by *A. awamori* BTMFW 032

Development of an efficient bioprocess for obtaining maximal tannase production and gallic acid by *A. awamori* BTMFW 032 was carried out by employing slurry state fermentation, submerged fermentation, and solid state fermentation and selected the suitable one based on the results obtained. The optimization studies were conducted employing statistical approaches, Plackett-Burman (PB) Design, and Response Surface Methodology (RSM), for optimization of cultivation medium for achieving maximal enzyme production by the fungus. The statistical software package Design-Expert® 6.0 (Stat Ease Inc., Minneapolis, and USA) was used. Variables that significantly influence tannase production were first selected employing the Plackett-Burman method (PB) and they were later applied to Response surface methodology in order to evaluate the individualized influence of each variable, as well as the effect of their interactions on enzyme production.

3.7.1 Slurry state fermentation

In general substrate for slurry sate fermentation (SLF) was prepared with a coarsely ground matrix of the selected natural substrate. Cultivation of the fungus for tannase production was carried out in 250 ml Erlenmeyer flasks and the study was conducted using both Czapek Dox minimal medium and seawater as

3.4.2 Protein estimation

Protein content was determined according to Bradford (1976) using Bradford kit (Biogene, USA). The samples were diluted to 100µl with 0.15N NaCl. One ml of Bradford reagent was added, vortexed, allowed to stand for 2 min and the absorbance was read at 595nm. Protein was quantified in comparison with a standard curve plotted with Bovine Serum Albumin (BSA) as standard and was expressed as mg/ml.

3.4.3 Tannin estimation

Tannin like compounds reduces phosphotungstomolybdic acid in alkaline solution to produce a highly colored blue solution, the intensity of which is proportional to the amount of tannins (Folin-Denis method of Schanderl, 1970). 0.5 g of powdered material was diluted with 75 ml of distilled water, boiled for 30 min, centrifuged at 2000 rpm for 20 min; supernatant was collected and made up to 100 ml. A fraction of one ml of the same was further diluted with 75 ml of distilled water, added with 5 ml of Folin-Denis reagent, 10 ml of sodium carbonate, and then made up to 100 ml. The intensity was measured in a spectrophotometer at 700 nm after 30 min. The tannin content of the sample was calculated as tannic acid equivalents from tannic acid standard graph.

3.4.4 Specific activity

Specific activity was calculated by dividing the enzyme units with protein content and was expressed as U/mg protein.

$$\text{Specific activity} = \frac{\text{Enzyme activity (U/ml)}}{\text{Protein (mg/ml)}}$$

3.4.5 Gallic acid estimation

Gallic acid accumulation was assayed by spectrophotometric method of methanolic rhodanine (Sharma *et al*., 2000). A standard curve was prepared using gallic acid.

3.5 Gallic acid extraction

The crude extract obtained after fermentation was equilibrated with different matrices like diatomaceous earth, silica gel and activated charcoal. Sodium chloride was used for eluting the gallic acid from the equilibrated culture filtrate. Initially the concentration of sodium chloride was standardized for the maximal recovery of gallic acid. Elute obtained was mixed well with two volumes of ethyl acetate and separated using a separating funnel. Later ethyl acetate was evaporated to recover gallic acid in powder form.

3.5.1 Fourier-Transform Infra red Spectroscopy (FT-IR Spectroscopy)

Fourier-Transform infra red spectroscopy is a technique that provides information about the chemical bonding or molecular structure of materials whether organic or inorganic. The bonds and group of bonds vibrate at characteristic frequencies. A molecule that is exposed to infra red rays absorbs infrared energy at frequencies which are characteristics to that molecule. During FT-IR analysis a spot on specimen is subjected to a modulated IR beam. The specimen's transmittance and reflectance of infrared rays at different frequencies is translated to an IR absorption plot consisting of reverse peaks. The resulting FT-IR spectral pattern is then analyzed and matched with known signatures of identified materials.

The parameters used in FT- IR analysis were: spectral range 4000-500cm^{-1}, resolution 4$cm.^{-1}$ The extracted gallic acid sample was subjected to FT-IR spectroscopic analysis (Thermo Nicolet, Avatar 370), equipped with KBr beam splitter with DTGS (Deuterated triglycine sulphate) detector (7800-350cm^{-1}), at Sophisticated Test and Instrumentation centre, Cochin University of Science and Technology, Cochin, Kerala.

3.5.2 Thin layer Chromatography (TLC)

The gallic acid extracted was also analyzed by thin layer chromatography

with silica gelG-60 F_{254} (E. Merck, Mumbai, India). The solvent system consisted of ethyl acetate, chloroform and formic acid (4:4:1). After drying the plates were developed by spraying a solution of $FeCl_3$ (Naidu *et al*., 2008). Standard gallic acid and sample were run in same plate. Retention factor (R_f) value was calculated according to the following equation from the chromatogram.

$$R_f = \frac{\text{Distance moved by the compound}}{\text{Distance moved by the solvent front}}$$

3.5.3 Gas Chromatography (GC)

The formation of gallic acid was analyzed by a gas chromatogram with flame ionization detector. The capillary column make was CPCL-24 with a length of 30 m and an internal diameter of 0.25µm. Helium was used as the carrier gas with a constant flow rate of 80 ml/min. Detector temperature was set at 250ºC. GC was performed at Sophisticated Test and Instrumentation centre, Cochin University of Science and Technology, Cochin, Kerala.

3.5.4 High Performance Liquid Chromatography (HPLC)

The Sample was loaded at a concentration of 1 mg/ml to the HPLC (Shimadzu). Sample was diluted in Acetonitrile:Water (50:50). Injection volume was 50µl. Absorbance was monitored at 265 nm and flow rate maintained at 1 ml/min. The sample was applied to Jupiter C 18 column (250x10 mm and 10 micron pore size) and eluted with a linear gradient of acetonitrile containing 0.1 % TFA (trifluroacetic acid). The sample and standard gallic acid were run identically. The reaction was performed at Molecular Biophysics Unit, IISC, Bangalore.

3.6 Selection of natural substrates for enzyme production by *A. awamori* BTMFW 032

Univariable strategy for natural carbon source was employed to select the ideal natural substrate for induction of tannase production by the fungus as a substitute/supplement to synthetic tannic acid which is currently used for the

3.7.1.3 Experimental validation of the optimized conditions

In order to validate the response surface model, a random set of experiments were setup according to conditions predicted by the model. The responses obtained from the trials conducted as above following the Box-Behnken design model for five variables, was used to estimate the coefficients of polynomial models using standard regression techniques. The estimate of Y was used to generate an optimal combination of factors that can support maximal enzyme production using predictive models from response surface methodology. The software Design Expert® 6.0 was used to fit the response surface-Box-Behnken model to the experimental data. All the experiments were carried out independently in triplicates.

3.7.1.4 Time course study under optimal condition

Time course experiment was conducted with the optimized conditions determined after statistical optimization of various variables. The conditions selected included the following:

Garcinia	-	26 % (w/v)
Seawater	-	32 % (v/v)
Sodium nitrate	-	33 mM
Potassium chloride	-	6.3 mM
Magnesium sulphate	-	1 mM
Zinc sulphate	-	0.011 mM
Ferrous sulphate	-	0.30 mM
Dipotassium hydrogen phosphate	-	15 mM
Calcium chloride	-	2.18 mM
Glucose	-	3.2 mM
Tannic acid	-	1 % (w/v)
Temperature	-	40ºC
Inoculum	-	3 % (v/v)

cultivation media. Fresh leaves dried at 60°C for 24 h, were coarsely ground and used as substrates for SLF (Zhong and Cen, 2005). The coarsely ground matrices of the substrates (5 g in 50 ml) and commercially available tea powder were moistened with Czapek Dox minimal medium and seawater (5 g in 50ml (w/v) in minimal medium), inoculated with the prepared spore inoculum (7.6 x 10^7 cfu/ml) at a concentration of 1 % (v/v), and incubated for 2 days at room temperature (28±2 °C) at 100 rpm in a rotary shaker. After incubation the fermented slurry was centrifuged at 4°C and 10,000 rpm for 15 min, and the supernatant was collected and assayed for tannase activity.

3.7.1.1 Optimization of physico-chemical and nutritional parameters using Plackett-Burman Design (PB Design) under slurry state fermentation

Optimization of process variables using statistical approach for maximal tannase under slurry state fermentation was carried out using Plackett-Burman design (Plackett-Burman, 1946) with selected 18 variables listed in Table 3.1.The parameters were varied over two levels, the minimum and maximum for each of the parameter. The statistical software package Design-Expert® 6.0 (Stat Ease Inc., Minneapolis, U.S.A) was used to generate a set of experimental designs. Production was set up by inoculating the media with respective inoculum percentages as suggested by the model and incubated for specified incubation period (12-48 h), at specified temperature (25-40ºC), at 150 rpm. For each experiment, the tannase production was calculated in terms of U/ml. The experiments were done in triplicate. Regression analysis of the experimental data was conducted using statistical software.

Based on results obtained from Plackett-Burman design the fitted first order model is

$$Y=\beta o+{}^{k}\Sigma_{i=1}\,\beta iXi$$

Y- Predicted response, βo, βi -Constant coefficients, Xi-Coded independent factors.

This model does not describe interaction among factors and it is used to screen and evaluate the important factors that influence the response. Effect of each variable on production was determined by calculating their respective E-values (Gupta *et al.*, 2004).

$$E = \frac{\text{(Total response at high level) – (Total response at low level)}}{\text{(Number of trials)}}$$

Table 3.1 Variables and their experimental levels adopted in Plackett-Burman method for selection of variables for tannase production by *A. awamori* BTMFW 032

SI. No	Variable	Upper level (+)	Lower level (-)
1	Garcinia leaves	30 % (w/v)	10 % (w/v)
2	Seawater	75 %(w/v)	25 %(w/v)
3	Sodium nitrate	50 mM	10 mM
4	Potassium chloride	10 mM	5 mM
5	Magnesium sulphate	10 mM	5 mM
6	Zinc sulphate	0.05 mM	0
7	Copper sulphate	0.025 mM	0
8	Ferrous sulphate	0.5 mM	0.1 mM
9	Di potassium hydrogen phosphate	15 mM	5 mM
10	Glucose	10 mM	0
11	Tannic acid	0.1 % (w/v)	0
12	pH	5	2
13	Temperature	40°C	25ºC
14	Inoculum	3 %(v/v)	0.5 %(v/v)
15	Agitation	150 rpm	0
16	Illumination	Dark	Light
17	Incubation hours	48 h	12 h
18	Calcium chloride	5 mM	0

3.7.1.2 Optimization of physico-chemical and nutritional parameters for tannase production using RSM under slurry state fermentation

The variables selected by the Plackett-Burman method were applied to Response surface methodology to evaluate the individualized influence of each variable, as well as the effect of their interactions on enzyme production. Table 3.2 shows the variables and the experimental levels studied with Box Behnken method of the response surface methodology. Five independent variables were used to obtain the combination of values that optimizes the response within the region of three-dimensional (3D) observation spaces, which allows one to design a minimal number of experiments. The components (independent variables) selected for the optimization were concentration of the substrate (garcinia leaves), tannic acid, glucose, inoculum concentration, and temperature. This is a second order design for the estimation of quadratic effect and a two level factorial design. Each factor was studied at three different levels. The levels coded in units with values -1, 0, and 1 representing lower, middle, and higher values respectively. Quadratic model chosen to represent the relationship fitted for the 5 variables:

$$Y=\beta o+ {}^{5}\Sigma_{i=1}\, \beta iXi + {}^{5}\Sigma\, i=1\, \beta iiXi2+ {}^{5}\Sigma_{i=1}\, {}^{5}\Sigma_{j=1}\, \beta ijXiXj$$

Y-Variable enzyme yield, X1, X2, X3, X4, X5 are independent variables selected. The experiments were carried out in triplicate. Replicates at the centre of the domain in three blocks permit the checking of the absence of bias between several sets of experiments. The effect of variables and their interactions and all the coefficients were calculated by the software package Design Expert Version® 6.0. Analysis of variance (ANOVA) was performed.

Table 3.2 Variables and their experimental levels adopted in Box-Behnken method

Variables	Low level (-1)	Medium level (0)	High level(+1)
Garcinia leaf	10 % (w/v)	20 % (w/v)	30 % (w/v)
Tannic acid	0	0.05 %(w/v)	0.1 %(w/v)
Glucose	0	5 mM	10 mM
Temperature	25°C	32.5°C	40°C
Inoculum	0.5 % (v/v)	1.75 % (v/v)	3 % (v/v)

(v/v), and incubated for 2 days at room temperature (28±2 ºC) at 100 rpm in a rotary shaker. After incubation, the biomass was separated by centrifugation at 4ºC and 10,000 rpm for 15 min, and the cell free fermented broth was collected and assayed for tannase activity.

3.7.2.1 Optimization of physico-chemical and nutritional parameters for tannase and gallic acid production using Plackett-Burman Design (PB Design) under submerged fermentation

Optimization studies were conducted employing the Plackett-Burman method and the variables which had the higher influence on tannase and gallic acid production were selected. The variables and their experimental levels studied using the Plackett-Burman statistical method for selection of the variables that had the largest influence on the experimental response is presented in Table 3.4.

Table 3.4 Values of independent variables used and levels used in experimental design

Independent variables	Different levels		Units
	Upper level	Lower level	
Tannic acid	4	0.5	%(w/v)
Sodium chloride	1	0	M
Sodium nitrate	50	10	mM
Potassium chloride	10	0	mM
Magnesium sulphate	5	0	mM
Ferrous sulphate	0.5	0	mM
Di potassium hydrogen phosphate	15	1	mM
pH	7	2	
Incubation hours	48	12	h
Inoculum	4	0.5	%(v/v)
Agitation	150	0	rpm

Incubation hours	-	24 h
pH	-	5
Agitation	-	nil
Illumination	-	light

3.7.1.5 Optimization of physico-chemical and nutritional parameters for gallic acid production using RSM under slurry state fermentation

From the Plackett-Burman design for enzyme activity, gallic acid present in the culture filtrates were estimated. Based on the results obtained for the Plackett-Burman design experiment factors which were found to be critical for gallic acid production were selected for experiments with Response surface methodology. Four independent variables were used to obtain the combination of values that optimizes the response within the region of three-dimensional (3D) observation spaces, which allows one to design a minimal number of experiments. The experiments were designed using the software, Design Expert Version® 6.0. The components (independent variables) selected for the optimization were sea water, tannic acid, glucose, and zinc sulphate.

This is a second order design for the estimation of quadratic effect and a two level factorial design. Each factor studied at three different levels. The levels coded in units with values -1, 0, +1 representing lower, middle, and higher values respectively. Quadratic model chosen to represent the relationship fitted for the 4 variables:

$$Y=\beta o+{}^{4}\Sigma_{i=1}\,\beta iXi + {}^{4}\Sigma_{i=1}\,\beta iiXi2+ {}^{4}\Sigma_{i=1}\,{}^{4}\Sigma_{j=1}\,\beta ijXiXj$$

Y-Variable enzyme yield, X1, X2, X3, X4 are independent variables selected. Analysis of variance (ANOVA) was performed and three dimensional response surface curves were plotted by Design Expert software to study the interaction among various physico-chemical factors. To examine the combined effect of four independent variables on maximum gallic acid production, Box Behnken design

was performed. The coded and actual values of independent variables are given in Table 3.3. The experiments were carried out in triplicate. Replicates at the centre of the domain in three blocks permit the checking of the absence of bias between several sets of experiments. The effect of variables and their interactions and all the coefficients were calculated by the software package Design Expert Version® 6.0

Table 3.3 Variables and their experimental levels adopted in Box-Behnken method

Variables	Low level (-1)	Medium level (0)	High level (+1)
Sea water	25 % (v/v)	50 % (v/v)	75 % (v/v)
Tannic acid	0	0.05 % (w/v)	0.1 % (w/v)
Glucose	0	5 mM	10 mM
Zinc sulphate	0.01 mM	0.03 mM	0.05 mM

3.7.1.6 Experimental validation of the optimized conditions

A random set of experiments were set up according to the conditions predicted by the model in order to validate the response surface model. The responses obtained from the trials conducted as mentioned above, following the Box-Behnken design model for four variables, were used to estimate the coefficients of the polynomial model using standard regression techniques. The estimate of "Y" was used to generate an optimal combination of factors that can support maximal enzyme production using predictive models from response surface methodology. The software Design-Expert® 6.0 was used to fit the response surface-Box-Behnken model to the experimental data. All the experiments were carried out independently in triplicates.

3.7.1.7 Time course study under optimal condition

Time course experiment was conducted with the optimized conditions determined after statistical optimization of various variables. The conditions selected include the following:

Garcinia	-	22.2 % (w/v)
Seawater	-	73.58 % (v/v)
Sodium nitrate	-	11 mM
Potassium chloride	-	7 mM
Magnesium sulphate	-	4.3 mM
Copper sulphate	-	0.017 mM
Zinc sulphate	-	1.25 mM
Ferrous sulphate	-	0.22 mM
Dipotassium hydrogen phosphate	-	11.83 mM
Glucose	-	7.94 mM
Tannic acid	-	0.09 % (w/v)
Temperature	-	32 ºC
Inoculum	-	2 % (v/v)
Incubation hours	-	36 h
pH	-	3.5
Agitation	-	100 rpm
Illumination	-	light

3.7.2 Submerged fermentation

Submerged fermentation (SmF) production of tannase was conducted using Czapek Dox minimal media. 40 ml of the medium without tannic acid taken in a 250 ml Erlenmeyer flask, was autoclaved at 121 ºC for 20 min, cooled, added filter sterilized tannic acid solution containing 0.5 g tannic acid (final concentration 1 %), inoculated with spore inoculum (7.6 x 10^7 cfu/ml) at a concentration of 1 %

nitrogen, added with 10 ml of citrate buffer pH 5, mixed well and centrifuged at 5000 rpm for 20 min. The supernatant was collected, made up to 50 ml with citrate buffer, and was assayed for intracellular tannase activity.

3.10 Purification of Tannase

Tannase produced by marine *Aspergillus awamori* BTMFW 032 under SMF was purified employing standard protein purification protocols, which included ammonium sulphate precipitation; followed by dialysis, gel filtration and concentration by ultra filtration. All the experiments were done at 4ºC unless otherwise specified.

3.10.1 Ammonium sulphate precipitation

Ammonium sulphate precipitation was done according to Englard and Seifter (1990). Ammonium sulphate (Sisco Research Laboratories Pvt. Ltd., India) required to precipitate tannase was optimized by its addition, at varying levels of concentrations (20, 40, 60, 80 and 100 % saturation), to the crude extracts. To precipitate the protein, ammonium sulphate was slowly added initially at 20 % saturation to the crude extract while keeping in ice with gentle stirring. After complete dissolution of ammonium sulphate, the solution was kept at 4ºC for overnight. Precipitated protein was collected by centrifugation at 10000 rpm for 15 min at 4ºC. The precipitate was resuspended in minimum quantity of 0.1M phosphate buffer (pH 7.0). To the supernatant ammonium sulphate, required for next level of saturation, was added and the procedure mentioned above was repeated. This precipitation process was continued up to 100 % ammonium sulphate saturation.

3.10.2 Dialysis

The precipitate obtained after ammonium sulphate precipitation was further dialyzed against the phosphate buffer 0.1 M (pH 7) in order to remove the

3.7.2.2 Optimization of physico-chemical and nutritional parameters for tannase and gallic acid production using RSM under submerged fermentation

The variables selected by the Plackett-Burman method were applied to Response surface methodology to evaluate the individualized influence of each variable, as well as the effect of their interactions on gallic acid production (Box *et al*., 1978). To examine the combined effect of six independent variables on maximum tannase production, Box Behnken design was performed. The coded and actual values of independent variables are given in Table 3.5. The experiments were carried out in triplicate. Replicates at the centre of the domain in three blocks permit the checking of the absence of bias between several sets of experiments. The effect of variables and their interactions and all the coefficients were calculated by the software package Design Expert Version 6.0.

Quadratic model chosen to represent the relationship fitted for the 6 variables:

$$Y=\beta o+{}^{6}\Sigma\ \beta_i X_{i\ i=1}+{}^{6}\Sigma\ \beta_{ii}X_i^{\ 2}{}_{\ i=1}+{}^{6}\Sigma\ {}^{6}\Sigma\ \beta_{ij}X_{i\ i=1}\ X_{j\ j=1}$$

Where Y is the production of tannase and gallic acid and X_1, X_2, X_3, X_4, X_5, X_6 are the 6 independent variables for tannic acid, sodium chloride, ferrous sulphate, dipotassium hydrogen phosphate, incubation hours and agitation and βo is the intercept term. Analysis of RSM was performed by analysis of variance.

Table 3.5 Variables and their experimental levels adopted in Box-Behnken method

Variables	Low level (-1)	Medium level (0)	High level (+1)
Tannic acid	0.5 % (w/v)	2.25 %(w/v)	4 %(w/v)
Sodium chloride	0	0.5 M	1 M
Ferrous sulphate	0	0.25 mM	0.5 mM
Di Potassium Hydrogen Phosphate	1 mM	8 mM	15 mM
Incubation hours	12 h	30 h	48 h
Agitation	0	75 rpm	150 rpm

3.7.2.3 Experimental validation of the optimized conditions

A random set of experiments were setup according to conditions predicted by the model in order to validate the response surface model. The responses obtained from the trials conducted as above following the Box-Behnken design model for five variables, were used to estimate the coefficients of polynomial models using standard regression techniques. The estimate of Y was used to generate an optimal combination of factors that can support maximal enzyme production using predictive models from response surface methodology. The software Design Expert 6.0 was used to fit the response surface-Box-Behnken model to the experimental data. All the experiments were carried out independently in triplicates.

3.7.2.4 Time course Experiment

A time course study was conducted with the optimized parameters in shaken flasks in triplicate.

Tannic acid	-	2.6 % (w/v)
Sodium chloride	-	0.45 M
Sodium nitrate	-	47 mM
Potassium chloride	-	5.1 mM
Magnesium sulphate	-	4.9 mM
Ferrous sulphate	-	0.27 mM
Dipotassium hydrogen phosphate	-	9.14 mM
Inoculum	-	0.5 % (v/v)
Incubation hours	-	36 h
pH	-	5.6
Agitation	-	93 rpm

3.7.3 Solid state fermentation

Solid state fermentation (SSF) production of tannase was carried out using Czapek Dox minimal medium and coconut pith and fiber as solid substrates. 10 g each of coir pith and dry fiber taken separately and in combination in 250ml Erlenmeyer flask was added with 6 ml of minimal media containing filter sterilized tannic acid at a concentration of 0.1% (w/v) and mixed thoroughly. The protocols for inoculation and fermentation parameters were as described for submerged fermentation process, except for incubation of flasks in inclined position under stationary conditions.

Since the solid state fermentation studies for tannase production did not show satisfactory results no attempt was made to proceed with optimization of variables as it was done in the case of slurry state and submerged fermentation

3.8 Comparative evaluation of suitability of the designed media for tannase and gallic acid production by *A. oryzae* ATCC9362 *A. awamori* ATCC44733 and the marine *A. awamori* BTMFW 032

A comparative evaluation was performed on the suitability of the designed media for tannase and gallic acid production by *A. oryzae* (ATCC9362), *A.awamori* (ATCC44733) and the marine *A.awamori* BTMFW 032 were conducted under the optimized conditions.

3.9 Intracellular enzyme production

A study was also conducted to evaluate the efficiency of the fungus to secrete tannase as intracellular enzyme as described here. After fermentation the mycelia was filtered from 50 ml of fermented broth, washed several times with distilled water, and pressed to remove the water. The intracellular enzyme was recovered from washed mycelial mat by grinding the mat with excess of liquid

Trypsin Inhibitor - 20.1 kDa

K_{av} (partition coefficient) of each protein was calculated by the formula

$$K_{av} = \frac{V_e - V_0}{V_t - V_0}$$

Where V_e is the elution volume of each protein, V_t is the total bed volume, V_0 is the void volume of the column, which was calculated by running Blue dextran 2000. Molecular weight of the active fraction was calculated from the semi logarithmic graph plotted for the K_{av} v/s molecular weight of standard proteins.

Active fractions pooled from gel filtration was lyophilized and stored for characterization.

$$\text{Yield of enzyme activity} = \frac{\text{Total activity of the fraction x 100}}{\text{Total activity of crude extract}}$$

$$\text{Fold of purification} = \frac{\text{Specific activity of the fraction}}{\text{Specific activity of crude extract}}$$

All the experimental data were statistically analyzed using Microsoft Excel.

3.11 Characterization of purified enzyme

Purified protein was further characterized for their biophysical and biochemical properties by subjecting the purified protein to molecular mass determination, zymogram analysis, isoelectric focusing, enzyme kinetics etc as described in the following sections.

3.11.1 Electrophoretic methods

All the samples obtained at various stages of purification namely, ultra filtration, and gel filtration chromatography were run on Native-PAGE gel and SDS-PAGE gel prepared using 10 % polyacrylamide gel according to the method of Laemmlli (1970). SDS page was performed under reductive and non reductive conditions i.e. with and without β mercaptoethanol.

ammonium sulphate from the precipitate. Dialysis tube (Sigma-Aldrich) was first treated to remove the humectants and protectants like glycerin and sulphur compounds present in it and to make the pores of the tube more clear. The pretreatment involved washing of the tube in running water for 3-4 h, dipping in 0.3 % (w/v) sodium sulfide at 80ºC for 1 min, further washing with hot water (60ºC) for 2 min followed by acid wash in 0.2 % (v/v) sulphuric acid. Finally the tube was rinsed with distilled water. The precipitated protein was dialyzed in the pretreated dialysis tube for 48 h at 4ºC with several changes of buffer and assayed for tannase activity, protein content, and specific activity. Yield was calculated. The treated tube retained most of the proteins of molecular weight 12 kDa and more.

3.10.3 Acetone precipitation

Required volume of acetone was cooled to -20°C. The extract was placed in acetone compatible tube, added with 4 volume of cold acetone to the tube, vortexed, and incubated for 60 min at -20°C. It was then centrifuged for 10 min at 10000 rpm at 4°C. The supernatant was discarded carefully without dislodging the protein pellet. Acetone was allowed to evaporate from the uncapped tube at room temperature for 30 min. Care was taken not to over dry the pellet. Phosphate buffer 0.1 M (pH 7) was added and vortexed thoroughly to dissolve the protein.

3.10.4 Ethanol precipitation

To 1 volume of the extract 9 volumes of cold ethanol (100 %) was added, mixed, and kept at -20°C overnight. It was centrifuged for 15 min at 10000 rpm at 4°C. Supernatant was discarded carefully, and retained the pellet. The tubes were dried by inversion on tissue paper. The pellets were washed with 90 % cold ethanol (kept at -20 °C), vortexed, and repelleted for 5 min. The samples were air dried to eliminate any ethanol residue. Phosphate buffer 0.1 M (pH 7) was added and mixed thoroughly to dissolve the protein.

3.10.5 Sucrose concentration

Concentration of the protein sample was carried out by covering the dialysis tube, containing 100ml of the extract, with excess of sucrose such that the water molecules diffused out through the membrane leading to the concentration of the crude extract to 12 ml.

3.10.6 Clarification and removal of tannic acid from culture filtrate

Clarification and removal of tannic acid from the culture filtrate was carried out with aluminium oxide acidic having Brockmann activity. To 100 ml of the crude culture filtrate 50 g of aluminium oxide was added, mixed well for 30 min, kept at 4ºC for 2 h with intermittent shaking, and the clear solution was filtered through What man No.1 filter paper. The clear filtrate obtained was used as the enzyme for further studies. The aluminium oxide was later recovered by several washes in 1N sodium hydroxide followed by several washes in 1N hydrochloric acid and several washes in distilled water before reuse.

3.10.7 Concentration of tannase by ultrafiltration

Tannase clarified was further concentrated by ultrafiltration. 1.5 l of the clarified extract was concentrated to 45 ml by ultrafiltration using membranes (Amicon ultra, Millipore) with a molecular weight cutoff of 30,000 Da followed by 100,000 Da.

3.10.8 Gel filtration chromatography

Purification of enzyme protein by Gel filtration chromatography was performed using sephadex G200 (Sigma-Aldrich) column.

3.10.8.1 Preparation of column

a. A fraction of 3.5 g of Sephadex G-200 (Sigma-Aldrich) was suspended in distilled water, allowed to hydrate for 3 h at 100ºC in a water bath, and fine particles were removed by decantation.
b. Hydrated gel suspension was degassed under vacuum to remove the air bubbles.
c. Filled the glass column with eluent (HCl-KCl buffer, pH-2) without air bubble.
d. Prepared Gel suspension was carefully poured into the column without trapping air bubbles and allowed to settle under gravity while maintaining a slow flow rate through the column.
e. Column was stabilized by allowing two times the bed volumes of eluent to pass through the column bed in descending eluent flow.

3.10.8.2 Sample preparation and application on the column

An aliquot of 1.5 ml of crude extract was applied to the prepared Sephadex-G200 column (70 x 1.5 cm). Care was taken to make sure that the sample was completely free of undissolved substances. After the complete entry of sample in to the column the proteins were eluted using 0.05 M HCl-KCl buffer, pH-2 at a flow rate of 50 drops per tube in a fraction collector (Biorad, USA). Tannase activity was assayed and protein content was estimated.

Calculation of molecular weight of eluted protein was determined by calibrating the column with molecular weight gel filtration markers (Amersham Biosciences) mentioned as below.

The markers used included

Catalase	-	240 kDa
Alcohol dehydrogenase	-	150 kDa
Bovine serum albumin	-	67 kDa

3.11.1.5 Sample preparation for reductive SDS PAGE

Added 100 µl of 1X sample buffer to the concentrated sample, mixed well, boiled for 5 min in a water bath, cooled to room temperature. A mixture containing 25 µl of this sample and 5 µl of low molecular weight marker was loaded onto the gel.

Procedure:

1. Cleaned and assembled the gel plates
2. Resolving gel - Added all the components except APS in to a beaker, mixed gently and finally added APS. Immediately poured the mixture into the cast and a layer of water over the gel, and allowed to solidify at least for half an hour
3. Stacking gel - Added the components of stacking gel, except APS, into a beaker, mixed gently, and finally added APS. Poured the contents into the cast above the resolving gel and immediately inserted the comb between the glass plates. Allowed it to solidify at least for 30 min.
4. Gel was placed in the electrophoresis apparatus, and then the upper and lower reservoirs were filled with reservoir buffer for SDS-PAGE
5. The gel was pre run for 1 h at 80 V
6. Loaded the gel with the protein sample
7. The gel was run at 80 V till the sample entered the resolving gel
8. When the dye front entered the resolving gel, increased the current to 100 V
9. Stopped the current when the dye front reached 1cm above the lower end of the glass plate
10. Removed the gel from the cast and stained for at least 1 h in the staining solution
11. Destained till the bands became clear and observed under a transilluminator.

3.11.1.1 Reagents for Polyacrylamide Gel Electrophoresis

(1) Stock acrylamide solution (30:0.8)

Acrylamide (30 %)	-	60.0 g
Bis-acrylamide (0.8 %)	-	1.6 g
Distilled water (DW)	-	200.0 ml

Stored at a temperature of 4ºC in amber colored bottle.

(2) Stacking gel buffer stock (0.5 M Tris-HCl, pH 6.8)

Tris buffer - 6 g in 40 ml DW

Titrated to pH 6.8 with 1M HCl and made up to 100ml with DW.

Filtered with Whatman No.1 filter paper and stored at 4°C.

(3) Resolving gel buffer stock (3MTris-HCl, pH 8.8)

Tris buffer - 36.3 g

Titrated to pH 8.8 with 1 M HCl and made up to 100 ml with DW. Filtered with Whatman No.1 filter paper and stored at 4 °C.

(4) Reservoir buffer for SDS-PAGE

Tris buffer	-	3.0 g
Glycine	-	14.4 g
SDS	-	1.0 g

Dissolved and made up to 1L with DW.

Prepared in 10X concentration and stored at 4 °C.

(5) Sample buffers

(a) Sample buffer for non-reductive SDS-PAGE

Tris -HCl (pH6.8)	-	0.0625 M
Glycerol	-	10 % (v/v)

SDS	-	2 %
Bromophenol blue	-	0.01 %

(b) Sample buffer for reductive SDS-PAGE

Tris-HCl (pH 6.8)	-	0.0625 M
Glycerol	-	10 % (v/v)
SDS	-	2 %
Dithiothreitol	-	0.1 M
Bromophenol blue	-	0.01 %

Prepared as 2X concentrations and stored at 4 °C

(6) SDS (10 %) - 1gm in 10 ml DW

(7) Sucrose (40 %) - 4gm in 10ml DW

(Autoclaved at 121 °C for 15 min and stored at 4 °C)

(8) Protein staining solution

Coomassie Brilliant blue (0.1 %)	-	100 mg
Methanol (40 %)	-	40 ml
Glacial acetic acid	-	10 ml
DW	-	50 ml

(9) Destaining Solution

Methanol (40 %)	-	40 ml
Glacial acetic acid (10 %)	-	10 ml
DW	-	50 ml

(10) Protein marker for SDS-PAGE

Myosin, rabbit muscle	-	205 kDa
Phosphorylase b	-	97.4 kDa
Bovine serum albumin	-	66 kDa

Ovalbumin	-	43 kDa
Carbonic anhydrase	-	29 kDa
Soyabean trypsin inhibitor	-	20.1 kDa

3.11.1.2 Gel preparation

Resolving Gel (10 %)

Acrylamide: bis-acrylamide	-	5 ml
Resolving gel buffer stock	-	1.875 ml
SDS (10%)	-	0.15 ml
DW	-	7.975 ml
TEMED	-	15µl
Ammonium per sulphate (APS)	-	A pinch

Stacking gel (2.5 %)

Acrylamide: bis-acrylamide	-	1.25 ml
Stacking gel buffer stock	-	2.5 ml
SDS (10%)	-	0.1 ml
DW	-	6.15 ml
TEMED	-	15 µl
Ammonium per sulphate (APS)	-	A pinch

3.11.1.3 Sample buffer

Sample buffer (2X)

50 % Sucrose

DW

3.11.1.4 Sample preparation for non reductive SDS PAGE

Added 20 µl of sample buffer and 10 µl of 50 % sucrose to lyophilized sample mixed well and 10 µl of marker mix were loaded on to the gel.

3.11.5 Glycoprotein staining

This procedure detects glycoprotein containing atleast 50ng of carbohydrate (Gander, 1984). Glycoprotein bearing hexosyl, hexuronosyl or pentosyl residues react with sulphuric acid to form furfural derivatives, which in turn react with thymol to form a chromogen that is stable for only a few hours at ambient temperature. Furfural derivatives are not formed when 2-deoxy or 2-acetamido-2-deoxy hexosaminyl residues are allowed to react with sulphuric acid. Gels must be washed free of low molecular weight contaminants before they are treated with acid.

Procedure

In sample buffer, instead of sucrose, glycerol was used. After electrophoresis the gel was washed twice for 2 h in a glass tray with isopropanol-acetic acid-distilled water mixture (25:10:65) to fix the proteins and remove low molecular weight substances. Glass tray was kept in gel rocker (Genei, Bangalore) for shaking. Additional washes were performed to remove large concentrations of sucrose or other soluble carbohydrates from protein samples. A final wash for 2 h in the same solvent containing 0.2 % (w/v) thymol was done which resulted in the formation of stable gel. The solution was decanted and gels were allowed to drain. A solution of concentrated sulphuric acid-absolute ethanol (80:20) at ambient temperature was added to the gel. The gel was shaken gently at room temperature for 2.5 h or until the opalescent appearance of gel disappeared. Zones containing glycoprotein stained red while the background was yellow. Other proteins did not form visible zones when treated in this manner.

3.11.6 Carbohydrate content

The carbohydrate content of the enzyme was determined by phenol sulphuric acid method using glucose as standard (Dubois *et al*., 1956). An aliquot

3.11.2 Native Polyacrylamide gel electrophoresis

3.11.2.1 Gel preparation

Resolving gel (10 %)

Acrylamide: bis-acrylamide (30:0.8)	-	10.0 ml
Resolving gel buffer stock	-	3.75 ml
Ammonium persulphate (APS)	-	pinch
Water	-	16.25 ml
TEMED	-	15.0 µl

Stacking Gel (2.5%)

Acrylamide: bis-acrylamide (30:0.8)	-	2.5 ml
Stacking gel buffer stock	-	5.0 ml
Ammonium persulphate (APS)	-	a pinch
Water	-	12.5 ml
TEMED	-	`15.0 µl

Sample buffer (1X)

Native-PAGE sample buffer (2X)	-	1.0 ml
50 % Sucrose	-	0.4 ml
DW	-	0.6 ml

3.11.2.2 Sample preparation

Sample mix was prepared with 25 µl sample and 5 µl marker. To this 10 µl of 1X sample buffer was added mixed well and loaded to the gel.

Procedure

The gel plates were cleaned and assembled.

a. Resolving gel -All the components, except APS, were added in to a beaker, mixed gently, and finally added APS. The mixture was immediately poured into the gel assembly and a layer of butanol was added over the gel and allowed to polymerize

at least for 1 h. Removed the butanol and washed with water to remove all the traces of butanol.

b. Stacking gel -All the components of stacking gel except APS were added into a beaker, mixed gently. Finally APS was added and the contents were poured into the gel assembly above the resolving gel, and the comb was immediately inserted between the glass plates. This was allowed to polymerize at least for 30 min.

Gel was placed in the electrophoresis apparatus, and upper and lower reservoirs were filled with reservoir buffer for Native-PAGE. The gel was pre run for 1 h at 80 V.The protein sample was loaded to the gel and was run at 80V until the sample entered the resolving gel. When the dye front entered the resolving gel, the current was increased to 100 V. The run was stopped when the dye front reached 1 cm above the lower end of the glass plate.The gel was removed from the cast and stained for at least one hr in the staining solution.The gel was destained till the bands became clear and were then observed using the transilluminator.

3.11.3 Silver staining

Solutions and reagents for silver staining

1. Wash solution - 20 % ethanol in distilled water
2. Sensitizer - 12.5 % gluteraldehyde in distilled water
3. Stain - 2 ml of 20 % aqueous solution of silver nitrate
   1 ml $(NH_4)_2OH$
   5 ml of 5 N NaOH
   Made up to 100 ml with solution 1. Mixed and used freshly.
4. Developer - 100 µl of 37% formaldehyde solution
   25 µl 2.3 M citric acid
   Made up to 100 ml with solution 1. Mixed and used freshly.

5. Fixing solution - 100 ml acetic acid and 400 ml methanol made up to 1 liter with DW
6. Preserving solution - 50 ml glycerol and 50 ml acetic acid made up to 500 ml with DW

Procedure

1. The gel was run till the dye front reached 1 cm above the lower end of the glass plate, and was transferred to the fixing solution and kept for 10 min on a Gel rocker.
2. This was followed by washing with distilled water for 10 min.
3. The sensitizer was added to the gel and allowed to react for 10 min.
4. The gel was washed twice with distilled water for 10 min each, followed by wash solution for 15 min.
5. The gel was stained for 15 min, followed by washing twice with wash solution for 10 min each.
6. The bands were then allowed to develop by rinsing in the developer solution for 2-10 min, after which the gel was transferred to the preserving solution.

3.11.4 Zymogram

The sample with tannase activity was mixed with native sample buffer. Samples were loaded and run at 70 V per gel, for 2.5 h at 4 °C, in a Mini Protean II electrophoresis cell (Bio-Rad). The location of tannase activity within the gel was determined essentially as described by Aoki *et al*., (1979). The gel was washed for 45 min twice with 100 ml of 10 mM acetate buffer pH 5.5 with constant shaking. This was followed by incubation of the gel in 0.1 M acetate buffer pH 5.5 containing tannic acid 0.5 % (w/v) at 30°C, with constant shaking. The tannic acid solution was discarded and replaced with 100 ml of 0.5 % (w/v) quinine hydrochloride solution in 0.05 M acetate buffer pH 5.5 at room temperature. Tannase activity appeared as a clear band on a white background.

determined by incubating the enzyme in the enzyme reaction buffer containing different concentrations of methyl gallate (0.01 to 0.2 M) at pH 2 for 5 min at 30ºC. Enzyme assay was done as described in section 3.4.1. The initial velocity data was plotted as the function of the concentration of substrate by the linear transformation of Michaelis-Menten equation and usual nonlinear curve fitting of Michaelis-Menten equation for calculation of K_m and V_{max} of the reaction.

3.11.16 Effect of various metal ions on enzyme activity

Effect of various metal ions on enzyme activity was evaluated by incubating the enzyme along with different concentrations (1, 5, 10, 15 and 20 mM) of various metal ions in the enzyme reaction mixture for 1h followed by measuring the residual enzyme activity. The metals studied included sodium chloride, calcium chloride, magnesium sulphate, zinc sulphate, potassium sulphate, cobalt chloride, manganese chloride, ferric chloride, cupric sulphate, sodium molybdate, barium chloride, lithium chloride, cadmium sulphate, aluminium sulphate and mercuric chloride which contributed the metal ions Na^{+}, Ca^{2+}, Mg^{2+}, Zn^{2+}, K^{+}, Co^{2+}, Mn^{2+}, Fe^{3+}, Cu^{2+}, Mo^{6+}, Ba^{2+}, Li^{+}, Cd^{2+}, Al^{3+} and Hg^{2+} respectively.

3.11.17 Effect of various detergents and chelator on enzyme activity

Effect of various non-ionic and ionic detergents such as tritonX-100, tween-80, tween-20 and brij-35 (w/v) on enzyme activity was determined by conducting enzyme assay in the presence of each detergent and residual activity was calculated. After incubation of enzyme in different concentration of each detergent 0.2, 0.4, 0.8, 1, and 5 % for 1 h, assay was conducted.

Effect of EDTA, enzyme chelator, on tannase activity was evaluated by preparing a mixture at 2 mM level with tannase enzyme at 1:1 (v/v) ratio and estimating the residual tannase activity.

of 0.1 ml of enzyme was diluted to 1ml with distilled water and 1ml of phenol was added to it. Five ml of 96 % sulphuric acid was added, mixed well and kept at 28ºC for 20 min. Absorbance was read at 490 nm.

3.11.7 Activity staining for esterase

Chromogenic substrate plates were prepared with phenol red (0.01 %) along with 1 % substrate tributyrin, 10 mM $CaCl_2$, and 2 % agar. The pH was adjusted to 7.3-7.4 by using 0.1 N NaOH. Crude extract was impregnated onto a small disc of filter paper (diameter 5 mm) and placed on the top of chromogenic substrate plates. The plates were incubated at 37 ºC for 20 min. Discs with heat inactivated enzymes served as control (Singh *et al.*, 2006).

3.11.8 Isoelectric focusing

Active fractions obtained after gel filtration chromatography and lyophilization were used for isoelectric focusing. 1 ml aliquot of the lyophilized protein sample was resuspended in 0.1ml buffer and used. Protean®IEF Cell, Biorad, U.S. was used for isoelectric focusing.

3.11.8.1 Reagents

Rehydration buffer

8 M Urea

2 % CHAPS

50 mM DTT

0.2 % Biolyte ampholyte

IPG strip of 7 cm (pI 3-10 range) was used. 125 µl rehydration buffer mixed with 30µl of sample containing169µg protein was applied in the rehydration tray and the strip was placed with gel upside down in the tray. The setup was left for rehydration overnight. 3 drops of mineral oil was overlaid on this to prevent the evaporation. Paper wicks were placed over the electrodes of IEF tray. 10 µl of

deionised water was added over each wick. Rehydrated gel after draining the mineral oil was placed again in the IEF tray with gel side down. 3 drops of mineral oil were over laid and care was taken to avoid air bubble getting trapped. IEF apparatus was closed and Isoelectric focusing was done in 3 steps.

	Volt	Time	Volt h	Ramp
1.	250V	20 min		Linear
2.	4000V	2 h		Linear
3.	4000V	-	10,000Vh	Rapid

A total of 5 h was taken with 14000 V h at 20ºC for the completion of Isoelectric focusing.

3.11.9 Optimal temperature

Temperature optimum for maximal tannase activity was determined by incubating the enzyme at various temperatures in the range of 5 to 100 ºC under assay conditions already mentioned, except temperature. Enzyme activity was calculated as described in section 3.4.1.

3.11.10 Enzyme stability at different temperatures

Thermal stability of tannase enzyme was determined by incubating the enzyme sample at various temperatures ranging from 30-80ºC over a total period of 24 h, and the enzyme assay was conducted after 1h, 2h, 4h, 6h, and 24h. Enzyme activity of the sample kept at 4ºC was taken as control. Residual activity of enzyme was calculated as described under section 3.11.21.

3.11.11 Optimal pH for tannase activity

Optimum pH for maximal enzyme activity was determined by conducting enzyme assay at various levels of pH in the range of 1-10. The enzyme assay was essentially same as described in section 3.4.1 with a few modifications. The substrate methyl gallate was prepared in respective buffer of each pH. The buffer

system used included HCl-KCl buffer (pH 1-2), Citrate buffer (pH 3-6), Phosphate buffer (pH 7), Tris buffer (pH 8-9), Carbonate bi carbonate buffer (pH 10).

3.11.12 Stability of tannase at different pH

Stability of the enzyme over a range of pH was determined by measuring the residual activity after incubating the enzyme in different buffer systems of pH 1-10 for 1 h and 24 h at RT. 0.2 ml of enzyme incubated in 3.8 ml of different buffer systems which included HCl-KCl buffer (pH 1-2), Citrate buffer (pH 3-6), Phosphate buffer (pH 7), Tris buffer (pH 8-9), Carbonate bi carbonate buffer (pH 10). After incubation enzyme assay was conducted and residual activity was calculated as described in section 3.11.21.

3.11.13 Effect of inhibitors on tannase activity

Effect of enzyme inhibitors phenyl methylsulfonyl fluoride (PMSF), 1, 10-O Phenanthroline, and Sodium deoxy cholate on tannase activity was evaluated. Each inhibitor solution was prepared at 2mM level and mixed at 1:1 (v/v) ratio with tannase enzyme. Residual tannase activity was calculated as described in section 3.11.21.

3.11.14 Substrate specificity

The efficiency of the enzyme to hydrolyze various substrates was evaluated by conducting enzyme assay with methylgallate, propylgallate and tannic acid as substrates at a concentration of 0.01, 0.05 and 0.1 M in solution prepared in HCl-KCl buffer- pH 2. Enzyme activity was expressed in U/ml.

3.11.15 Kinetic studies

The enzyme kinetic studies were conducted, towards determining the K_m and V_{max}. K_m, the substrate concentration at which the reaction velocity is half maximum and V_{max}, the velocity maximum of the enzyme reaction were

temperature. 5 µl of DNA (in 6X loading buffer) was loaded on to gel and electrophoresis was carried out at 80 V for 1 h. Double digest of Lambda DNA with *EcoR1*/*HindIII* (Genei, Bangalore) was used as marker. The gel was stained in freshly prepared 0.5mg/ml ethidium bromide solution for 10 min and viewed on an UV transilluminator and the image was captured with the help of gel documentation system (Bio rad, U.S).

3.13 Cloning of the tannase gene fragments from *A. awamori BT*MFW 032

3.13.1 Primers

3.13.1.1 Primers for amplifying and sequencing of the tannase gene from *A. awamori* BTMFW 032

The only available sequences for tannase from *Aspergillus* sp. were the cDNA sequence of *A. oryzae*, *A. niger* and *A. fumigatus.* The sequences were obtained from Gen Bank. From those sequences two upstream and two downstream primers were designed for the amplification of the tannase gene from *A. awamori.* All the primers used in the study were synthesized by Integrated DNA Technologies, Inc.USA.

Designed forward and reverse primers for the amplification of the tannase gene from *A. awamori.*

Primer Name	**Forward primers**
TF1	**5' – GACTACTGCAATGTGACTGTC-3'**
T F2	**5' – TACTACCCTCCTCCATGTGAG-3'**
	Reverse primers
TR1	**5' – GTCAGATTCACCGTGGTAGTG-3'**
TR2	**5' – AACAGTGGCATTGAGACGGGA-3'**

3.11.18 Effect of an oxidizing agent on enzyme activity

Activity and stability of the enzyme in the presence of oxidizing agent, hydrogen peroxide, was studied by measuring the residual activity after 1 h of incubation of the enzyme in different concentrations of hydrogen peroxide (1, 2, 3, 4, 5, 10 and 20 % (v/v)). The residual enzyme activity was calculated as described under section 3.11.21.

3.11.19 Effect of reducing agents on enzyme activity

Activity and stability of the enzyme in the presence of reducing agents were studied by incubating the enzyme solution with 0.2, 0.4, 0.6, 0.8, 1 and 5 % (v/v) of dithiothreitol, β-mercaptoethanol and sodium thioglycollate for 1 h and estimating the residual activity as described under section 3.11.21.

3.11.20 Effect of organic solvents on enzyme activity

Impact of various organic solvents on enzyme activity was evaluated by incubating the enzyme with each organic solvent for 1 h and 24 h and assaying the residual activity as described under section 3.11.21. The organic solvents studied included acetone, ethanol, methanol, dimethyl sulphoxide, hexane, benzene, butanol, acetonitrile, isopropanol and chloroform at 10, 30 and 60 % (v/v).

3.11.21 Residual activity

Residual activity is the percent enzyme activity of the sample with respect to enzyme activity of control sample.

$$\text{Residual Activity} = \frac{\text{Activity of sample (U/ml) x100}}{\text{Activity of control (U/ml)}}$$

All experimental data were statistically analyzed using Microsoft Excel.

3.12 Genetic characterization of tannase

3.12.1 Strains and vectors used

Fungus *Aspergillus awamori* BTMFW032, *E.coli* DH5α and the plasmid pGEM® T Easy Vector (Promega) were used in the present study.

3.12.2 Enzymes and chemicals used in the study

The enzymes used included Taq DNA polymerase (Sigma), T_4DNA ligase (Promega) and *Eco*R1 (Genei, Bangalore).

All chemicals were of analytical or molecular biology grade. The chemicals used were sodium chloride (NaCl), sodiumdodecyl sulphate (SDS), 2-amino-2-hydroxy methyl-1, 3-propane diol (Tris) and ethylenediamine tetraacetic acid (EDTA). The agarose (Qbiogene) molecular biology grade was used for electrophoresis. Ethidium bromide and ampicillin were procured from Genei, Bangalore. X-Gal (5-bromo-4chloro-3-indolyl-β-Dgalactopyranoside) and IPTG (Isopropyl-β-D-thiogalacto pyranoside) were obtained from Sigma-Aldrich.

3.12.3 Cultivation of fungus

The fungus *A. awamori* BTMFW 032 was cultivated in the Czapek Dox medium with glucose as carbon source. Medium preparation was done as described in section 3.2. Instead of tannic acid, glucose was used as carbon source in 50 ml of the medium in a 250 ml Erlenmeyer flask and cultivated at 28±2°C on a rotary shaker at 150 rpm for 4 days. Inoculation and incubation were performed as mentioned earlier under section 3.3.3.

3.12.4 Isolation of genomic DNA

Genomic DNA was isolated from 4 days old culture of *A. awamori* (Mini prep method, Lee and Taylor, 1990). The mycelia were harvested by means of filtration through Whatman® No.1 filter paper. The harvested mycelia were washed with phosphate buffered saline (PBS) and again filtered to remove residual

media. The mycelia were then ground with a pre-cooled (-20°C) mortar and pestle with liquid nitrogen until a fine powder obtained. Ground mycelia (2.5 g) was resuspended in extraction buffer (12.5 ml) consisting of 200 mM Tris - HCl (pH 8.5), 250 mM NaCl, 25 mM EDTA and 0.5 % SDS, after which phenol (pH 7.9) (8.75ml) preheated to 60°C was added followed by the addition of chloroform/isoamylalcohol [24:1 (v/v)] (3.75 ml)]. The suspension was carefully inverted a few times. After centrifugation (12000 rpm in a centrifuge, Sigma-Laboratory Centrifuge, Germany) for 60 min at 4°C the top liquid phase was removed containing the DNA. To remove excess RNA from the liquid phase 500 µl (5 mg/ml) RNase H was added and incubated for 15-20 min at 37°C. Equal volume of phenol was added to the mixture after incubation with the RNase and the mixture was again centrifuged (12000 rpm for 20 min) at 4°C. The liquid phase was removed and the DNA was precipitated with 0.54 volumes of isopropanol. The mixture was centrifuged (12000 rpm for 15 min) at 4°C and the resulting pellet was washed with 70 % (v/v) ethanol. The sample was centrifuged (12,000 rpm for 2 min) at 4°C after which the ethanol was aspirated and the pellet was dried under vacuum. The pellet containing the isolated DNA was then dissolved in 1 ml TE buffer 10 mM Tris (pH 7.8) and 1 mM EDTA and stored at -20°C for further manipulation.

The purity of DNA was checked by reading the absorbance ratioA_{260}/A_{280}. The quantification of DNA was done using DNA/Protein pack® software of Shimadzu UV-VIS spectrophotometer.

3.12.5 Agarose gel electrophoresis

The agarose gel electrophoresis was carried out for the visualization of DNA isolated. 1 % (w/v) agarose in 1X TAE buffer (0.1 M Tris, 0.05 M Na_2EDTA (pH 8.0) and 0.1 mM glacial acetic acid) was melted in a microwave oven. The molten agarose was poured into a gel casting tray and allowed to solidify at room

restriction analysis using E*coR*1 and reamplification of the plasmid DNA using the specific degenerate primers as described earlier section 3.13.2.

3.13.7 Restriction analysis

One µg of plasmid DNA was made up to 17 µl with deionized water. 2 µl of 10X reaction buffer was added, tapped well, and 1µl (4U/µl) of E*coR*1 enzyme was added, mixed well, and incubated at 37 °C overnight and reaction was arrested by heating the mix at 65 °C for 5 min. The mix was loaded in a 0.8 % (w/v) agarose gel.

3.13.8 *In-silico* analysis of the sequence

Sequencing was done at Chromous Biotech, Bangalore, using the big dye terminator kit. The nucleic acid and deduced protein sequences were analyzed by various online algorithms for structural prediction, presence of exons, motifs and domains, analysis of phylogenetic relation with other published tannase genes etc. The cloned and sequenced PCR products were analyzed online using BLAST (http://www.ncbi.nlm.nih.gov/blast). Nucleic acid and protein sequences of other tannase genes were obtained from NCBI.

Alignments of sequences were carried out using CLUSTALW 1.83(www.ebi.ac.uk/clustal), Dendrogram http://align.genome.jp/, ORF by http://www.ncbi.nlm.nih.gov, Genscan by www.genes.mit.edu/genscan/ and Motif scan by www.scansite.mit.edu/.

3.14 Application studies

3.14.1Tea cream Solubilisation

3.14.1.1 Preparation of tea extract:

A standard brew commercially available was prepared according to methodology of Rutter and Stainsby, (1975). 8 g of Kannan Devan tea

3.13.2 Gene amplification

A PCR was performed in a total reaction mix of 50µl of the isolated genomic DNA from *A. awamori* to amplify the tannase gene. The PCR was performed with the primer pairs TF1-TR1, TF1-TR2, TF2-TR1, TF2-TR2 combinations of the forward and reverse primers. The PCR mixture contained 1µl of isolated genomic DNA (200 ng) from *A. awamori*, 1 µl of each primer (100 pmol/µl), 2 µl 10 mM deoxyribonucleoside triphoshate, 5 µl 10X PCR buffer containing $MgCl_2$, and 1µ l of 5 U/µl *Taq* DNA Polymerase. The PCR reaction was conducted with the initial denaturation at 94°C for 2 minutes followed by denaturation at 94°C for 45 seconds, annealing at 60°C for 60 seconds and elongation at 72°C for 2 min. These cycles were then followed by 34 cycles of denaturation, Annealing and elongation was followed by an extended final elongation step at 72°C for 10 min.

The PCR product was electrophoresed in a 1 % (w/v) agarose gel stained with ethidium bromide and observed on an UV transilluminator. The amplicon was excised from the gel and the DNA was eluted from the gel slice by using the EZ-10 Spin column DNA Gel extraction kit obtained from Biogene, USA, according to the manufacturer's specifications.

The purified amplicon after gel extraction was cloned into pGEM®T vector of pGEM®T Easy vector system II-Promega as per manufacturer's specifications. The ligated plasmids were transformed in to $CaCl_2$ competent cells (*E coli* DH5α) as described below.

3.13.3 Ligation

The amplicon obtained was ligated to pGEM®T vector (pGEM®T Easy vector system II-Promega as per manufacturer's specifications). Ligation mix was prepared with 5 µl of 1X ligation buffer, 3µl of insert, 1µl of pGEM®T vector and

1 µl of T_4 ligase enzyme. All the components were mixed by pipetting and kept at 4ºC overnight.

3.13.4 Competent cell preparation

A single colony of *E.coli* host cell (DH5α) was inoculated in 5 ml of Luria Bertani (LB) broth and incubated overnight with a constant shaking of 150 rpm at 37°C. 1 % (v/v) of overnight culture was inoculated to 50 ml of LB broth and incubated at 37°C in a shaker at 150 rpm until the OD was 0.4 - 0.6. The cells were harvested at 10,000 rpm for 10 min at 4°C. The pellet was suspended in 10 ml of ice cold 0.1 M$CaCl_2$ and incubated in ice for 30 min. The cells were harvested again by centrifugation at 7,000 rpm for 5 min at 4ºC. The pellet was resuspended in 1ml of 0.1M $CaCl_2$.

This was aliquoted as 80µl fractions and added with chilled glycerol (20µl) and stored at -80ºC until use (Sambrook *et al*., 1989).

3.13.5 Transformation

Ten µl of ligation mix was added to 100 µl of stored competent cells and incubated in ice for 40 min. Then the cells were given a heat shock at 42°C for 90 seconds in a water bath followed by quick chilling on ice for 5 min. A volume of 250 µl LB was added to the transformed cells and incubated at 37°C for 1 h. 50 µl of the cells were plated on LB agar plates containing 50 µg/ml ampicillin, 100µg/ml IPTG and 40 µg/ml X-gal employing spread plate technique and incubated at 37°C. The plates were checked for the transformants after overnight incubation. Colonies containing plasmids with inserts were identified by blue/white colony selection. Isolated colonies were grown in 5 ml LB containing 50 µg/ml conc. of ampicillin at 37°C for 16 h, after which the plasmid DNA was isolated from the bacterial cells by using the alkaline lysis plasmid isolation procedure (Maniatis *et al*., 1982).

3.13.6 Plasmid Isolation

3.13.6.1 Reagents

Solution I

Glucose	-	50 mM
Tris-HCl (pH 8)	-	25 mM
EDTA (pH 8)	-	10 mM

Solution II

NaOH	-	0.2 N
SDS	-	1 % (w/v)

Solution III

Potassium acetate 5M (pH 7.5)	-	60.0ml
Glacial acetic acid	-	11.5 ml
Deionized water	-	28.5 ml
pH	-	5.4

The cultures were centrifuged at 12,000 rpm for 2 min at 4°C. The pellets were suspended in 100µl each of ice cold solution I for 5 min. 200 µl of freshly prepared solution II were added, mixed well by inversion and incubated for 10 min. This was followed by addition of 150 µl (-20°C) of ice cold solution III to each tube and then incubated in ice for 15 min. Centrifuged at 12000 rpm for 15 min and the clear supernatants were transferred to new tubes. 0.6 volumes of ice cold isopropanol was added to each tube, mixed well and incubated for 10 min. The plasmid DNA was pelleted by centrifugation and washed with 70 % ethanol; air dried, and dissolved each in 20 µl TE buffer (pH 8). The plasmid DNA samples were stored at -20ºC.

The prepared plasmid DNA was subjected to agarose gel electrophoresis on a 0.8 % (w/v) agarose gel. The presence of the insert DNA was confirmed by

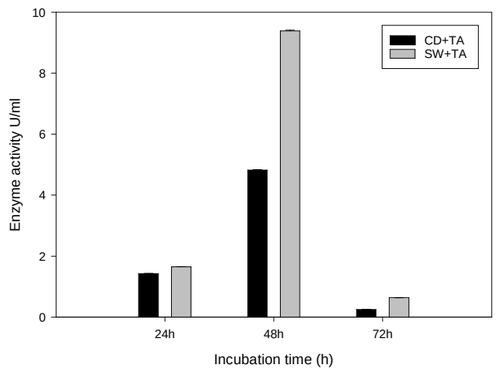


Fig 4.2 Evaluation of cell free extract obtained from *A. awamori* BTMFW 032 for tannase activity.

Optimal concentration of tannic acid required for supporting maximal tannase production was determined by growing the fungus in cultivation medium supplemented with various concentration of tannic acid varying from 1-15 % (w/v). The study was conducted in Czapek Dox medium prepared with 50 % sea water such that the original constituents of Czapek Dox medium were at their half strength and constituents of sea water at their half strength. Results presented in Fig 4.3 indicated that tannic acid at concentrations of 3-5 % supported maximal tannase activity (423 U/ml), while maximal specific activity could be recorded with 1-3 % tannic acid concentrations. Although enhanced levels of tannase activity was recorded for tannic acid concentrations up to 7.5 % (w/v) the specific activity obtained for the same level of concentration was comparatively minimal. It was also noted that in the presence of glucose and with induced inoculum, production of tannase was at enhanced level compared to that with uninduced inoculum. Gallic acid production in the fermented medium also recorded a gradual

(commercially available in market) was infused in to 100 ml distilled water at 90ºC for 6 min and filtered immediately through cotton.

3.14.1.2 Estimation of solid content

3.14.1.2.1 Total solids

Total solids were determined by incorporating an aliquot of infusion, in previously weighed dried petriplates, dried in an oven at 80ºC overnight (Rutter and Stainsby, 1975) and the final weight was calculated.

3.14.1.2.2 Cream solids

The brew was cooled over night at 4 ºC for cream formation. The brew was then centrifuged at 8,000 rpm for 15 min at 4 ºC. The clarified supernatant was then decanted from the cream layer and analyzed for total solid content. The weight of cream solids was then obtained by the difference from the total solid content of the original brew (Rutter and Stainsby, 1975).

3.14.1.3 Treatment of tea extract with tannase

Ten ml of tea extract was treated with 1 ml of the enzyme and incubated at 30ºC for 1 h. The solid contents were determined in an aliquot of enzyme treated infusion and control which inlcuded 1ml of distilled water instead of enzyme. The samples were kept at 4ºC overnight to allow cream formation. The infusion was centrifuged and solid contents were determined in the supernatant of the enzyme treated tea infusion and in control samples. The difference in weight of solid contents was reported as cream solubilised from the extract.

3.14.1.4 Determination of cream content

$$\text{Cream content (\%)} = \frac{(\text{Total solids in control} - \text{Total solids in control after centrifugation})}{(\text{Total solids in control})} \times 100$$

3.14.1.5 Determination of % cream solubilised

% cream solubilised =

$$\frac{(\text{Cream content in control} - \text{Cream content in enzyme treated sample})}{(\text{Cream content in control})} \times 100$$

3.14.2 Propyl gallate

Propyl gallate can be synthesized by transesterificationof tannic acid in presence of tannase (Sharma and Gupta, 2003). Two ml of tannase enzyme was added to 10 ml of 10 mM tannic acid in n-propanol, shaken at 100 rpm for 72 h and then it was extracted with double the volume of ethyl acetate and separated using a separating funnel. Ethyl acetate was evaporated to recover propyl gallate in powder form. This powder was further analyzed for the presence of propyl gallate.

3.14.2.1 Fourier-Transform Infra red Spectroscopy (FT-IR Spectroscopy)

This was done as described under section 3.9.1

3.14.2.2 Thin layer Chromatography

The propyl gallate obtained after transesterification of tannic acid with tannase was also analyzed by thin layer chromatography with silica gelG-60 F_{254} (E. Merck, Mumbai, India). The solvent system consisted of ethyl acetate, chloroform and formic acid (4:4:1). After drying, the plates were developed by spraying a solution of $FeCl_3$. Standard propyl gallate and sample were run in the same plate. R_f value was calculated.

Chapter 4

RESULTS

4.1.1 Preliminary evaluation of basal cultivation medium and process variables for tannase production by *Aspergillus awamori* BTMFW 032

Aspergillus awamori BTMFW 032 produced tannase on Czapek Dox minimal medium supplemented with tannic acid as sole source of carbon (Fig.4.1). Maximal tannase enzyme activity was observed after 48 h. Enhanced levels of tannase production were supported by sea water based medium compared to Czapek Dox medium. It was observed that about 95 % of enhancement in enzyme production was recorded with sea water. In Czapeks Dox minimal media supplemented with 1 % (w/v) tannic acid as sole source, and with uninduced inoculum 4.8 U/ml of enzyme activity was recorded at 48 h compared to 9.39 U/ml recorded with medium containing seawater added with 1 % (w/v) tannic acid as the sole carbon source and with uninduced inoculum (Fig 4.2).

Fig 4.1 *Aspergillus awamori* BTMFW 032 in tannicacid agar plate.

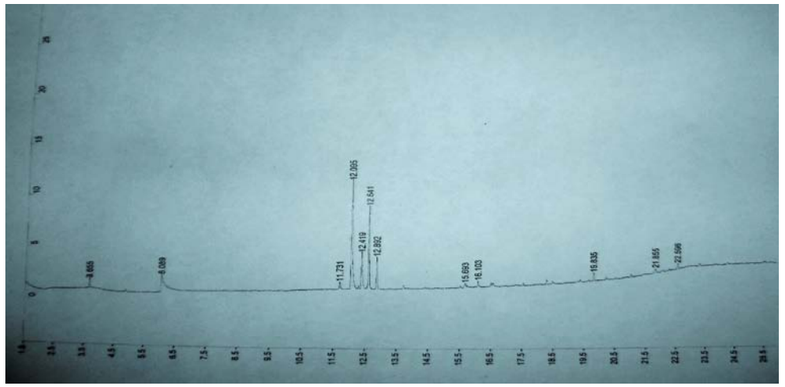


Fig.4.7 Gas chromatogram of gallic acid standard

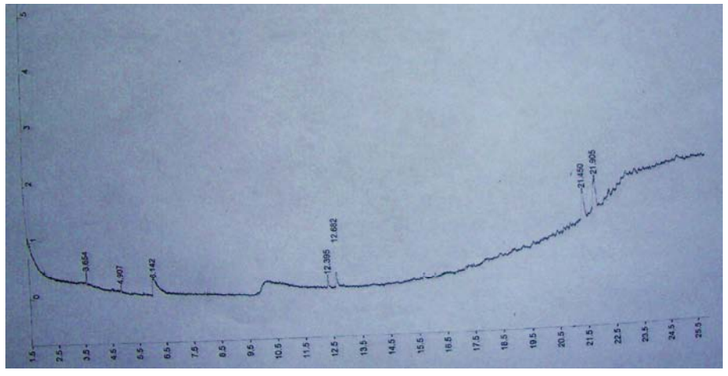


Fig.4.8 Gas chromatogram of gallic acid extracted from the fermented broth

increase along with increase in concentration of the tannic acid from 1 % to 4 % (w/v), which however later declined rapidly.

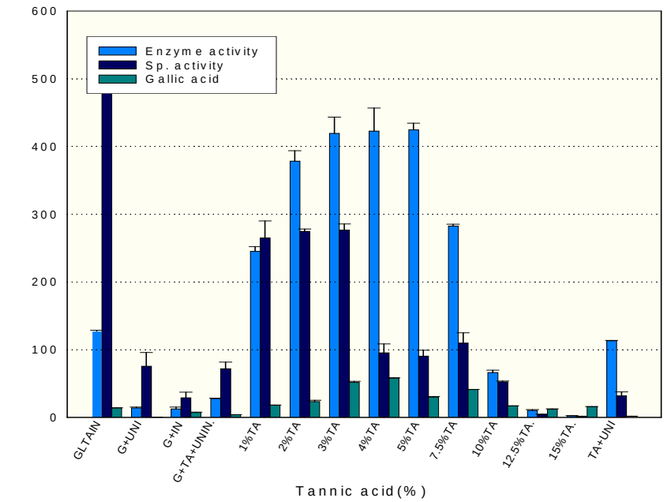


Fig.4.3 Optimization of concentrations of tannic acid, glucose and uninduced inoculum for maximal production of tannase by *A. awamori* BTMFW 032

4.1.2 Identification of gallic acid

The FT-IR absorption for gallic acid has strong bands at 1639.26 cm^{-1} and 1560.90 cm^{-1} representing the C=C bond (1900-1500 cm^{-1}), O-H bonds at 3442.21 cm^{-1} (3700-2500 cm^{-1}), C-C bond at 1413.68 and 1384.82 cm^{-1}. This correlates with the presence of these bonds in a commercially available gallic acid run as standard (Fig.4.4, 4.5).

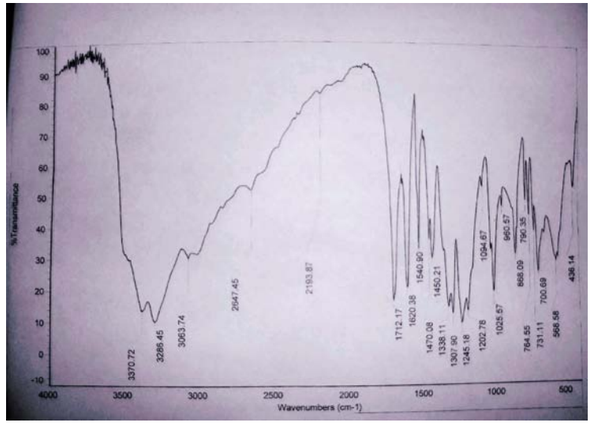


Fig.4.4 FTIR analysis of Standard gallic acid

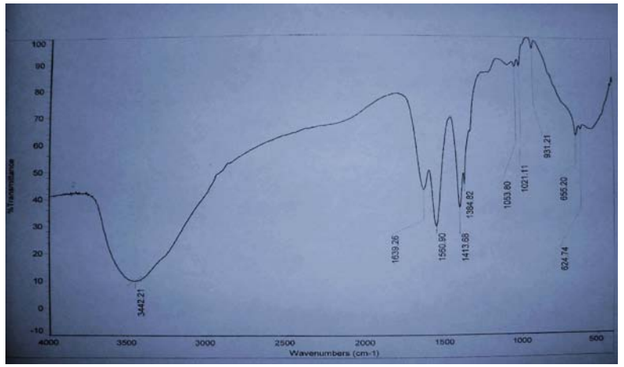


Fig.4.5 FTIR analysis of gallic acid extracted from the fermented broth

1. Standard gallic acid
2. Sample (after extraction)
3. Sample (before extraction)

Fig.4.6 TLC analysis of gallic acid extracted from the fermented broth

Thin layer chromatogram clearly indicated the presence of gallic acid in the sample when compared with standard and the R_f value was found to be 0.69 which correlated with the already reported value (0.698) (Fig 4.6). Gas chromatography results indicated the purity of the sample when compared with a commercially available gallic acid run as a standard Fig (4.7, 4.8). HPLC results also indicated the purity of extracted and purified sample of gallic acid and efficiency of the method in separating the gallic acid (Fig 4.9, 4.10).

4.2 Optimization of bioprocess variables for tannase production, by *A. awamori* BTMFW 032, employing statistical approach

Medium that could support maximum tannase production by *A. awamori* BTMFW 032 was optimized employing statistical approach. Initially process variables were optimized using Plackett-Burman design and in the second stage, Response surface methodology was adopted towards selection of optimal variables and understanding the probable interaction among the significant variables. Fermentation techniques slurry state fermentation (SLF), submerged fermentation (SmF) and solid state fermentation (SSF) were studied for optimization of suitable variables that support maximal tannase enzyme and gallic acid production.

4.2.1 Slurry state fermentation

4.2.1.1 Selection of variables that significantly affect tannase production

4.2.1.1.1 Plackett-Burman Design

Plackett-Burman design offers an effective screening procedure and computes the significance of a large number of variables in one experiment, which is time saving and maintains convincing information on each component. The data presented in Table 4.2 on tannase production with Plackett-Burman design experiments illustrate a wide variation of enzyme concentrations from 23.36 U/ml to 57.43 U/ml, which indicated the importance of medium optimization to attain higher yields. The statistical significance of the model equation, evaluated by F-test analysis of variance (ANOVA) (Table 4.3), revealed that the obtained regression is statistically significant. The model F value of 8.36 and values of Prob>F less than 0.05, evidence that the model terms were significant.

Further the results presented as pareto chart (Fig.4.11) for the effect of individual variables studied in Plackett-Burman design testify that tannic acid and inoculum concentration had a positive effect in enhancing enzyme production,

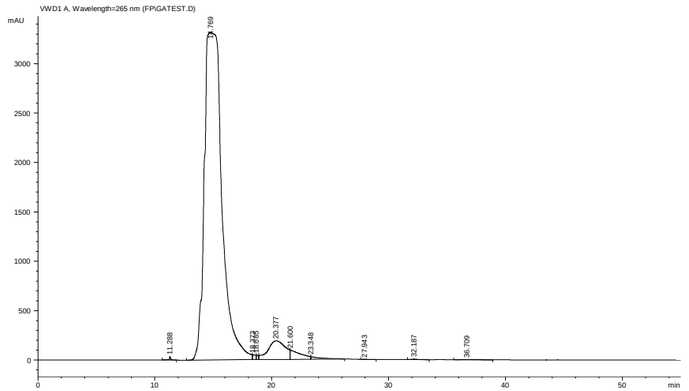


Fig. 4.9 High performance liquid chromatogram of gallic acid sample

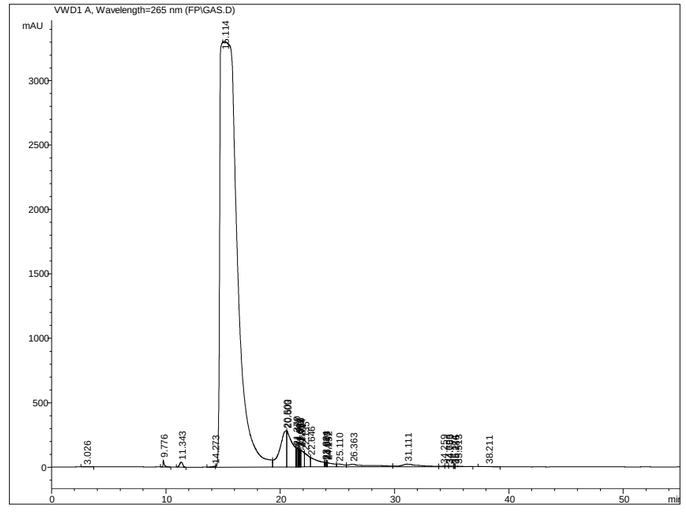


Fig. 4.10 High performance liquid chromatogram of gallic acid standard

4.1.3 Selection of natural substrates as carbon source for tannase production by *A. awamori* BTMFW 032

Various natural substrates were tried as carbon source, both independent and in combination with tannic acid, for tannase production by *A. awamori* BTMFW 032. It is evident from the data presented in Table 4.1 that irrespective of the substrates tried and the medium whether prepared with sea water or not, tannic acid influenced tannase synthesis by the fungus although tannase production was also observed in the absence of tannic acid in the medium in the case of a few substrates. In fact tamarind in combination with tannic acid in sea water medium recorded not only enzyme levels higher than the control (240U/ml) but also a maximum enzyme activity (374.67 U/ ml) followed by grapes wastes and tannic acid in Czapek Dox medium (369.21U/ml), and garcinia leaves and tannic acid in sea water medium (256.05 U/ml). Further, it was observed that garcinia leaves alone, without addition of tannic acid, could induce tannase production by the fungus (22.059 U/ml in Czapek Dox medium and 26.2090 U/ml in sea water) suggesting its potential for use as a substrate for tannase production. All other substrates were not effective and recorded tannase at very low level compared to control. Tamarind and grape wastes are not economical and not easily available even though they supported enhanced production of tannase when used as carbon source and inducer. Hence, garcinia leaves were selected as a potential substrate for tannase production. It was also noted that garcinia contains 5 mg tannic acid equivalents found to be present in 500 mg of leaf *i.e.*, 1 % Tannic acid equivalent present in garcinia leaves (As per Folin-denins method, Schanderl, 1970).

Table 4.1 Effect of various natural substrates as carbon source on tannase production by *Aspergillus awamori* BTMFW032 (enzyme activity assayed after 48 h of fermentation.)

S.No	Natural substrate	Media	Inducer	Enzyme activity U/ml
1	Control	Seawater50%(v/v) +Czapekdox50%(v/v)	Tannic acid 1% (w/v)	240.51
2	Mango leaves	Seawater Czapekdox	Tannic acid	13.4354
3	Mangoleaves	Seawater	Tannic acid	172.617
4	Grape wastes	Seawater	Tannic acid	369.208
5	Garcinia leaves	Czapekdox	Tannic acid	256.048
6	Garcinia leaves	Czapekdox	Tannic acid	188.727
7	Garcinia leaves	Seawater	-	22.059
8	Garcinia leaves	Seawater	-	26.2090
9	Cashew apple	Seawater	Tannic acid	154.779
10	Tea powder	Czapekdox	Tannic acid	98.679
11	Tamarind	Czapekdox	Tannic acid	374.67
12	Tamarind pods,	Czapekdox	Tannic acid	0.0
13	Psdium leaves,	Czapekdox	Tannic acid	0.0
14	Coconut fibre	Czapekdox	Tannic acid	0.0
15	Coconut pith	Czapekdox	Tannic acid	0.0
16	*Averrhoa bilimbi* fruits	Sea water	Tannic acid	0.0
17	*Averrhoa bilimbi* leaves		Tannic acid	0.0

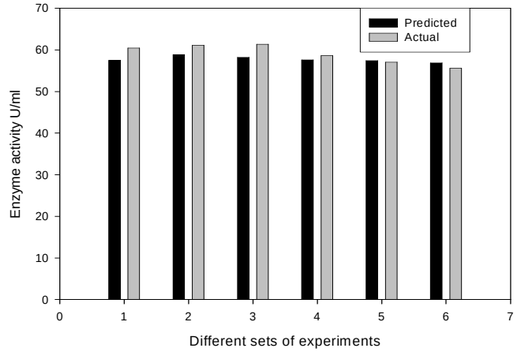


Fig 4.12 Validation of Plackett–Burman design

From the results obtained with Plackett-Burman design it is inferred that among the eighteen variables evaluated only five variables namely garcinia leaf, tannic acid, glucose, temperature and inoculum were found to be the most significant variables.

First order model equation:

Tannase activity Y U/ml= +44.14250-0.28315*garcinia-76.550*glucose+140.0100*tannic acid. 3.9740*temperature + 5.02600* inoculum.

4.2.1.1.2 Response Surface methodology

Response surface methodology using Box-Behnken design was adopted towards selection of optimal level of significant variables garcinia leaf, tannic acid, glucose, inoculum concentration and temperature based on Plackett-Burman design experiment. The results obtained for Box-Behnken design experiment were analysed by ANOVA, which yielded the following regression equation for the level of tannase production.

along with the increase in their concentrations. Whereas glucose, incubation temperature and garcinia leaves had a negative effect on enzyme production along with the increase in variable.

Validation of the PB design was carried out in shake flasks under conditions predicted by the model and it was noted that the experimental values were very close to the predicted values and hence the model was successfully validated (Fig.4.12).

Table 4.2 Studies on the effect of the variables on tannase and gallic acid production by *Aspergillus awamori* BTMFW 032

	Garcinia	Sea water	Sodium nitrate	Potassium chloride	Magnesium sulphate	Zinc Sulphate	Coppersulphate	Ferrous sulphate	Dipotassium hydrogen phosphate	Glucose	Tannic acid	pH	Temperature	Inoculum	Agitation	Illumination	Incubation	Calcium chloride	Dummy	Enzyme activity U/ml	Gallic acidμg/ml
1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	42.17	2.34
2	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	29.61	0.648
3	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	31.66	0.766
4	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	25.91	1.24
5	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	56.15	3.45
6	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	12.14	0.984
7	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	26.19	0.844
8	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	34.36	2.83
9	-1	-1	-1	-1	-1	1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	35.42	0.764
10	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	42.88	1.32
11	-1	-1	-1	1	-1	-1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	57.43	0.825
12	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	47.28	2.53
13	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	31.8	2.43
14	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	49.69	3.715
15	1	-1	-1	-1	-1	-1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	23.36	1.2
16	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	53.17	2.6
17	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	49.911	2.67
18	1	-1	-1	1	1	1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	16.47	1.23
19	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	39.11	0.680
20	-1	-1	1	1	-1	-1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	45.93	0.678

Table 4.3 ANOVA for the experiments with Plackett-Burman design for tannase production by *A. awamori* BTMFW 032 under slurry state fermentation.

Term	Tannase yield
Model F-value	8.36
Prob > F	0.0008
Mean	37.53
R-Squared	0.7492
Adj R-Squared	0.6596
Pred R-Squared	0.4882
Coefficient of Variance	20.19
Adeq Precision	9.683

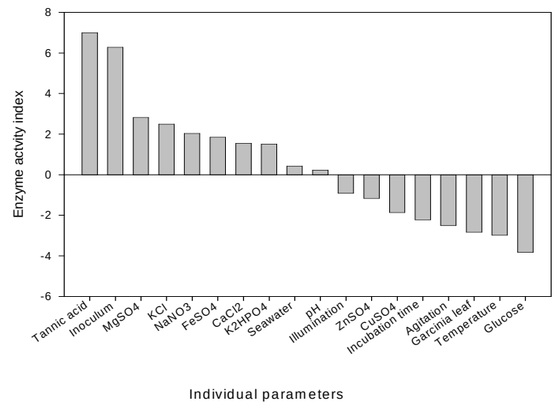


Fig.4.11 Pareto chart showing effect of individual factors on production of tannase enzyme by *A. awamori* BTMFW 032

Run	Block	Garcinia	Glucose	Tannic acid	Temperature	Inoculum	Enzyme activity U/ml
40	1	0	1	1	0	0	60.1651
41	1	0	0	0	0	0	64.1363
42	1	1	-1	0	0	0	52.6092
43	1	0	1	0	1	0	60.4462
44	1	0	1	0	-1	0	52.4718
45	1	0	0	0	0	0	64.6592
46	1	0	0	-1	0	1	42.098

4.2.1.1.3 Evaluation of variables influencing tannase production

Three dimensional response surface curves were plotted to study interaction among various physicochemical variables and to determine the optimum concentration of each individual factor for maximum tannase production. The model predicted 75.8 U/ml of tannase enzyme activity and obtained a maximum of 75.2 U/ml. It was noted that tannic acid which is used as an inducer has very high influence on the enzyme production and its higher concentration supported maximum enzyme production.

The pair wise interactions among the variables in terms of tannase production under the optimized conditions were assessed by examining the response surfaces. Three dimensional response surfaces were generated holding three factors constant at a time and plotting the response obtained for varying levels of the other two. With increase in tannic acid, enzyme activity increased but at higher and lower concentrations of garcinia, there was no interactive effect on production of enzyme (Fig 4.13).

Tannase activity Y (U/ml) = +1.29328 -0.18255*garcinia +1.81366*glucose +541.516*tannicacid +1.97696*temperature +1.24176*inoculum -0.047544*garcinia^2 -0.16906*glucose^2 -3269.474*tannic acid^2 -0.046906*temperature^2 -1.98237*inoculum^2 -.037727*garcinia*Glucose -0.88075*garcinia*tannic acid + 0.056280* garcinia temperature +0.21372* garcinia*inoculum - 14.45289* glucose*tannic acid +0.022286* glucose* inoculum + 1.18215* tannic acid*temperature +51.60360* tannic acid*inoculum.

The model F value of 14.84 and values of prob>F less than 0.05, obtained after ANOVA analysis of tannase production, indicated that the model terms are significant. Three linear and six quadratic terms were significant model terms for the response. The model coefficients estimated by multiple linear regressions and ANOVA (Table 4.4) testified that the model was significant with coefficient of determination R^2 of 0.9223. This ensured a satisfactory adjustment of the quadratic model to the experimental data and indicated that approximately 92.2 % of the variability in the dependent variable (response) could be explained by the model. The adjusted R^2 which is more suited for comparing models with different number of variables was 0.8601. All selected parameters were significant and varied levels of interactions were recorded for the variables in their cumulative effect on tannase production. The coefficient of variance was 6.53, and the adequate precision that measures the signal to noise ratio was 15.203. A ratio greater than 4 is desirable as it indicated an adequate signal. Thus this model could be used to navigate the design space. Experimental data on the effect of five selected physicochemical variables on production of tannase by *A. awamori* BTMFW 032 in a total of 46 experiments (Table 4.5) showed strong dependence on the presence and levels of selected variables since the enzyme production varied between 37.4 U/ml and 76.79 U/ml under experimental conditions studied. It was observed that in all the 46 experimental runs at instances (experimental runs 8, 14, 27, 41 and 45) when the variables were at mid level “0” the enzyme yield varied between 63.26 U/ml

and 64.75 U/ml with a mean value of 64.18 U/ml. Further among the variables evaluated tannic acid was observed to have a strong impact, compared to other variables, on the overall enzyme yield while effecting cumulative impact on enzyme yield. Thus it was noted that at instances when the tannic acid was at lower level "1", (the experimental runs- 2, 6, 9, 17, 21, 23, 33, and 46) the enzyme yield varied between 37.438 and 42.098 U/ml with a mean value of 39.36U/ml.

Table.4.4 ANOVA for the response surface experiments conducted using Box-Behnken design for tannase production by *A. awamori* BTMFW 032

Term	Tannase yield
Model F-value	14.84
Prob > F	<0.0001
Mean	55.92
R-Squared	0.9223
Adj R-Squared	0.8601
Pred R-Squared	0.6916
Coefficient of Variance	6.53
Adeq Precision	15.203

Table 4.5 Optimization of medium composition and physical parameters for production of tannase enzyme by *A. awamori* BTMFW 032under slurry state fermentation by Response surface methodology

Run	Block	Garcinia	Glucose	Tannic acid	Temperature	Inoculum	Enzyme activity U/ml
1	1	0	1	-1	0	0	37.438
2	1	0	0	1	0	0	62.3781
3	1	0	1	0	0	-1	47.6454
4	1	1	0	0	0	-1	43.3024
5	1	0	0	-1	-1	0	39.4036
6	1	0	0	1	1	0	62.3962
7	1	0	0	0	0	0	64.7497
8	1	0	-1	-1	0	0	39.6166
9	1	1	0	0	-1	0	52.6092
10	1	-1	-1	0	0	0	50.0532
11	1	-1	0	0	0	-1	57.508
12	1	-1	0	0	-1	0	59.6379
13	1	0	0	0	0	0	63.2588
14	1	-1	0	1	0	0	61.9808
15	1	0	-1	0	-1	0	55.3312
16	1	0	0	-1	0	-1	38.2045
17	1	1	0	0	1	0	66.0852
18	1	0	1	0	0	1	63.1757
19	1	0	0	0	1	1	65.262
20	1	0	0	-1	1	0	39.2226
21	1	1	1	0	0	0	53.9084
22	1	-1	0	-1	0	0	39.2119
23	1	0	0	0	-1	-1	52.656
24	1	1	0	1	0	0	60.656
25	1	-1	0	0	0	1	62.3163
26	1	1	0	0	0	1	58.7966
27	1	0	0	0	0	0	64.0927
28	1	0	0	0	1	-1	57.7316
29	1	-1	1	0	0	0	58.8978
30	1	0	-1	0	1	0	59.9627
31	1	0	-1	1	0	0	76.7966
32	1	0	0	-1	0	1	75.7859
33	1	1	0	-1	0	0	39.6486
34	1	0	-1	0	0	-1	51.3419
35	1	0	0	1	0	-1	58.9915
36	1	-1	0	0	1	0	56.23
37	1	0	0	1	-1	0	60.804
38	1	0	-1	0	0	1	59.9265
39	1	0	0	0	-1	1	58.5924

The parabola (Fig 4.17) indicated the interactive effect of glucose and temperature at higher concentration of tannic acid on tannase production. At higher and lower concentrations of glucose and temperature the enzyme productivity was less compared to the medium level.

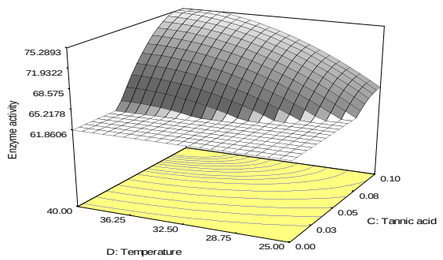


Fig.4.18 Effect of temperature and tannic acid on tannase production by *A. awamori* BTMFW 032

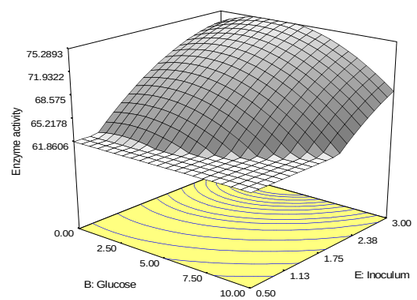


Fig.4.19 Effect of inoculum concentration and glucose on tannase production by *A. awamori* BTMFW 032

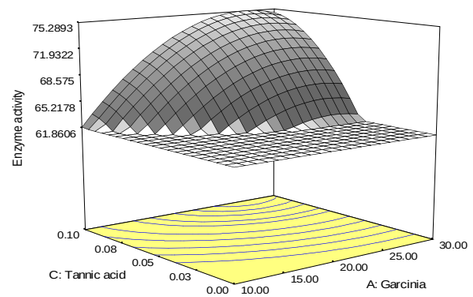


Fig.4.13 Effect of garcinia and tannic acid on tannase production by *A. awamori* BTMFW 032

At higher concentration of tannic acid, the medium level of inoculum concentration showed more enzyme activity compared to the higher and lower levels (Fig.4.14) where as garcinia showed lesser interaction with inoculum concentration at its higher, middle and lower levels (Fig 4.15).

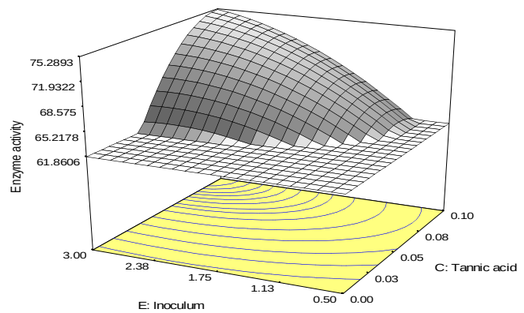


Fig.4.14 Effect of inoculum concentration and tannic acid on tannase production by *A. awamori* BTMFW032

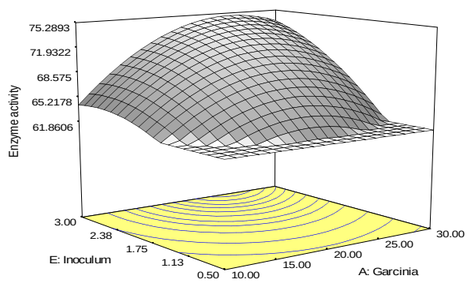


Fig. 4.15 Effect of inoculum concentration and garcinia on tannase production by *A.awamori* BTMFW 032

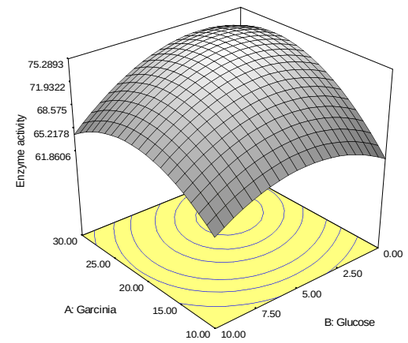


Fig. 4.16 Effect of garcinia and glucose on tannase production by *A. awamori* BTMFW 032

The parabolic nature of the graph indicated that the interactive effect of garcinia and glucose was very high at higher concentration of tannic acid, higher and lower levels of garcinia and glucose recording lesser enzyme activity compared to the medium level (Fig.4.16).

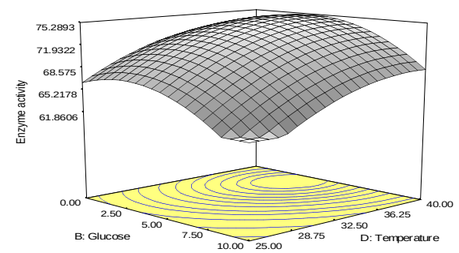


Fig.4.17 Effect of glucose concentration and temperature on tannase production by *A. awamori* BTMFW 032

zinc sulphate and glucose were found to be the most significant variables in gallic acid production.

First order model equation:

Gallic acid Y µg=0.9386+9.86600E-003*Seawater-8.53800*Zincsulphate-0.060610*Glucose+15.43100*Tannic acid.

Table.4.7 ANOVA for the experiments with Plackett-Burman design for gallic acid production by *Aspergillus awamori* BTMFW 032

Term	Gallic acid yield
Model F-value	16.23
Prob > F	<0.0001
Mean	1.69
R-Squared	0.8124
Adj R-Squared	0.7623
Pred R-Squared	0.6664
Coefficient of Variance	29.30
Adeq Precision	12.418

The statistical significance of the model equation was evaluated by F-test analysis of variance (ANOVA), which revealed that this regression is statistically significant (Table 4.7). The model F value of 16.23 and values of Prob>F less than 0.05 indicated that the model terms are significant. The effect of individual variables studied in Plackett –Burman design is presented as Pareto chart in Fig 4.23. The data evidence that tannic acid and seawater had a positive effect in enhancing gallic acid production, along with the increase in their concentrations while glucose and zinc sulphate recorded a negative effect on gallic acid production along with increase in the level of variable.

Temperature had poor interactive effect on tannase production with tannic acid at the range of temperature 25ºC-40ºC (Fig.4.18). At the same time interactive effect of glucose and inoculum seems to be negligible on enzyme production (Fig.4.19).

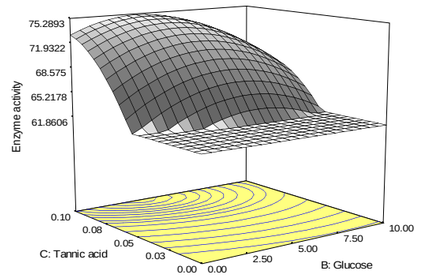


Fig.4.20 Effect of glucose and tannic acid on tannase production by *A.awamori* BTMFW 032

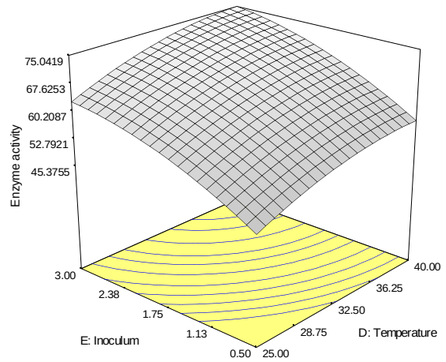


Fig.4.21 Effect of inoculum concentration and temperature on tannase production by *A.awamori* BTMFW 032

The glucose appeared to have poor interaction with tannic acid on enzyme production as the enzyme production increased with tannic acid concentration (Fig.4.20). The enzyme productivity was supported at higher concentration of inoculum and high temperature compared their lower range (4.21).

Table 4. 6 Mathematically predicted and experimental values for tannase production by *A. awamori* BTMFW032 in slurry state fermentation as per the design of RSM

Experiment No	Tannase production Predicted U/ml	Experimental U/ml
1	75.8309	75.245
2.	75.8136	75.189
3.	75.8038	75.019
4.	75.8015	75.007
5.	75.777	75.002
6.	75.6854	74.987
7.	75.459	74.836

4.2.1.1.4 Validation of the response model

Validation of the deduced response surface model based on the previous experiments was carried out in shake flasks under conditions predicted by the model. The experimental values were found very close to the predicted values and hence the model was successfully validated (Table 4.6).

Time course studies using *A.awamori* BTMFW 032 under final optimized conditions reveal that tannase production (Fig.4.22) increased rapidly during initial stages of fermentation and maximum enzyme activity (75.23 U/ml) was recorded at 24 h. Further incubation registered only a decline in enzyme activity**.**

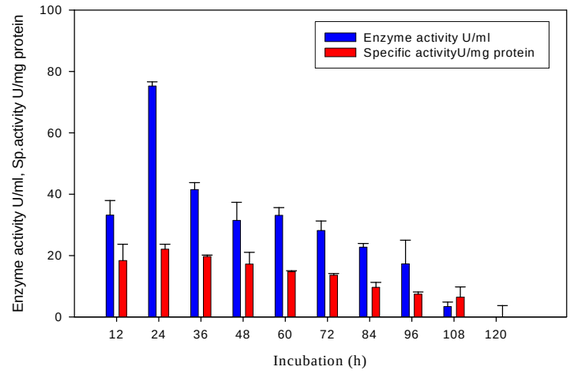


Fig.4.22 Time course study under optimized condition for tannase production by *A.awamori* BTMFW 032

4.2.1.2 Screening of the variables that significantly affect gallic acid production

A statistical approach was attempted for the optimization of gallic acid production. From the results obtained in Plackett –Burman design for tannase production under slurry state fermentation it was inferred that among the eighteen variables screened in the experiment four factors namely, tannic acid, seawater,

Table.4.9 Optimization of medium composition and physical parameters for gallic acid production by *A.awamori* BTMFW 032 under slurry state fermentation by Response surface methodology (RSM)

Run	Block	Seawater	Zinc sulphate	Glucose	Tannic acid	Gallic acid (µg/ml)
1	Block 1	75.00	1.00	5.00	0.05	3.35539
2	Block 1	50.00	3.00	10.00	0.00	0.72388
3	Block 1	25.00	1.00	5.00	0.05	2.10397
4	Block 1	50.00	3.00	10.00	0.10	2.90799
5	Block 1	75.00	3.00	0.00	0.05	2.90541
6	Block 1	50.00	1.00	10.00	0.05	2.73143
7	Block 1	50.00	3.00	5.00	0.05	2.99763
8	Block 1	50.00	5.00	5.00	0.10	2.91859
9	Block 1	75.00	3.00	5.00	0.00	0.9348
10	Block 1	25.00	3.00	5.00	0.00	0 .73789
11	Block 1	50.00	5.00	10.00	0.05	1.57161
12	Block 1	50.00	3.00	0.00	0.00	0.825222
13	Block 1	25.00	3.00	0.00	0.05	1.50541
14	Block 1	50.00	1.00	5.00	0.00	0.921715
15	Block 1	75.00	3.00	10.00	0.05	2.70046
16	Block 1	50.00	3.00	5.00	0.05	2.97229
17	Block 1	50.00	3.00	5.00	0.05	2.97017
18	Block 1	25.00	5.00	5.00	0.05	1.22019
19	Block 1	50.00	3.00	0.00	0.10	3.3913
20	Block 1	50.00	5.00	5.00	0.00	0.831559
21	Block 1	25.00	3.00	5.00	0.10	3.09818
22	Block 1	25.00	3.00	10.00	0.05	1.5094
23	Block 1	50.00	3.00	5.00	0.05	2.95682
24	Block 1	50.00	1.00	0.00	0.05	2.81906
25	Block 1	50.00	5.00	0.00	0.05	2.7045
26	Block 1	50.00	1.00	5.00	0.10	3.45517
27	Block 1	75.00	3.00	5.00	0.10	3.59552
28	Block 1	75.00	5.00	5.00	0.05	2.95404
29	Block 1	50.00	3.00	5.00	0.05	2.91107

The result obtained for Box-Behnken design experiment were analysed by ANOVA, which yielded the following regression equation for the level of gallic acid production.

Final Equation in Terms of Actual Factors:

Gallic acid =-1.80259+0.066749 * Seawater+0.25810 * ZnSO4+0.23620 * Glucose+51.13376 * Tannic acid+2.41215E-003 * Seawater * $ZnSO_4$-4.17880E-004 * Seawater * Glucose+0.060087 * Seawater * Tannic acid-0.026131 * $ZnSO_4$ * Glucose-1.11606 * $ZnSO_4$ * Tannic acid-0.38192 * Glucose * Tannic acid-5.39986E-004 * Seawater^2-0.054168 * $ZnSO_4^2$-0.015125*Glucose^2-248.94140*Tannicacid^2

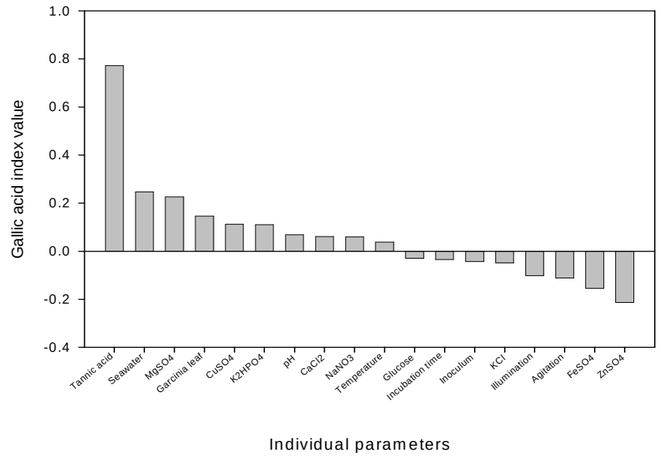


Fig.4.23 Pareto chart showing effect of individual factors on production of gallic acid production by *A. awamori* BTMFW 032

Validation of the PB design was carried out in shake flasks under conditions predicted by the model. The experimental values were found to be close to the predicted values and considered that model was successfully validated (Fig.4.24).

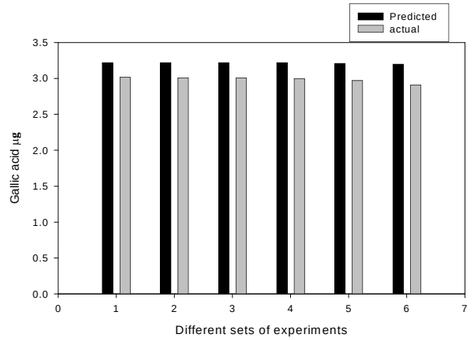


Fig 4.24 Validation of P-B design

4.2.1.2.2 Response Surface Methodology

Response surface methodology using Box-Behnken design was adopted towards selection of optimal level of significant variable seawater, tannic acid, glucose and zinc sulphate based on Plackett –Burman design experiment.

The model F value of 22.86 and values of Prob>F less than 0.05, obtained after ANOVA analysis of tannase production, indicates that the model terms are significant. Three linear and six quadratic terms were significant model terms for the response. The model coefficients estimated by multiple linear regressions and ANOVA showed that the model was significant with coefficient of determination R^2 of 0.9581. This ensured a satisfactory adjustment of the quadratic model to the experimental data and indicated that approximately 95.8% of the variability in the dependent variable (response) could be explained by the model. The adjusted R^2 which is more suited for comparing models with different number of variables, were 0.9162. All selected parameters were significant and varied levels of interactions were recorded for the variables in their cumulative effect on gallic acid production. The coefficient of variance found to be 12.14 and the adequate precision that measures the signal to noise ratio was 17.010. A ratio greater than 4 is desirable as it indicates an adequate signal. Thus this model could be used to navigate the design space (Table 4.8).

Table 4.8 ANOVA for the experiments with RSM design for gallic acid production by *A. awamori* BTMFW 032

Term	Gallic acid yield
Model F-value	22.86
Prob > F	<0.0001
Mean	2.32
R-Squared	0.9581
Adj R-Squared	0.9162
Pred R-Squared	0.7593
Coefficient of Variance	12.14
Adeq Precision	17.010

Experimental data on the effect of four selected media components on production of gallic acid by *A. awamori* BTMFW 032 in a total of 29 experiments (Table.4.9) showed strong dependence on the presence and levels of selected factors since gallic acid production varied between 0.723µg/ml and 3.6 µg/ml, under experimental conditions.. It was observed that increase in concentration of tannic acid in the medium had made proportionate increase in the concentration of gallic acid yield and thus for the tannic acid level “0.10” the gallic acid varied between 2.9 and 3.6 when compared to the values 0.72 -0.935 recorded for the level “0”. Whereas, both sea water and glucose could make an impact on gallic acid production at their mid-high levels in contributing to enhanced gallic acid production compared to zinc sulphate which had a positive impact on gallic acid production at their low-mid level.

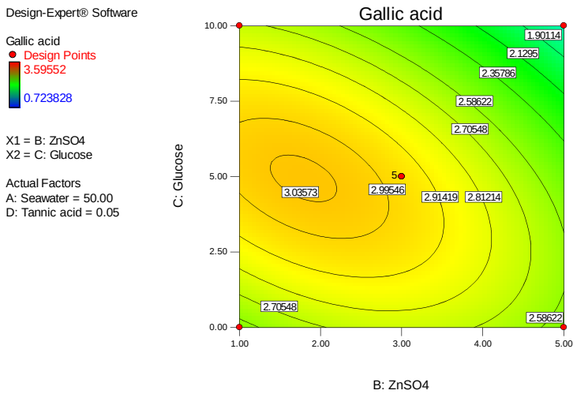


Fig.4.28 Effect of glucose and zinc sulphate on gallic acid production by *A. awamori* BTMFW 032

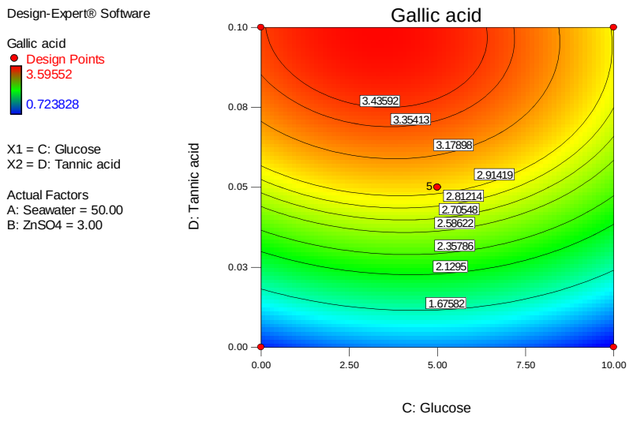


Fig.4.29 Effect of tannic acid and glucose on gallic acid production by *A. awamori* BTMFW 032

4.2.1.2.3 Evaluation of variables influencing gallic acid production by *A. awamori* BTMFW 032

The contour plots were plotted to study interaction among various media components and to determine the optimum concentration of each individual factor for maximum gallic acid production. The model predicted 3.65 µg/ ml of gallic acid and obtained a maximum of 3.60µg/ml, at 36 h of incubation. It was noted that tannic acid which was used as inducer had very high influence on gallic acid production and its higher concentration supported maximum gallic acid production.

4.4.2.3.1 Interaction between factors

The pair wise interaction among the variables in terms of gallic acid production under optimized conditions was assessed by examining the response surfaces. The contour plots were generated holding two factors constant at a time and plotting the response obtained for varying levels of the other two.

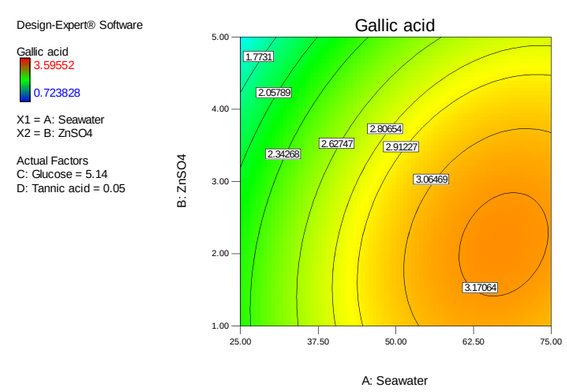


Fig.4.25 Effect of zinc sulphate and seawater on gallic acid production by *A. awamori* BTMFW 032

Higher levels of gallic acid production was recorded with lower concentration of zinc sulphate and higher concentration of seawater (Fig 4.25) while maintaining glucose and tannic acid at their medium level. Whereas, at medium concentration of tannic acid and zinc sulphate, gallic acid production was supported by medium concentration of glucose and higher concentration of sea water (Fig.4.26).

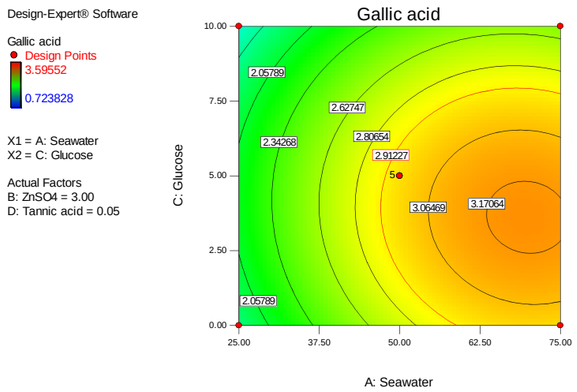


Fig.4.26 Effect of glucose and seawater on gallic acid production by *A. awamori* BTMFW 032

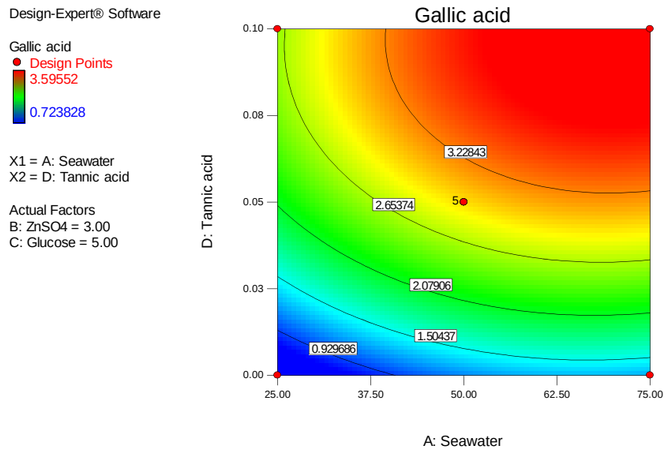


Fig.4.27 Effect of seawater and tannic acid on gallic acid production by *A .awamori* BTMFW 032

At medium levels of zinc sulphate and glucose, higher levels of gallic acid production was recorded with medium level of seawater and slightly higher than medium level concentration of tannic acid (Fig 4.27). When seawater and tannic acid levels were kept constant, low concentration of zinc sulphate and medium concentration of glucose supported enhanced levels of gallic acid production (Fig.4.28).

to the predicted values testifying successful validation of the model. From the results obtained in Plackett –Burman design it is inferred (Table.4.11) that among the eleven variables six factors namely tannic acid, incubation period, ferrous sulphate, dipotassium hydrogen phosphate, agitation, and sodium chloride were found to be the most significant.

Table 4.11 Studies on the effect of the variables on tannase production by *Aspergillus awamori* BTMFW032

Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Factor 6	Factor 7	Factor 8	Factor 9	Factor 10	Factor 11	Response 1	response 2
Tannic acid	NaCl	NaNO3	:KCl	MgSO4	:FeSO 4	K2HPO4	pH	Inoculu m	Incubati on	Agitati on	Enzyme activity	Gallic acid
%	M	mM	mM	mM	mM	mM		%	h	rpm	U/ml	µg/ml
0.50	0.00	50.00	10.00	5.00	0.00	15.00	2.0	0.50	48.00	0.00	359.247	45.6696
0.50	0.00	10.00	0.00	0.00	0.00	1.00	2.0	0.50	12.00	0.00	12.7796	16.3921
0.50	1.00	50.00	0.00	5.00	0.00	1.00	7.0	4.00	48.00	150.00	154.775	28.0524
0.50	1.00	10.00	0.00	0.00	0.50	15.00	7.0	0.50	48.00	0.00	556.62	31.5167
0.50	0.00	10.00	10.00	5.00	0.50	1.00	7.0	4.00	12.00	0.00	106.496	28.8551
4.00	1.00	50.00	0.00	5.00	0.50	1.00	2.0	0.50	12.00	0.00	3565.49	90.3675
4.00	0.00	50.00	10.00	0.00	0.50	1.00	2.0	0.50	48.00	150.00	5289.31	46.6624
0.50	1.00	50.00	10.00	0.00	0.50	15.00	7.0	4.00	12.00	0.00	29.819	0.83861
4.00	0.00	50.00	0.00	0.00	0.00	15.00	7.0	4.00	12.00	150.00	4241.39	3.81707
4.00	1.00	10.00	10.00	0.00	0.00	1.00	2.0	4.00	48.00	150.00	3632.23	3.42205
4.00	0.00	10.00	0.00	5.00	0.50	15.00	2.0	4.00	48.00	150.00	5246.72	4.22476
4.00	1.00	10.00	10.00	5.00	0.00	15.00	2.0	0.50	12.00	150.00	4239.97	3.818

First order model equation:

Enzyme activity Y U/ml= -542.93250+1190.25646 * Tannic acid-512.83870 * NaCl+718.02107 * FeSO4+22.76992*K2HPO4-83.72973*pH + 14.08774 * Incubation h

At medium levels of seawater and zinc sulphate, maximum gallic acid production was supported by higher concentration of tannic acid and glucose at less than medium level (Fig.4.29). Maximum gallic acid production was recorded with higher concentration of tannic acid and less than medium level of zinc sulphate, while glucose and sea water were maintained at their medium level (Fig.4.30).

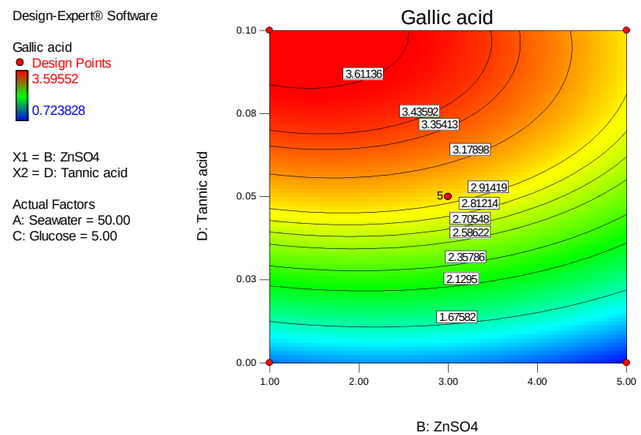


Fig.4.30 Effect of tannic acid and zinc sulphate on gallic acid production by *A. awamori* BTMFW 032

4.2.1.2.4 Validation of the response model

Validation of the deduced response surface model based on the previous experiments was carried out in shake flasks under conditions predicted by the model. The experimental values were found to be very close to the predicted values and hence the model was successfully validated.

Time course studies using *A. awamori* BTMFW032 under final optimized conditions indicated that gallic acid production increased rapidly up to 36h of fermentation when a maximum of 4.016µg/ml was recorded, which however declined later (Fig.4.31). A total of 18 variables were checked and out of these four variables namely seawater (65.27 %), glucose (0.41 mM), tannic acid (0.09 %) and zinc sulphate (3.01mM) were optimized by response surface methodology.

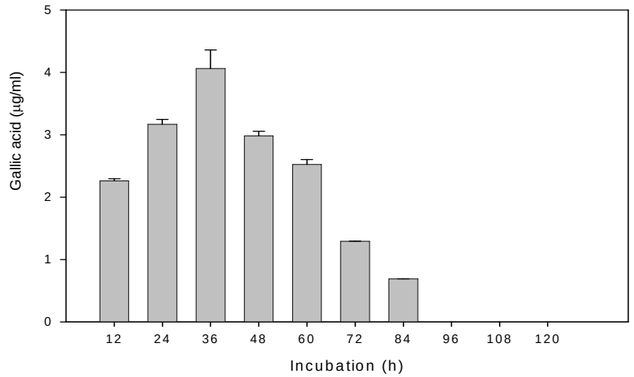


Fig. 4.31 Time course study under optimized condition for gallic acid production by *A. awamori* BTMFW 032.

4.2.2 Selection of the variables that significantly affect tannase enzyme and gallic acid production by *A. awamori* BTMFW 032 under submerged fermentation

4.2.2.1 Plackett - Burman Design

The statistical significance of the model equation was evaluated by F-test analysis of variance (ANOVA), (Table 4.10) which revealed that this regression is statistically significant. The model F value of 158.66 and values of Prob>F less than 0.05 indicated that the model terms are significant.

Table.4.10 ANOVA for the experiments with P-B design for tannase production by *A. awamori* BTMFW032under submerged fermentation

Term	Tannase yield
Model F-value	158.66
Prob > F	<0.0500
Mean	2286.24
R-Squared	0.9948
Adj R-Squared	0.9885
Pred R-Squared	0.9699
Coefficient of Variance	10.50
Adeq Precision	29.514

The effect of individual parameters studied in Plackett –Burman design and presented as paretochart (Fig 4.32) evidence that tannic acid, incubation period, ferrous sulphate, dipotassium hydrogen phosphate and agitation had a positive effect in enhancing enzyme production, along with the increase in their concentrations. However, sodium chloride, and pH had a negative effect on enzyme production along with increase in the respective variable.

Validation of the P-B design was carried out in shake flasks under conditions predicted by the model. The experimental values were found to be close

Table.4.13 Optimization of medium composition and physical parameters for tannase and gallic acid production by *A.awamori* BTMFW032 under SmF by Response surface methodology (RSM).

Run	Block	A:Tannic acid %	B:NaCl M	C:FeSO4 mM	:K2HPO4 mM	E:Incubation h	F:Agitation rpm	Enzyme act U/ml	Gallic acid µg/ml
1	1	-1	0	0	-1	-1	0	413.206	20.9125
2	1	-1	-1	0	-1	0	0	434.505	19.7719
3	1	1	-1	0	1	0	0	1523.25	105.065
4	1	0	1	0	0	-1	1	1268.02	136.206
5	1	-1	0	1	0	0	-1	885.41	22.5602
6	1	0	0	1	-1	0	1	3522.19	58.847
7	1	0	-1	-1	0	1	0	2227.9	98.849
8	1	0	0	-1	-1	0	1	1346.11	74.174
9	1	-1	1	0	-1	0	0	354.988	21.4195
10	1	-1	0	1	0	0	1	647.497	35.5049
11	1	0	1	0	0	1	-1	247.071	96.7047
12	1	1	0	0	1	-1	0	255.591	32.4039
13	1	0	0	0	0	0	0	4870.43	125.057
14	1	0	-1	-1	0	-1	0	1269.44	99.714
15	1	-1	0	-1	0	0	-1	52.5382	62.4842
16	1	-1	0	0	1	-1	0	536.741	52.0025
17	1	0	-1	0	0	1	-1	846.29	81.633
18	1	1	1	0	-1	0	0	593.717	79.61
19	1	0	0	0	0	0	0	4874.97	127.068
20	1	0	1	1	0	1	0	846.29	122.18
21	1	1	1	0	1	0	0	800.852	106.21
22	1	1	-1	0	1	0	0	212.993	46.5568
23	1	0	-1	1	0	-1	0	3441.96	100.084
24	1	0	-1	0	0	-1	1	2690.81	131.812
25	1	0	0	0	0	0	0	4769.44	133.443
26	1	0	1	0	0	-1	-1	840.611	79.685
27	1	1	0	1	0	0	-1	842.031	87.705
28	1	0	0	0	0	0	0	4792.3	133.832
29	1	1	-1	0	-1	0	0	530.32	105.218
30	1	0	0	-1	1	0	1	2891.73	102.471
31	1	0	0	-1	1	0	-1	802.272	85.57
32	1	0	1	0	0	1	1	1509.76	107.106
33	1	0	1	1	0	-1	0	2178.2	104.52
34	1	1	0	-1	0	0	-1	852.538	88.524
35	1	0	0	1	-1	0	-1	451.544	89.757
36	1	0	0	-1	-1	0	-1	614.838	80.675
37	1	1	0	0	-1	1	0	433.085	102.522
38	1	0	-1	0	0	-1	-1	2695.07	75.928
39	1	0	0	1	1	0	1	3342.21	102.864
40	1	1	0	1	0	0	1	739.794	105.036
41	1	1	0	0	1	1	0	2233.01	106.003
42	1	1	0	-1	0	0	1	839.191	106.041
43	1	-1	0	0	1	1	0	553.781	49.7254
44	1	0	1	-1	0	-1	0	2384.1	109.206
45	1	0	-1	0	0	1	1	724.175	101.352
46	1	1	0	0	-1	-1	0	1718.14	101.519
47	1	0	0	0	0	0	0	4727.8	128.374
48	1	0	0	1	1	0	-1	742.634	80.249
49	1	0	-1	1	0	1	0	1644.3	108.264
50	1	0	1	-1	0	1	0	985.446	76.6371
51	1	-1	1	0	1	0	0	149.095	42.8137
52	1	0	0	0	0	0	0	4814.59	133.815
53	1	-1	0	0	-1	1	0	201.633	28.5594
54	1	-1	0	-1	0	0	1	259.851	32.5729

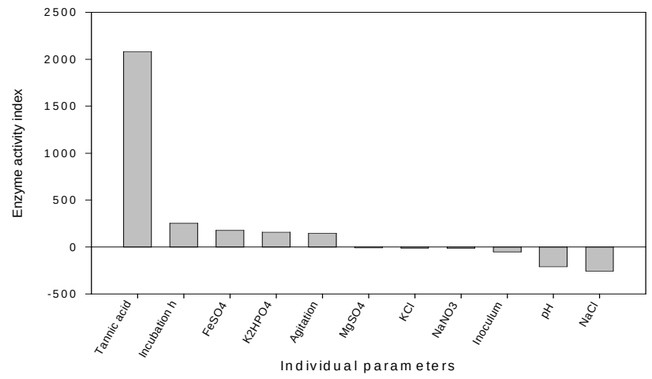


Fig.4.32 Pareto chart showing effect of individual factors on production of tannase production by *A. awamori* BTMFW 032 under submerged fermentation

The model coefficients estimated by multiple linear regressions and ANOVA showed that the model was significant with coefficient of determination R^2 of 0.9948. This ensured a satisfactory adjustment of the factorial model to the experimental data and indicated that approximately 99.5% of the variability in the dependent variable (response) could be explained by the model. The adjusted R^2 which is more suited for comparing models with different number of variables was 0.9885. The coefficient of variance was found to be 10.50 and the adequate precision that measures the signal to noise ratio was 29.514. A ratio greater than 4 is desirable as it indicated an adequate signal. Thus this model could be used to navigate the design space.

Experimental validation of the solutions obtained showed only very less decrease from those predicted by the software.

4.2.2.2 Response surface methodology

Response surface methodology using Box-Behnken design adopted towards selection of optimal level of significant variables tannic acid, incubation period, ferrous sulphate, dipotassium hydrogen phosphate, agitation, and sodium chloride based on Plackett –Burman design experiment.

The result obtained for Box-Behnken design experiment were analysed by ANOVA, which yielded the following regression equation for the level of tannase production.

Tannase activity, Y(U/ml)= -5665.96440+3162.10823 * Tannic acid4303.68305 * NaCl+11546.33008 * $FeSO_4$+249.26724 * K_2HPO4+134.10436 * Incubation h +32.03982 * Agitation-73.65519 * Tannic acid * NaCl-380.12070 * Tannic acid * $FeSO_4$+7.59779 * Tannic acid * K_2HPO_4+3.51945 * Tannic acid * Incubation h-0.080937 * Tannic acid * Agitation-1933.97231 * NaCl * $FeSO_4$-27.50626* NaCl * K_2HPO_4+10.91833 * NaCl * Incubation h+6.05490 * NaCl * Agitation-115.85298 * $FeSO_4$ * K_2HPO_4-74.70516 * $FeSO_4$ * Incubation h+7.71791 * $FeSO_4$ * Agitation+3.46338 * K_2HPO_4 * Incubation h+0.21122 * K_2HPO_4 * Agitation+0.066429 * Incubation h *Agitation -673.77590 * Tannic acid^2 -4722.98821 *NaCl^2-12683.83607*$FeSO_4^2$-20.18579*$K_2HPO_4^2$-2.97087*Incubationh^2-0.23328*Agitation^2

Table.4.12. ANOVA for the experiments with RSM design for tannase production by *A. awamori* BTMFW 032 under submerged fermentation

Term	Tannase yield	Gallic acid yield
Model F-value	7.38	6.53
Prob > F	<0.0500	<0.0500
Mean	1563.45	84.37
R-Squared	0.8845	0.8714
Adj R-Squared	0.7646	0..7379
Pred R-Squared	0.7549	0..7218
Coefficient of Variance	45.28	21.29
Adeq Precision	10.184	9.921

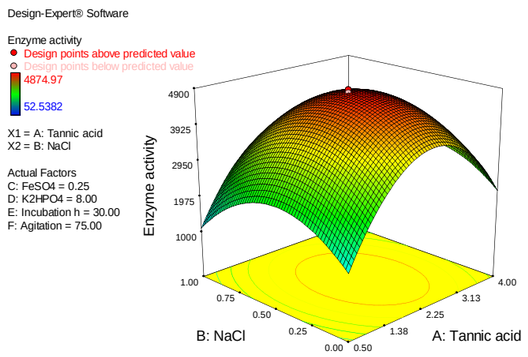


Fig.4.34 Effect of tannic acid and sodium chloride on tannase production by *A. awamori* BTMFW 032

An interactive effect of factors agitation and tannic acid showed a variation in activity from 52.53U/ml to 4874.97 U/ml, when levels of sodium chloride, ferrous sulphate, dipotassium sulphate and incubation period were maintained at their medium level. The interaction is represented by a parabola which defines the maximum enzyme productivity at medium concentration (Fig.4.35).The interactive effect of tannic acid and incubation on enzyme production indicated that they support maximum enzyme production at their medium level rather at their higher and lower levels presenting a parabola (Fig.4.36).

The model F value of 7.38 and values of Prob>F less than 0.05 obtained after ANOVA analysis of tannase production, indicated that the model terms are significant. Two linear and six quadratic terms were significant model terms for the response. The model coefficients estimated by multiple linear regressions and ANOVA showed that the model was significant with coefficient of determination R^2 of 0.8845. This ensured a satisfactory adjustment of the quadratic model to the experimental data and indicated that approximately 88.45 % of the variability in the dependent variable (response) could be explained by the model. All selected parameters were significant and varied levels of interactions were recorded for the variables in their cumulative effect on tannase production. The coefficient of variance was found to be 45.28 and the adequate precision that measures the signal to noise ratio was 10.184. A ratio greater than 4 is desirable as it indicated an adequate signal (Table 4.12). Thus this model could be used to navigate the design space.

Experimental data on the effect of six selected physicochemical variable on production of tannase by *Aspergillus awamori* in a total of 54 experiments (Table.4.13) showed strong dependence on the presence and levels of selected factors as the enzyme production varied between 212.99U/ml and 4870.4.U/ml under experimental conditions studied.

The results obtained for Box-Behnken design experiment were analysed by ANOVA, which yielded following regression equation for the level of gallic acid production.

Y Gallic acid µg/ml=-39.14147+51.63814 * Tannic acid+1.42028 * NaCl-8.61670 * $FeSO_4$+8.57933 * K2HPO4+2.30230 * Incubation h+0.37107 * Agitation-3.19531 * Tannic acid * NaCl+10.04787 * Tannic acid * $FeSO_4$-0.24807 * Tannic acid * K_2HPO_4+0.63584 * Tannic acid * Incubation h+0.049348 * Tannic acid * Agitation+31.07188 *NaCl* $FeSO_4$+0.76295 * NaCl * K_2HPO4-0.066315 * NaCl * Incubation h-0.028935 * NaCl * Agitation+0.094071 * $FeSO_4$ * K_2HPO_4+1.64650 *

$FeSO_4$ * Incubation h+0.079915 * $FeSO_4$ * Agitation-0.028102 * K_2HPO_4 * Incubation h+0.018316 * K_2HPO_4 * Agitation-7.61897E-003 * Incubation h * Agitation-13.12423 * Tannic $acid^2$-3.27192 * $NaCl^2$-171.11859 * $FeSO_4{}^2$-0.47797 * $K_2HPO_4{}^2$-0.050371*$Incubationh^2$-2.10192E-003*$Agitation^2$

The ANOVA analysis of gallic acid production showed the model F value of 6.53 and values of Prob>F less than 0.05 indicated that the model terms are significant. Two linear and six quadratic terms were significant model terms for the response. The model coefficients estimated by multiple linear regressions and ANOVA showed the model was significant with coefficient of determination R^2 of 0.8714. This ensured a satisfactory adjustment of the quadratic model to the experimental data and indicated that approximately 87.14% of the variability in the dependent variable (response) could be explained by the model. All selected parameters were significant and varied levels of interactions were recorded for the variables in their cumulative effect on gallic acid production. The coefficient of variance was found to be 21.29 and the adequate precision that measures the signal to noise ratio was 9.921. A ratio greater than 4 is desirable as it indicates an adequate signal. Thus this model could be used to navigate the design space.

Experimental data on the effect of six selected physicochemical factors on production of gallic acid by *A. awamori* BTMFW 032 in a total of 54 experiments showed strong dependence on the presence and levels of selected factors as the gallic acid production varied between 19.77 and 136.206µg/ml under the studied experimental conditions.

4.2.2.3 Analysis of factors influencing tannase production

Three dimensional response surface curves were plotted to study interaction among various physicochemical factors and to determine the optimum concentration of each individual factor for maximum tannase production. The model predicted 4824.61U/ml of tannase enzyme activity and a maximum of 4874.97U/ml was obtained from the

experiment. It was noted that tannic acid which was used as inducer had very high influence on the enzyme and gallic acid production and at higher concentration supported maximum enzyme yield. Even though it was observed that the individual effect of tannic acid in the bioprocess increased along with concentration in Plackett Burman design, the interactive effect of tannic acid with other media components and process parameters like ferrous sulphate, sodium chloride, agitation, and incubation showed an optimum enzyme yield at the medium level of tannic acid concentration in Response surface methodology. Tannic acid and ferrous sulphate supported maximum enzyme production at their medium concentration level when all other variables were maintained at their medium concentration. This resulted in a parabola (Fig.4.33). The interactive effect of sodium chloride and tannic acid indicated that maximum activity was supported at medium concentrations of these variables when levels of ferrous sulphate, disodium hydrogen phosphate, incubation, and agitation were maintained at their medium value (Fig.4.34).

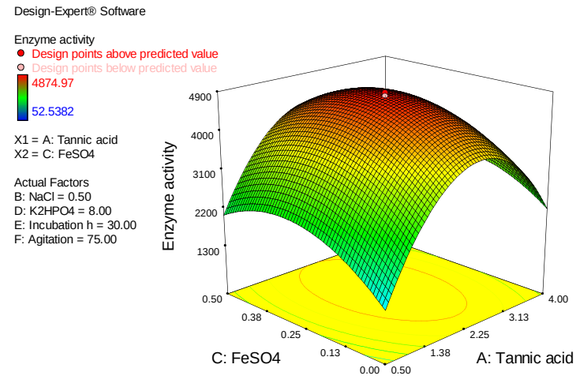


Fig.4.33 Effect of tannic acid and ferrous sulphate on tannase production by *A. awamori* BTMFW 032

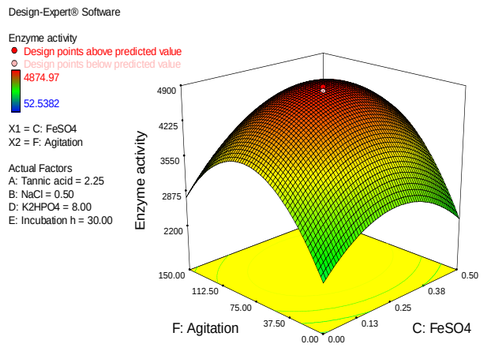


Fig.4.39 Effect of agitation and ferrous sulphate on tannase production by *A. awamori* BTMFW 032

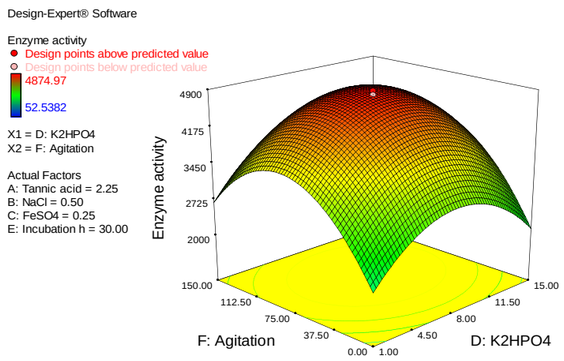


Fig.4.40 Effect of agitation and dipotassiumhydrogen phosphate on tannase production by *A. awamori* BTMFW 032

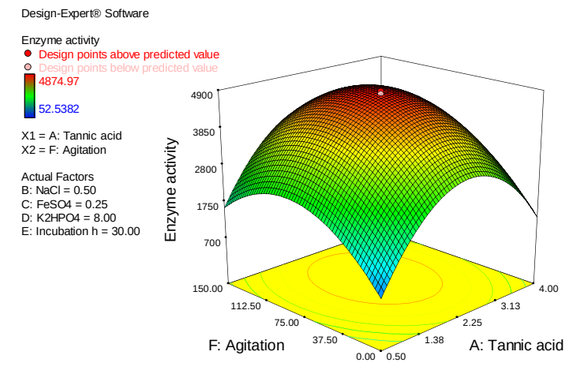


Fig.4.35 Effect of tannic acid and agitation on tannase production by *A. awamori* BTMFW 032

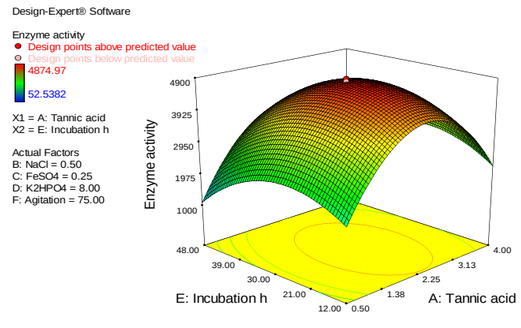


Fig.4.36 Effect of incubationh and tannic acid on tannase production by *A. awamori* BTMFW 032

From the data presented in Fig.4.37 it was inferred that medium levels of sodium chloride concentration and agitation supported maximum enzyme production and their lower and higher levels led to decreased enzyme production. Further it was observed that the enzyme production decreased with higher concentrations of sodium chloride and dipotassium hydrogen phosphate and the medium level of both the factors supported maximum enzyme activity (Fig.4.38).

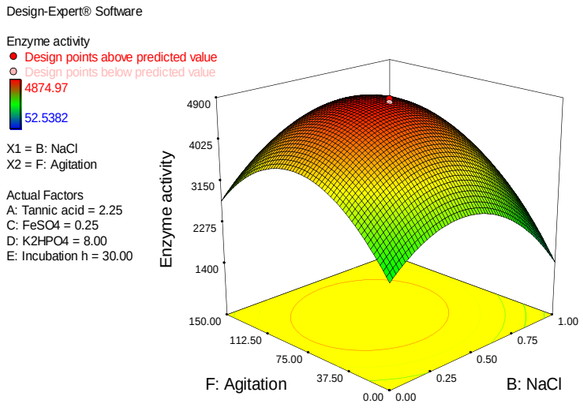


Fig.4.37 Effect of agitation and sodium chloride on tannase production by *A. awamori* BTMFW 032

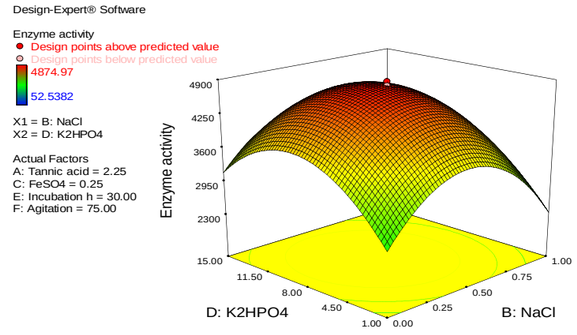


Fig.4.38 Effect of dipotassium hydrogen phosphate and sodium chloride on tannase production by *A. awamori* BTMFW 032

Similarly agitation and ferrous sulphate supported maximum tannase production at the medium levels of both the variables, and when other 4 variables were maintained constant at their medium level (Fig.4.39). Medium level of agitation and dipotassium hydrogen phosphate concentration supported maximum enzyme activity (Fig.4.40). When all other variables were maintained at medium level, higher level of ferrous sulphate concentration and medium level of incubation period supported maximum enzyme activity (Fig.4.41).

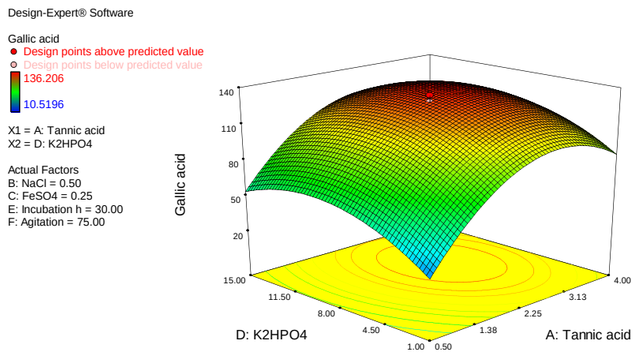


Fig.4.45 Effect of dipotassium hydrogen phosphate and tannic acid on gallic acid production by *A. awamori* BTMFW 032

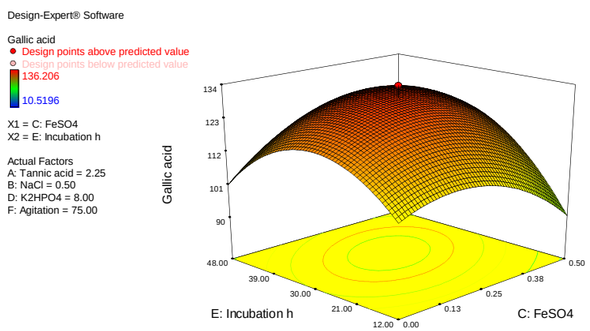


Fig.4.46 Effect of incubation and ferrous sulphate on gallic acid production by *A. awamori* BTMFW 032

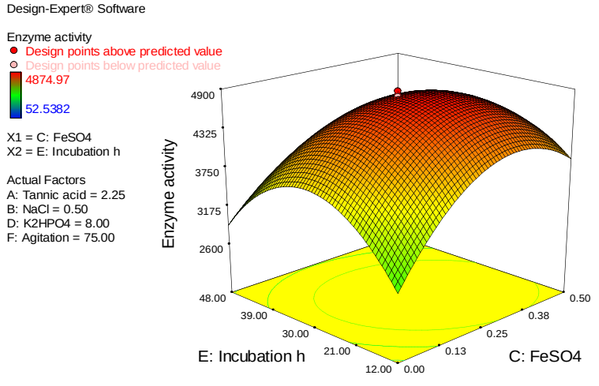


Fig.4.41 Effect of incubation and ferrous sulphate on tannase production by *A. awamori* BTMFW 032

4.2.2.4 Evaluation of variables influencing gallic acid production by *A .awamori* BTMFW 032

Relative effect of two variables on gallic acid production has been depicted in the Response surface plot presented in Fig.4.42 testify that maximum gallic acid production was supported by medium levels of agitation and ferrous sulphate when tannic acid, sodium chloride, dipotassium hydrogen phosphate, and incubation period were maintained at their medium level. The interactive effect of sodium chloride and tannic acid supported maximum gallic acid production at higher level of tannic acid and lower level of sodium chloride (Fig.4.43).

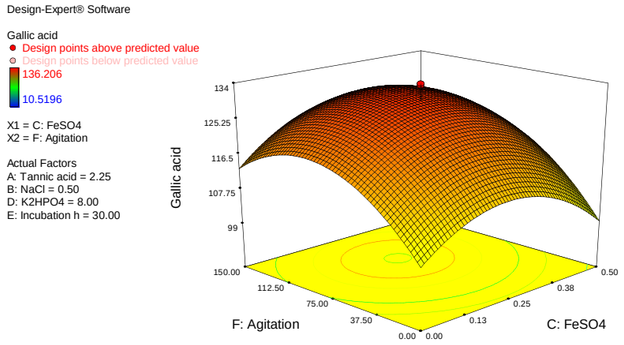


Fig.4.42 Effect of agitation and ferrous sulphate on gallic acid production by *A. awamori* BTMFW 032

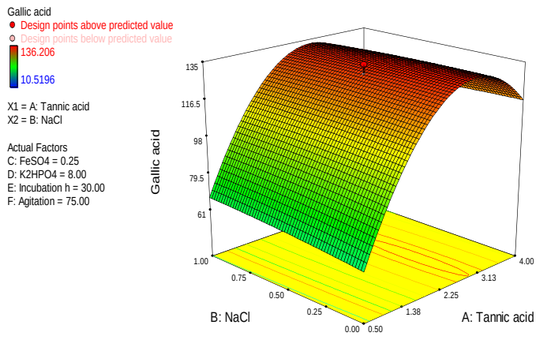


Fig.4.43 Effect of sodium chloride and tannic acid on gallic acid production by *A. awamori* BTMFW 032

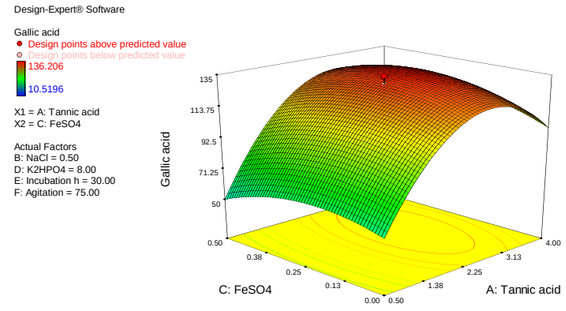


Fig.4.44 Effect of ferrous sulphate and tannic acid on gallic acid production by *A. awamori* BTMFW 032

Maximum gallic acid production (Fig.4.44) was supported by higher concentration of tannic acid and there was no much interactive effect of ferrous sulphate with tannic acid in enhancing production of gallic acid. It is evident from the Fig.4.45 that the medium level of dipotassium hydrogen phosphate and higher level of tannic acid supported maximum gallic acid production.

4.2.2.5 Validation of the response model

Validation of the deduced response surface model based on previous experiments was carried out in shake flask under conditions predicted by the model. The experimental values were very close to the predicted values and hence it was concluded that the model was successfully validated. The optimized conditions were as follows: Tannic acid 2.56 % (w/v), Inoculum 0.5 % (v/v), sodium chloride 0.45M, pH 5.6, ferrous sulphate 0.27mM, incubation period 36h, Dipotassium hydrogen phosphate 9.14mM, agitation- 93 rpm, Sodium nitrate 47mM, potassium chloride 5.1mM, and magnesium sulphate 4.9mM.

4.2.3 Solid state fermentation

Very low production and growth was observed in solid-state fermentation with coconut fiber and coconut pith as support (Table 4.14) and hence further studies were not conducted.

Table 4.14 Effect of various natural substrates as support for tannase production by *Aspergillus awamori* BTMFW 032 (enzyme activity assayed after 4 days of incubation).

Substrate used	Tannase activity U/gds
Coconut husk fibre	0.00
Coconut husk fiber +0.1% tannic acid	2.3U/gds
Coconut husk- pith	0.00
Coconut husk pith+ 0.1% tannic acid	1.32 U/gds
Coconut husk (pith+fibre)	0.00
Coconut husk (pith+fibre +0.1% tannic acid)	1.685U/gds

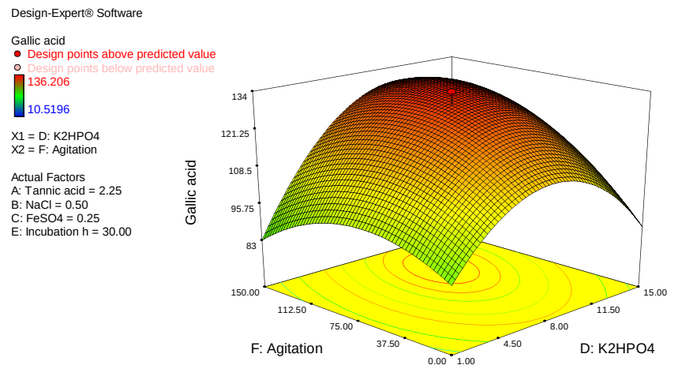


Fig.4.47 Effect of agitation and dipotassium hydrogen phosphate on gallic acid production by *A. awamori* BTMFW032

From the data presented in Fig.4.46 it was inferred that medium levels of ferrous sulphate and incubation period supported maximum production of gallic acid when all other variables were maintained at their medium level. Further agitation and dipotassium hydrogen phosphate also, at their medium levels, supported maximum gallic acid production (Fig.4.47). Whereas at lower levels of incubation period and higher concentrations of tannic acid supported maximum gallic acid production (Fig.4.49).

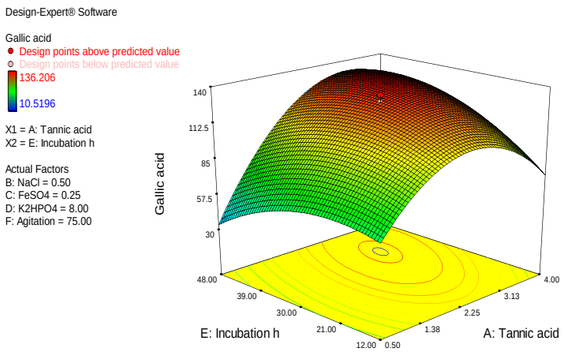


Fig.4.48 Effect of incubation h and tannic acid on gallic acid production by *A. awamori* BTMFW 032

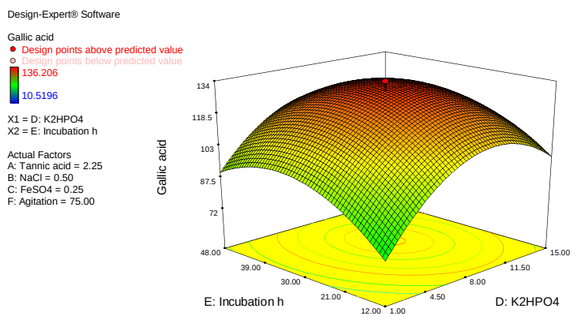


Fig.4.49 Effect of incubation h and di potassium hydrogen phosphate on gallic acid production by *A. awamori* BTMFW 032

The parabolic nature of the response surface plot, depicted in Fig.4.49, evidence that medium levels of incubation period and dipotassium hydrogen phosphate supported maximum gallic acid production.

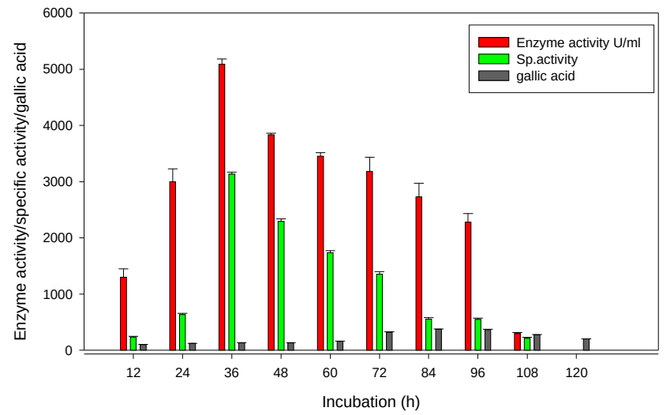


Fig.4.50 Time course study under optimized condition for tannase and gallic acid production by *A. awamori* BTMFW 032 under submerged fermentation

The model predicted 4824.61U/ml of tannase enzyme activity and 136.206 µg/ml gallic acid after 48 h of incubation. Whereas, it was noted that during the time course experiment 5085U/ml tannase activity was obtained after 36 h of incubation and 372.6µg/ml gallic acid was obtained after 84 h of incubation (Fig.4.50). The production attained under optimized conditions was nearly 15 times higher than that obtained under the non optimized condition.

Table 4.16 Purification of tannase produced by *A. awamori* BTMFW032 *(Protocol 1)*

Fractions	Vol (ml)	Tannase activity U/ml	Protein mg/ml	Specific activity U/mg	Total activity	Total protein	Yield (%) of activity	Purification fold
Crude	100	2047.87	4.5903	446.1590	204787	459mg	100	1
Crude dialysed	100	1963.679	1.658	1190.10	196367.9	165mg	95.88	2.667
Sucrose concentrated	10	19064.54	3.68	5180.58	190645.4	36.8 mg	93.09	11.61
Sephadex G200	2	7920.33	1.06	7409.869	31681.32	6.9 mg	15.47	16.6

4.5.5 Enzyme clarification

The enzyme extract obtained was dark brown in colour, which showed the probable presence of phenols and pigments produced by *A. awamori* during growth and sporulation, besides unused tannic acid present in the filtrate. Treatment with acidic aluminium oxide having Brockmann activity removed the colour leaving the enzyme colourless and transparent (visual observation) even though only 55% of activity was retained. The clear filtrate obtained was used for further studies.

4.5.6 Ultra filtration through Amicon ultra

Ultra filtration of the enzyme sample was carried out using ultra filter membranes in two steps. In the first step, a membrane with a molecular weight cut off of 30,000 dalton was used which facilitated concentration of the enzyme more

4.3. Comparative evaluation of tannase and gallic acid by the three different fungal strains

A comparative evaluation of tannase and gallic acid production in the optimized medium by *A. oryzae* (ATCC9362*), A. awamori (*ATCC44733) and the marine *A. awamori* BTMFW 032 is presented in (Table 4.15). From the data it is evident that the marine *A. awamori* BTMFW 032 could produce enhanced levels of tannase (2990 U/ml) as extracellular fraction compared to the terrestrial *A. awamori* ATCC although it was very close to levels produced by *A. oryzae* (ATCC9362*).* In the case of gallic acid marine *A. awamori* could record very high levels of gallic acid (136.28µg/ml) compared to other strains. These observations testify the suitability of the designed medium for production of tannase and gallic acid by the species of *Aspergillus* in general and particularly for marine *A. awamori.*

Table 4.15 Comparative evaluation of tannase and gallic acid by the three different fungal strains

Culture (48 h of incubation)	Tannase activityU/ml		Gallicacid µg/ml
	Extracellular	Intracellular	
Aspergillus oryzae (ATCC9362*)*	2929(±11)	217.25(±4.5)	94.789(±3.2)
*Aspergillus awamori (*ATCC44733)	464.32 (±8.2) 2990.4(±12.4)	174.6(±3.8) 178.9(±1.17)	99.845(±1.78) 136.28(±2.3)
Aspergillus awamori (BTMFW 032*)*			

4.4 Intracellular enzyme production

From the results presented in Table 4.15 it is evident that all the three fungal strains recorded intracellular tannase production in the medium and maximal enzyme activity was recorded by *A. oryzae* ATCC 9362 (217.25U/ml) followed by marine *A. awamori* BTMF W0 32(178.9 U/ml) and *A. awamori*

ATCC 44733(174.6 U/ml). These observations also suggest the suitability of the designed medium after statistical optimization.

4.5 Purification

4.5.1 Ammonium sulphate precipitation

Fractional precipitation with ammonium sulphate, showed that initial fractionation up to 60% ammonium sulphate removed some of the non-enzyme proteins and tannase was precipitated at and above 80 % saturation with ammonium sulphate (Fig.4.51). Only 32% yield was obtained. On further dialysis of the precipitated enzyme protein it was observed that the dialyzed fractions in spite of recording some activity did not show any clear bands on PAGE and hence it was inferred that only minimum recovery of the enzyme is possible by this technique.

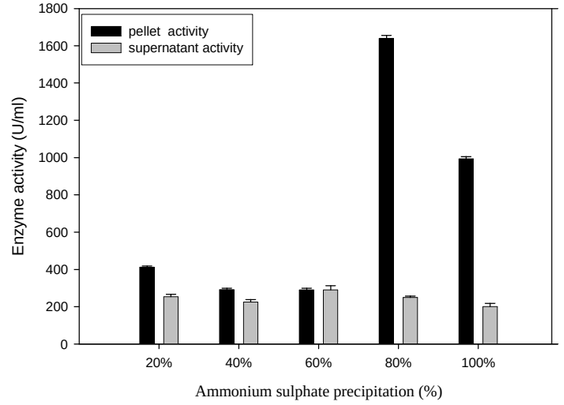


Fig.4.51. Ammonium sulphate precipitation of the cell free extract obtained from the fermented broth

4.5.2. Solvent precipitation

In the case of solvent precipitation with acetone and ethanol, the precipitated fraction showed negligible activity indicating the unsuitability of this method for fractionation of this enzyme.

4.5.3. Dialysis and Sucrose concentration

Dialysis of the crude enzyme fraction towards removing tannic acid present in the crude extract did not show much impact since only very less amount of tannic acid was removed through the dialysis membrane (12 kDa cut off) indicating the presence of enzyme bound to the tannic acid forming certain complexes. The sucrose concentration of the crude enzyme also was not very successful since the sample contained tannic acid, which could affect the further characterization of the enzyme even though significant purification fold was recorded by Protocol1 (Table 4.16).

4.5.4. Gel filtration

Two ml of sucrose concentrated sample was loaded on a gel filtration column and 16.6 foldpurification was obtained. However, the sample was not fully free of tannic acid and hence another method was tried.

4.6.2. Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophoresis (SDS PAGE)

The sample on analysis with SDS PAGE showed a single band on staining with coomassie Brilliant blue R250 indicating the homogeneity of the preparation. SDS PAGE analysis of partially purified enzyme performed under nonreducing and reducing condition yielded a single band of 230 kDa and 37.5 kDa respectively (Fig. 4.56 a,b) which indicated the presence of six identical monomers of 37.5kDa. Silver staining of reduced condition was performed which yielded only a significant single band at 37.5 kDa (Fig.4.57). In SDS PAGE the zymogram was not visible. The zymography showed significant clearing zone in a native gel which showed the activity of the enzyme in its native state rather than in its denatured condition.

than 3 fold removing very small molecules. In the second step, a membrane with a molecular weight cut off of 100000 dalton was used which enabled further concentration of the enzyme more than ten times achieving a total concentration of more than 30 fold by Protocol 2 (Table.4.17).

Table 4.17 Purification of tannase produced by *A. awamori* BTMFW032 *(Protocol 2)*

Fractions	Vol (ml)	Total Tannase activity U/ml	Total Protein mg	Specific activity U/mg	Yield of activity (%)	Purification fold (%)
Crude	100	204980	469.3648	436.722	100	1
Aluminium oxide treated	74	113167.164	160.136	704.45932	55.2	1.613
Amicon ultra 30kDa cutoff	20	24867.96	33.726	737.3528	12.131	1.688
Amicon ultra 100kDa cutoff	3	2532.2685	3.288	770.154	1.2	1.763
Sephadex G200	1.5	1049.52	0.3795	2765.53	0.5120	6.33

4.5.7. Gel filtration and molecular weight determination

Gel filtration chromatography of the partially purified enzyme protein using Sephadex G200, yielded single peak with tannase activity. The molecular weight was calculated from the standard graph plotted for K_{av} versus log molecular weight of the standard proteins. From the K_{av} value the molecular weight of the enzyme was determined as 230 kDa (Fig.4.52).

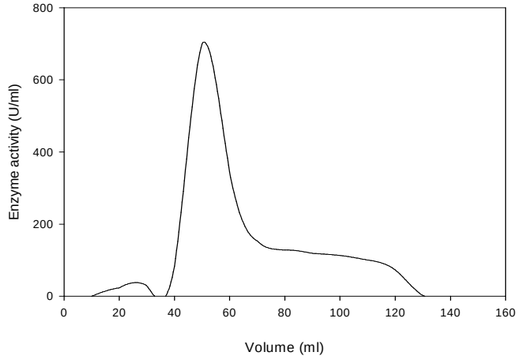


Fig.4.52 Elution profile of tannase on sephadex G-200

4.6. Characterization of the enzyme

4.6.1. Native Poly acrylamide gel electrophoresis (Native PAGE) and Zymogram

Results presented for the Native Polyacrylamide gel electrophoresis (Fig.4.53) and zymography (Fig.4.54) of the partially purified enzyme containing tannic acid showed smiling face band. Whereas the purified sample without tannic acid showed a single band in Native PAGE (Fig.4.55). From these results it was inferred that the enzyme extract may contain certain complexes formed of tannic acid and enzyme.

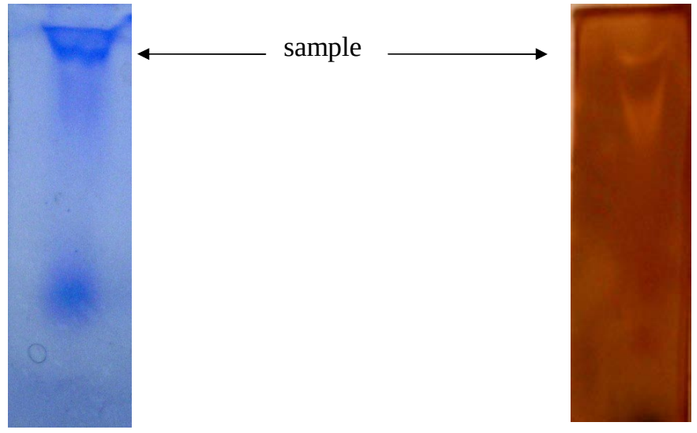


Fig.4.53 Native PAGE of sample with tannic acid

Fig.4.54 Zymogram of sample

Fig.4.55 Native PAGE of sample without tannic acid

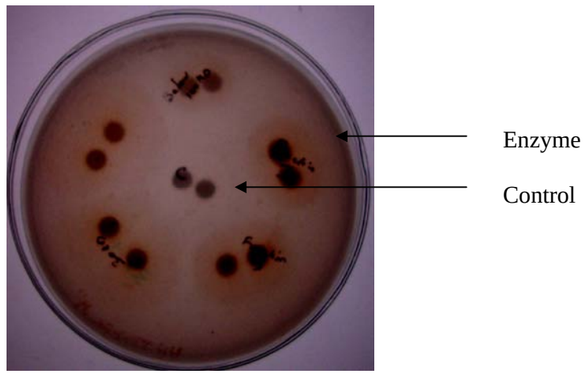


Fig.4.59 Plate assay for determining esterase activity

4.6.6 Isoelectric focusing

The purified tannase enzyme appeared as a single band with a pI value of 4.4 on isoelectric focusing (Fig.4.60)

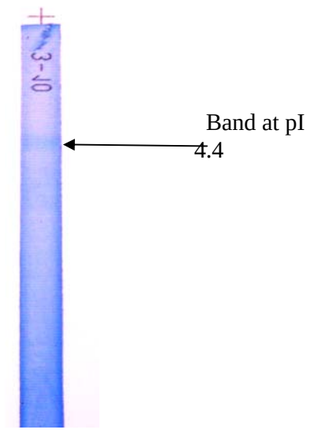


Fig.4.60 Isoelectric focusing of purified enzyme

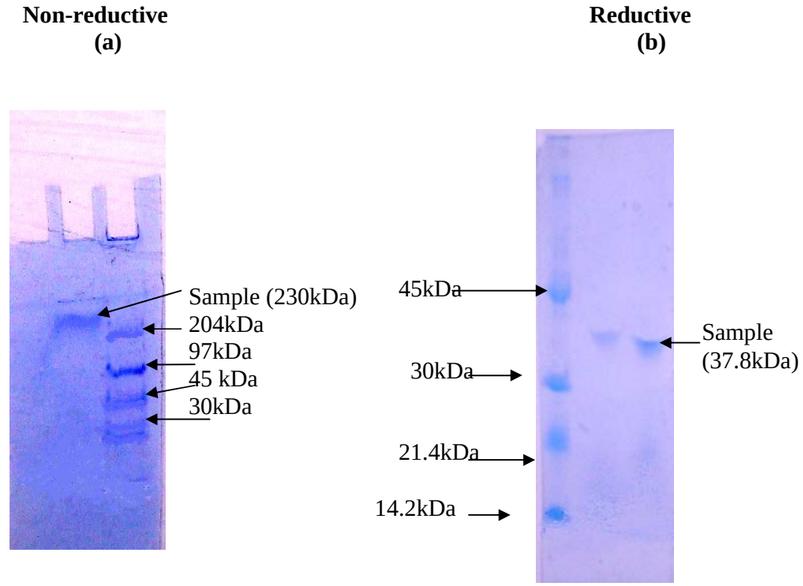


Fig. 4.56 SDS-PAGE of purified enzyme sample

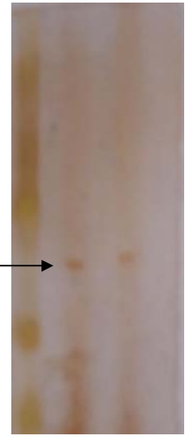


Fig.4.57. Silver staining of SDS-PAGE of purified enzyme sample

4.6.3 Glycoprotein staining

Glycoprotein staining of the tannase enzyme was performed with Thymol-H_2SO_4 reagent in order to assess the glycoprotein nature. A purple band obtained after staining indicated the glycoprotein nature of marine *A. awamori* tannase (Fig.4.58).

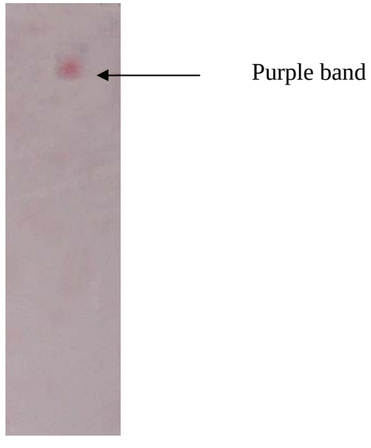


Fig.4.58 Glycoprotein staining of purified tannase

4.6.4 Carbohydrate content

The carbohydrate content of the purified enzyme was determined by phenol sulphuric acid method. It was found that the marine *A. awamori* tannase was glycosylated to an extent of 8.02 %.

4.6.5 Esterase activity

Results presented in Fig 4.59 for the esterase activity of tannase on plate assay indicated that the enzyme has esterase activity since a clearing zone was recorded for the sample while no such clearing zone was observed with heat inactivated enzyme

4.6.11 Effect of inhibitors on tannase activity

Results presented in Table.4.18 for the studies conducted on the effect of enzyme inhibitors on tannase activity evidence that the enzyme was inhibited significantly by phenyl methyl sulphonide fluoride, followed by sodium deoxy cholate and phenanthroline retaining only 4.5%, 26.4% and 61.04% respectively. Results suggested that the enzyme is a serine or cysteine hydrolase.

Table.4.18 Effect of Inhibitors on Tannase

Inhibitors (1mM)	Residual activity (%)
PMSF	4.587532
Phenanthroline	61.01417
Sodium deoxy cholate	26.41108

4.6.12. Effect of various metal ions on enzyme activity

Among the various metal ions evaluated for their effect on tannase activity, ferric chloride alone contributed to enhanced enzyme activity which however declined along with increase in concentration in the medium (Table 4.19). However there was no inhibition of enzyme activity even at the highest concentration tested. Thus a maximum of 271.2 U/ml could be recorded for 1mM concentration compared to other concentrations. Potassium sulphate also supported enhanced enzyme activity at concentrations from 1mM to10mM. However at higher concentrations there was a decline in enzyme activity. NaCl presented interesting observation. While there was very low enzyme activity at low concentration of NaCl, the enzyme activity increased along with increase in the concentration of NaCl.

4.6.7 Optimal temperature for tannase activity

The results presented in Fig. 4.61 indicated that the enzyme was active over a broad range of incubation temperature (5 - 80ºC) with maximal activity at 30ºC. The enzyme was active up to 60ºC and at temperatures above 80ºC the enzyme activity declined. It was also noted that the enzyme was active even at 5ºC.

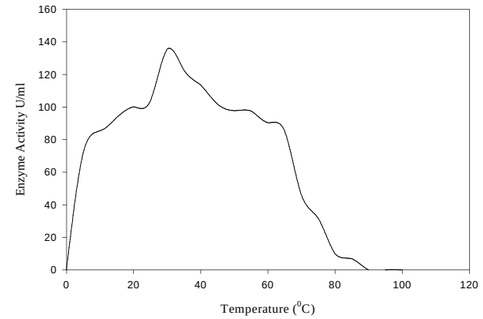


Fig.4.61 Effect of temperature on tannase activity

4.6.8 Thermostability of tannase at different temperatures

From the results presented in Fig.4.62 for the temperature stability studies conducted with tannase it is inferred that the enzyme has thermal stability over a broad range of temperatures (30-70ºC) for one h since more than 50 % of the relative enzyme activity was retained after incubation. Nevertheless even at 80ºC 43% of activity was retained after 1 h. More than 50 % of activity was retained by the enzyme even after 2 h up to 60ºC. It may be suggested that the enzyme is thermostable up to 60ºC**.**

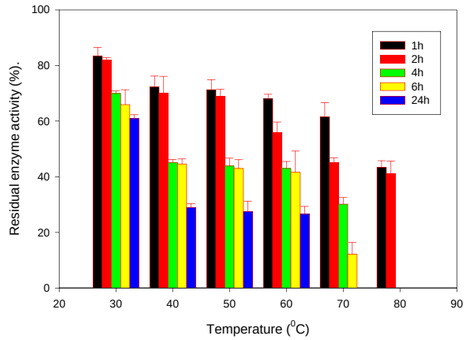


Fig.4.62 Thermostability of tannase at various

4.6.9. Optimal pH for tannase activity

Results depicted in Fig. 4.63 indicate that tannase has dual pH optima, one at acidic and another slightly at alkaline conditions, for showing maximal enzyme activity. Similar levels of enzyme activity (126.5 U/ml) were recorded at both pH 1.0 and pH 8.0 although maximal enzyme activity was recorded at pH 2 (156.3U/ml). It was also observed that the enzyme was almost inactive around netural pH.

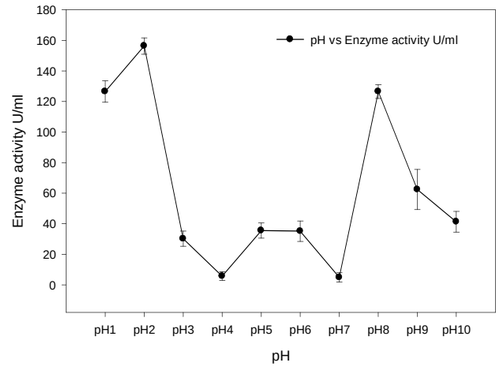
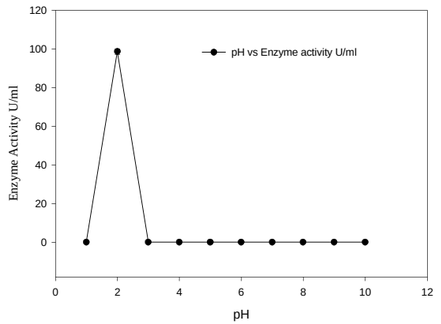


Fig.4.63 Activity profile of tannase at different pH

4.6.10 Stability of tannase at different pH

The data presented in Fig. 4.64 very clearly testify that the enzyme was stable only at pH 2 even after 24 h of incubation at room temperature. Hence it is inferred that the pH optimum of the enzyme is pH 2



pH

Fig.4.64 Stability profile of enzyme in different pH

Table 4.20 Residual activity of tannase enzyme in presence of different detergents

Detergent	Residual activity at different concentrations (%)				
	0.2%	0.4%	0.6%	0.8%	1%
TritonX	112.1215	171.3584	79.05904	59.77261	54.2622
Tween 80	156.7405	157.5823	157.8885	91.84012	83.49798
Tween 20	100.4884	98.11586	147.4034	179.9301	246.7438
Brij35	27.7051	48.36913	81.89077	67.57902	66.89022

4.6.15 Effect of various detergents and chelator on enzyme activity

Results obtained for the study on the effect of various detergents on enzyme activity are presented in Table 4.20. Among the four detergents studied both tween 20 and 80 and tritonX supported enhanced enzyme activity at certain levels of concentrations. Thus tween 20 supported maximal enzyme activity at 1% (246.7438U/ml). It was also observed that enzyme activity increased along with increase in concentration in tween 20 from 0.6% to 1.0%. Similarly triton X also supported enhanced enzyme activity along with increase in concentration from 0.2 % to 0.4 %. However at all other concentrations, decrease in enzyme activity was recorded. In the case of tween 80 the enzyme activity was stable, but at enhanced level at concentrations varying from 0.2 % to 0.6 %. Among the detergents brij 35 significantly reduced the enzyme activity brij 35 did not supported enhanced enzyme activity. However at concentrations 0.6-1 % the enzyme activity was relatively higher compared to that at concentrations less than 0.6 %. The EDTA

In general more than 50% residual activity was recorded for NaCl at 1mM-15mM, magnesium sulphate at 10mM, potassium sulphate at 15mM, cobalt chloride at 5mM, copper sulphate at 5mM, sodium molybdate at 5mM-15mM, lithium chloride at 10mM and 15mM, and calcium chloride at 15mM and mercuric chloride at all concentration tested, except at 10mM when there was no enzyme inhibition. Both aluminium sulphate and barium chloride almost effected complete enzyme inhibition while cadmium sulphate and manganese chloride affected significant level of enzyme inhibition.

Table 4.19 Effect of various metal ions on tannase activity

	Residual activity at different concentrations (%)				
Metal ions	**1mM**	**5mM**	**10mM**	**15mM**	**20mM**
Sodium chloride (Na^+)	54.4159	60.244	60.96338	97.97258	120.3697
Calcium chloride (Ca^{2+})	6.946711	30.54157	36.60997	78.13054	0.838396
Magnesium sulphate (Mg^{2+})	34.05485	45.31332	65.63444	9.461899	7.026558
Zinc sulphate (Zn^{2+})	32.45791	55.85315	33.69554	33.09669	30.54157
Potassium sulphate (K^+)	127.2765	127.9153	176.6221	75.09634	26.07013
Cabalt chloride (Co^{2+})	39.48447	89.34908	44.63461	40.20309	39.04531
Manganese chloride (Mn^{2+})	24.35341	20.36105	23.79448	14.25273	10.18052
Ferric chloride (Fe^{3+})	271.2012	156.1812	144.4437	131.0693	100.7273
Cupric sulphate (Cu^{2+})	33.97501	71.10398	47.18973	24.15379	10.57976
Sodiummolybdate (Mo^{6+})	48.42736	53.8969	56.97101	60.1649	22.477
Barium chloride (Ba^{2+})	0.598854	**0**	**0**	**0**	**0**
Lithium chloride (Li^+)	28.58532	43.07759	55.33415	64.11734	3.074119
Cadmium sulphate (Cd^{2+})	0	18.80403	18.36487	15.33067	11.81739
Aluminium sulphate (Al^{3+})	**0**	**0**	**0**	**0**	**0**
Mercuric chloride (Hg^{2+})	72.10207	96.21594	100.7672	53.01858	53.01858

4.6.13 Kinetic studies

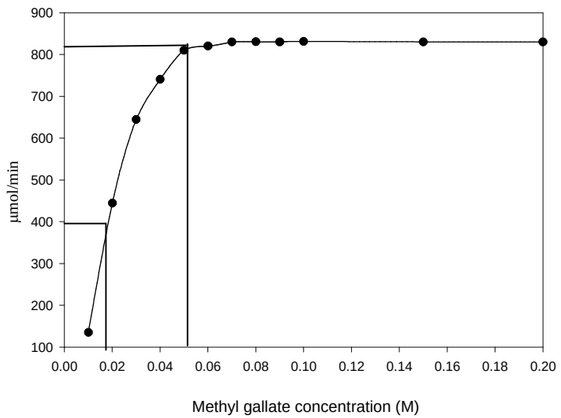


Fig.4.65 Kinetic studies with methyl gallate as substrate

Kinetic studies were performed for tannase using methyl gallate as the substrate and the data obtained is presented in Fig.4.65 K_m and V_{max} were estimated by plotting the initial velocity data as function of the concentration of the substrate. K_m and V_{max} were recorded as 19mM and 830µmol/min. respectively.

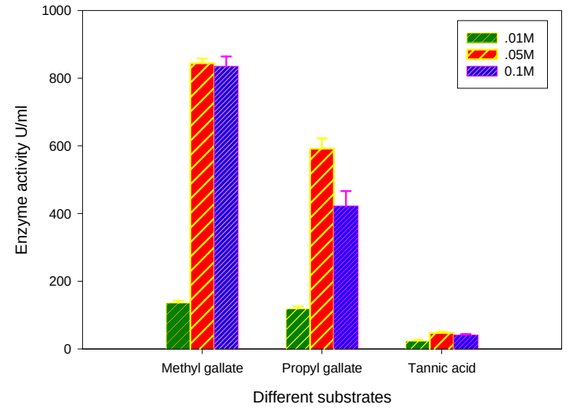


Fig.4.66 Substrate specificity of *Aspergillus awamori* BTMFW 032 with different known substrates

4.6.14 Substrate specificity

Substrate specificity for tannase were tested with three different substrates at 0.01, 0.05 and 0.1M concentration and the results are presented in Fig. 4.66. High level of affinity was recorded with methylgallate at 0.05 and 0.1M concentration followed by 0.01M propyl gallate compared to tannic acid.

incubation time. All the other solvents showed the same pattern of decreasing activity with increase in time and concentration. Only DMSO could retain 61 % activity after incubating for 24 h at 60 % concentration.

4.7 Genetic characterization

4.7.1 Genomic DNA

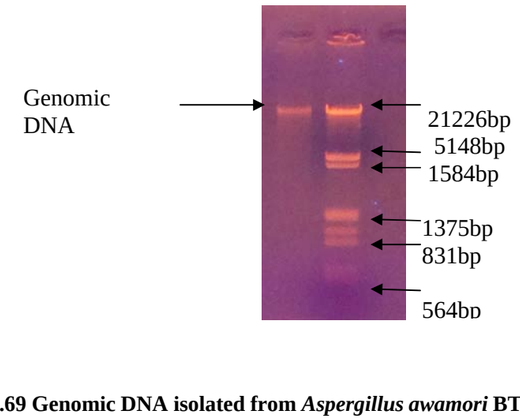


Fig.4.69 Genomic DNA isolated from *Aspergillus awamori* BTMFW 032

Genomic DNA was isolated from *A. awamori* BTMFW 032. Approximately 100µg of DNA was obtained from 2g of fresh mycelia. The gel picture presented in Fig.4.69 indicates the purity of isolated genomic DNA without RNA and protein contamination.

had a little inhibitory effect on this tannase enzyme giving 76 % of residual activity at 1mM concentration.

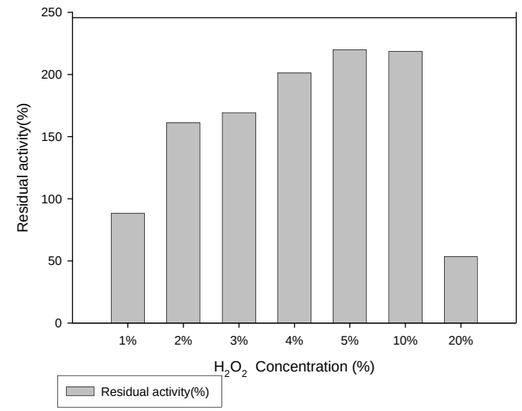


Fig.4.67 Effect of oxidizing agents on enzyme activity

4.6.16 Effect of oxidizing agent on enzyme activity

Effect of the oxidizing agent on enzyme activity was studied using hydrogen peroxide and the results obtained are presented in the Fig. 4.67. From the data it is evident that the oxidizing agent has enhanced the activity

Table.4.21 Residual activity of tannase enzyme in presence of different reducing agents

Reducing Agent	Residual activity at different concentrations (%)					
	0.2%	**0.4%**	**0.6%**	**0.8%**	**1%**	**5%**
β-mercapto ethanol	**0**	40.9094	22.3638	16.54558	2.909113	**0**
Di -thiothreitol	108.0917	110.000	111.819	102.0917	49.45493	**0**
Sodium thioglycollate	23.09109	36.7275	94.6370	114.7282	321.1843	126.0919

more than two fold at concentrations from 4 % to 10 %, compared to lower levels and at a very high level of concentrations.

4.6.17 Effect of reducing agents on enzyme activity

Results presented in the Table 4.21 indicated that the reducing agents have very high inhibitory effect on enzyme activity except in the case of sodium thio glycollate. The residual activity was reduced to its minimum in the case of β mercaptoethanol. In the case of DTT, at lower concentration, no inhibitory effect was noted but with increasing concentration of the reducing agent inhibitory effect was noted. Sodium thioglycollate at higher concentrations supported enhanced enzyme activity rather than inhibiting activity unlike other reducing agents.

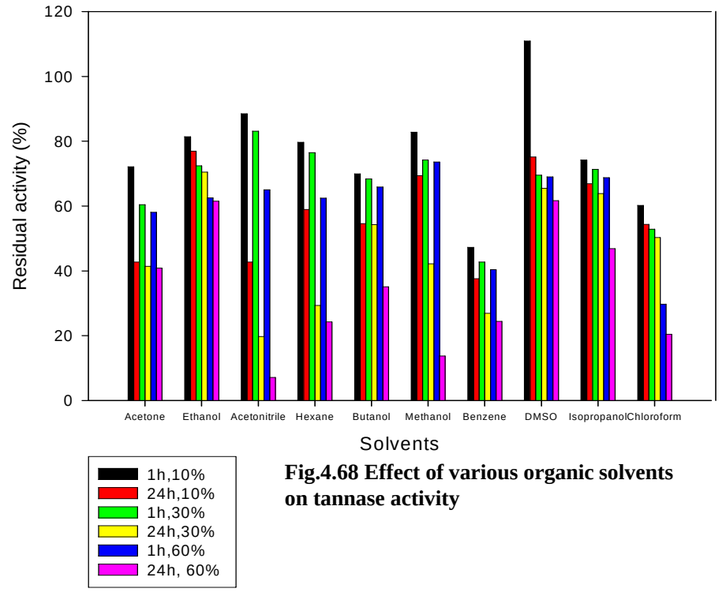


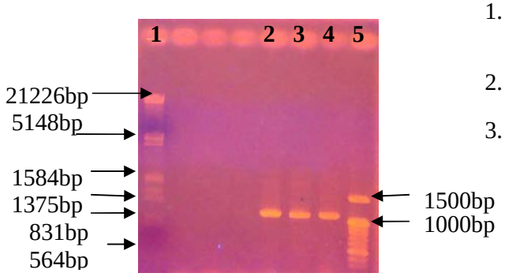
Fig.4.68 Effect of various organic solvents on tannase activity

4.6.18 Effect of organic solvents on enzyme activity

Effect of organic solvents on tannase activity were tested using 10 different solvents. In general all the solvents at different concentration reduced the enzyme activity when incubated for 1h, except DMSO which did not inhibit enzyme activity at very low concentration and instead original activity was retained (Fig.4.68). In case of acetone only 58 % of activity was retained at a concentration of 60 %, when incubated for 1 h and only 40 % was retained when incubated for 24h. Ethanol retained 81% of activity after 1 h of incubation at a level of 10 % concentration and the activity decreased with increase in concentration and

The comparison of nucleotide sequence information and the deduced amino acid sequence with the known proteins from Genbank indicated that this gene could encode tannin acyl hydrolase. A total of 1.232kb of tannase gene sequence was obtained from the genomic clone and sequence analysis revealed an open reading frame consisting of 1122bp (374 amino acids) of one stretch in -1 strand .The longest ORF shows homology to *Aspergillus* tannase. Comparison of Tan 1 with other tannase in the data bank showed that it has higher similarity of 82 % identity with *Aspergillus niger* tannase (Tan Ani) mRNA complete cds, 77 % identity with *Aspergillus flavus* NRRL3357 tannase putative mRNA sequence, 77% identity with *Aspergillus oryzae* DNA for tannase complete cds, 77% identity with *Aspergillus oryzae* tannase partial cds and tannase partial sequences of *Aspergillus fumigates* and *Aspergillus terreus* with 76% and 74% identity respectively. This result confirms the unique nature of this tannase as it shows variability among other *Aspergillus* species. The phylogram which indicates the phylogenetic relationship of various *Aspergillus* tannases with that of marine *Aspergillus awamori* Tan1 revealed it belongs to the cluster of *Aspergillus niger*. *Aspergillus awamori* is an industrial filamentous fungus, widely used for brewing Japanese traditional spirits, *A. awamori* is genetically very close to *Aspergillus niger* and close to *Aspergillus oryzae (*Machida *et al*., 2009) (Fig.4.75). Genscan predicted a sequence of 338 amino acids, which is an internal exon as the predicted peptide without introns. Expasy protopram predicted the molecular weight of protein to be 37.8 kDa and theoretical pI 4.31 which correlates with the protein characteristics as evidenced from SDS PAGE and isoelectric focussing. GOR secondary structure prediction indicates the presence of alpha helix, extended strand and random coil and no beta structures.

4.7.2Gene Amplification

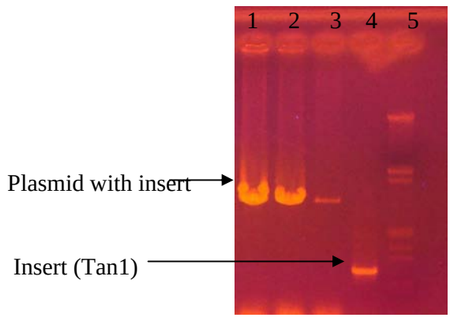


1. Lane1. λ DNA/*Eco*R1/*Hind* III Double digest
2. Lane 2, 3, 4. Amplicon 1.2kb
3. Lane 5. 1500bp ladder

Fig.4.70 PCR amplified partial tannase gene from *Aspergillus awamori*

The degenerate primers were designed from the already available sequence for tannase from *Aspergillus sp*. deposited at Genbank . Amplification of genomic DNA with degenerate primers forward TF1 and reverse TR1 yielded an amplicon (TAN1) of approximately 1.2 kb at an annealing temperature of 60ºC (Fig.4.70).The band was eluted and used for transformation.

The amplicon was cloned into the pGEMT plasmid vector, transformed in *E.Coli* DH5α and plated. From the plates of blue white colonies, white colonies were selected and checked for recombinant plasmids. Plasmids were isolated from white colonies. The isolated plasmids were checked for true recombinants. It was observed that true recombinant plasmids had insert DNA approximately 1.2 kb size which was of the same size of the amplicon obtained from PCR with the primer combination TF1 and TR1 (Fig.4.71,4.73). Restriction analysis of the recombinant plasmid yielded two separate bands corresponding to the vector and released insert (Fig.4.72). The insert was sequenced with T7 primer and a sequence of 1247bp was obtained. (Table 4.22).



1. Lane1, 2, 3 Plasmid with insert
2. Lane 4 Insert (Tan 1)
3. Lane 5 λ DNA/*Eco*R1/*Hind* III Double digest

Fig.4.71 Plasmid isolated and the amplicon

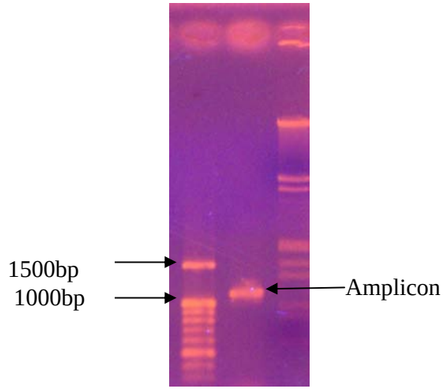


Fig.4.73 Reamplification of the gene with TF1 and TR1

1. Lane 1.λ DNA/*Eco*R1/*Hind* III Double digest
2. Lane 2. Restricted plasmid
   A. Vector
   B. Insert
3. Lane 3.Unrestricted plasmid

.

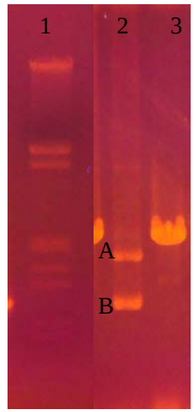


Fig.4.72 Restriction analysis of the plasmid with *EcoR1*

Table 4.21 Sequence obtained for TAN1.

CGATGGGCCGACGTCGCATGCTCCCGGCCGCCATGGCGGCCGCGGGAATT
CGATTGTCAGATTCACCGTGGTAGTGGATCATCTTACCACCGGACTTCTGG
AAGGTGGTGAGGTCGGGAATGGTGGTCTGGAGACTGTCGATGTAGCGGAT
CATACCGATGTTCATCCACTCGACCAGGGTGTCGTAGGCGACATTATCGAG
GTTCTCCAGGTTGTCGATGTTAAGAAGCTGCACGAACTTGGTCACGTACTC
GCCGCCAGTGGAGGGGATGCTCAAAGTCCAGGAGTCAGTGGTGGAGTCAT
ACTCGGTGTCACCGTCAGACAGCTCGGCGGCGATCTGCCACGAGAGGTAG
GCGCGCTTGCCGTTGGAGTCGTGGAGACCGTCGTAGATGGCCTGGGCGAG
GGCGACACCTTCGGCGGTGACGGAGCCATTCTGGGCGGGCTGGTAGCTAG
TAGTGCTGCCTTCAGCGCGCTTGCTGAAGCCGAAGCCCAGGGAGGTGTAGT
TCTCTGCAGCACAGTAGTAGGGCTCGCCGATGATGGAGGTGAGATTGAAGT
TCAGCATGCAGAGGTCAGTACGGGAGACAACGCCATCGGTACGGCCGTCG
AGAGGGTCACAGGCTTCGATGGTAGCGTTGACGATCTTGTCAAGCTCGCAA
GGGGGAGGGTAGTAATCCATGGTGTGCTCGATGGTGGCAGGGAAGACGTG
GTGGACCTGCTGCTGAGCAAAACGGAAGGCAGGGGCACCAGCGATAACAC
CGTCATATTCATCTCCCCAGCGCTGAACCTGACTCATACCCTCACGACCAC
CATCGGAACAGCCCTCGTAGTAGGTGTAGATCTTCTTGTCGCTGGAGAGGC
CGTAGAAGCCACGGGTCAGGGGCTTGGCGATCTTGGTCATTTCACCCAGAG
CCTGGTAGCCAAACATGTAAGTGGCATCCCAGTTGATCGAGCCGTTGCCAT
AGAGGACGACTTCGTCGTAGCTGTAGGAGAAGGCGTCGTAGCCGGCATCG
GTGGCACCCGAGGCAGCACCGTACTCGAGACCGCCAGTAGCATCGCTGGA
GAGAGAGAAACCACCACCACCGGCGACGTAGAAACGGTTCTTGAAATCAGA
AGGAGCGGGCAGGGCGTACTTTACGACCACCTTGTCACCCTTGCCGGTGTG
GGTGTAGGTGACAGTCACTTTATTCTATCAATCACTAGTGAATTCGCGGCC
GCC

Accession No. GQ337O57

```
A.niger          CTCGCCCAG--CAGCAGGGG--------------GGACCACGGCTT-----CCCGGCACC 2530
A.awamori        CTCACACAGGTCCCCAAGTATTCCTGCCCCCATTGACCCCCCATTTTTACTCCCTTCACC 568
                    * *                                 *          *

A.flavus         AGTGGAG-------------------------CAAACTCTGGACT------ACTACCCGC 736
A.Oryzae         ATCAACG-------------------------ATTGGAACTGGAG------GTGATTGGA 682
A.oryzae         AGTGGAG-------------------------CAAACCCTGGACT------ACTACCCGC 682
A.fumigatus      TGTGGAG-------------------------AAGACTCTGGACT------ACTACCCTC 757
A.niger          ATCGGA--------------------------CACACCATGGACT------ACTACCCTC 2558
A.awamori        TTCAAAAGTTTGAAAAATATCAACCTTCCTTTCAAGCTTTGGACCCTCCCCACCCCCTTC 628
                                                    *

A.flavus         CTCCATGTGAGTTGAAGAAGATCGTGAA-----CGCCACC--ATTGCTGCTTGC------ 783
A.Oryzae         GACCAAATTCTTCAGCATCTTATCTTTG-----ATTGTTA--ACTCCGAGGGCT------ 729
A.oryzae         CTCCATGTGAGTTGAAGAAGATCGTGAA-----CGCCACC--ATTGCTGCTTGC------ 729
A.fumigatus      CCCCGTGCGAACTGGCCAAGATCGTCAA-----CGCCACC--ATTGAGGCCTGC------ 804
A.niger          CCCCTTGTGAACTCGAGAAGATCGTCA-----CCGAGACC--ATCTCCGCCTGC------ 2605
A.awamori        CGTTTGTTGCCCCCCAAATGACCCCTGGCATTCCAAAATTCAATCTCCCCCCCCTTATGG 688
                                                     *

A.flavus         ---GACCCGC--TTG------------ATGGAAGAACCGACGGTGTT---GTGTC-CCG 821
A.Oryzae         ---CGGGAAT--AGT------------TACCCGTTTCCTCTTAGCGG---ATGCA-ATA 767
A.oryzae         ---GACCCGC--TTG------------ATGGAAGAACCGACGGTGTT---GTGTC-CCG 767
A.fumigatus      ---GATCCTC--TCG------------ACGGCCGCACTGACGGGGTG---GTCTC-CCG 842
A.niger          ---GACCCTC--TTG------------ACGGCCGTACCGACGGCGTT---GTCTC-CCG 2643
A.awamori        GGAAACCCCCAATTGGGTGCGTATAAAAAAAACCCCCCCCTGGGGATTTCGGTCTCTCCG 748
                                             *          *

A.flavus         GACG---------GATCTTTG-----------CAAGCTT-AACTTCAATTTGACCTCTA 859
A.Oryzae         GAGC---------AAGAAAAC-----------GTGCCAA-AATACTCAAGAAAGACCGC 805
A.oryzae         GACG---------GATCTTTG-----------CAAGCTC-CACTTTAATTTGACTTCCA 805
A.fumigatus      CACC---------GACCTCTG-----------CAAGCTG-CACTTTGACCTTTCGAAGA 880
A.niger          TACC---------GATCTCTG-----------CATGCTG-AACTTCAACCTCACCTCCC 2681
A.awamori        CATCACAGCTCGCGCTCTTTAAAGGGGGGACCACATACTACAAATTCTTCCACACCTCCT 808
                  *                                 *    *
```

```
A.flavus         TCATCGGTGAG------CCTTACTACTGTG------------------------------ 883
A.Oryzae         GTCAGACAAGA------TGAGTGCCAAGAG------------------------------ 829
A.oryzae         TCATTGGTGAG------CCTTACTACTGTG------------------------------ 829
A.fumigatus      TCATCGGGGAG------CCGTACTACTGCG------------------------------ 904
A.niger          TCATCGGCA------CCCCCTACTACTGCG------------------------------ 2705
A.awamori        CCCCCCCCAAAAAAGCCTCCTGCCGCCAGGAAGACACATATCTCACAAAAGATGGTGTCC 868
                                    *

A.flavus         ----------------------------------------CTGCGGGAACTAGCAC 899
A.Oryzae         ----------------------------------------AGAGCCAAATCTCGGT 845
A.oryzae         ----------------------------------------ATGCGGAGACCAGCAC 845
A.fumigatus      ----------------------------------------CCGCAAAGACCAGCAC 920
A.niger          ----------------------------------------CCGCCCAGAACTACAC 2721
A.awamori        CCAATAACACACAAAGGAGAGAGAGGGGGCCCATCCCACCCCCCGCAGAGAACTACAC 928
                                                          *

A.flavus         TTCGCTTGGTTTCGGCTTCAGCAATGGCAAGCGCAGCAATGTCAAGCGTCAGGCCGAGGG 959
A.Oryzae         CATTGTATCTCCCCTTGAATGTTGCTGACATGGTGGCTCGATCATGGATAGCTTTGCACGC 905
A.oryzae         TTCGCTTGGTTTCGGCTTCAGCAATGGCAAGCGCAGCAATGTCAAGCGTCAGGCTGAGGG 905
A.fumigatus      CTCCCTCGGCTTCGGCTTCAGCA---------------------AACGCCAGGCAGCCGG 959
A.niger          CTCCCTGGGCTTCGGCTTAAGAA---------------------AGCGCAAAGCCAAAGG 2760
A.awamori        CTCCCTGGGCTTCGGCTTCAGCA---------------------AGCGC---GCTGAAGG 964
                       *     *                                      *

A.flavus         CAGCACCACCAGCTACCAGCCCGCCCAGAACGGCACGGTCACCGCACGTGGTGTAGCTGT 1019
A.Oryzae         GCAAGGGTCAGGGCTGCATGGAGAGATCAGATAAGGCCGGATCTCAGCCGAACCGGAACA 965
A.oryzae         CAGCACCACCAGCTACCAGCCCGCCCAGAACGGCACGGTCACCGCACGCGGTGTAGCTGT 965
A.fumigatus      CAGCACGACCAGCTACCAGCCTGCACAAAACGGCACCGTCACCAAGGAGGGGGTGGCTGT 1019
A.niger          CAGCACCACCAGCTACCAGCCCGCCCAGAATGGCACCATCACCGCCGAGGTGTCGCTTT 2820
A.awamori        CAGCACTACTAGCTACCAGCCCGCCCAGAATGGCTCCGTCACCGCCGAAGGTGTCGCCCT 1024
                        *  *   **    *     *           * *     *    *
```


Fig.4.74 Phylogenetic tree showing diversity of *Aspergillus awamori* BTMFW 032 tannase gene among various *Aspergillus* sp. tannase gene

CLUSTAL W 2.0.11 multiple sequence alignment of tannase (TAN1) amino acid sequences

```
A.flavus         MRQHSRMAVAALAAGANAASFTDVCTVSNVKAALPANGTLLGISMLPSAVTANPLYN--Q 58
A.oryzae         MRQHSRMAVAALAAGANAASFTDVCTVSNVKAALPANGTLLGISMLPSAVTANPLYN--Q 58
pA.oryzae        ------------------ASFTDVCTVSNVKAALPANGTLLGISMLPSAVTASPLYN--Q 40
A.fumigatus      MRISYGSAVAALAAAANAASLADVCTISHVQSVLPSNGTLLGINVIPSAVTASAVYNSTS 60
A.niger          MRQHSRSVRCSGSSTANAASLSDVCTVSNVQSALPSNGTLLGIDLIPSAVTANTVTD--A 58
A.awamori        ----------------------------------------CFEPCQAAAN------- 10
                                                          . *  .:*.

A.flavus         SAGMG-----STTTYDYCNVTVAYTHTGKGD---KVVIKYAFPKPSDYENRFYVAGGGGF 110
A.oryzae         SAGMG-----STTTYDYCNVTVAYTHTGKGD---KVVIKYAFPKPSDYENRFYVAGGGGF 110
PA.oryzae        SAGMG-----STTTYDYCNVTVAYTHTGKGD---KVVIKYAFPKPSDYENRFYVAGGGGF 92
A.fumigatus      SGGMGGMGGSNSANYPYCNVTVTYTHPGKGD---KVVVKYAFPQPSDFKNRFYVAGGGGY 117
A.niger          TAGMG-----STTTYDYCNVTVTYTHTGKGD---QVVVKYAFPAPSDFENRFYVAGGGGF 110
A.awamori        ----------SLVIDYCNVTVTYTHTGQGQGGRKVRPARSFFQEPFLRRRWWWFSLSPA 59
                           :    ******:***.*:*:   :*    :*   .  ..*::  . .

A.flavus         SLSSDATGGLAYGAVGGATDAGYDAFDNSYDEVVLYGNGTINWDATYMFAYQALGEMTRI 170
A.oryzae         SLSSDATGGLAYGAVGGATDAGYDAFDNSYDEVVLYGNGTINWDATYMFAYQALGEMTRI 170
PA.oryzae        SLSSDATGGLAYGAVGGATDAGYDAFDNSYDEVVLYGNGTINWDATYMFAYQALGEMTQI 152
A.fumigatus      SLSSDATGGLEYGAASGATDAGYDAFSYSYDEVVLYGNGSINWDATYMFAYQALGEMTTL 177
A.niger          SLSSDATGGLEYGAASGATDAGYDAFSYSYDEVVLYGNGFINWDATNMFGYQALGEMTKI 170
A.awamori        MLLAVSSTVLPRVPPMPATTPSPTATTKSSSMATARSTG----MPLTCLATRLWGEMTKI 115
                  * : ::  *   .  ** ..  *   * . ..  ..*     .   :. :  **** :

A.flavus         GKYITKGFYGQSSDSKVYTYYEGCSDGGREGMSQVQRWGEEYDGAITGAPAFRFAQQQVH 230
A.oryzae         GKYITKGFYGQSSDSKVYTYYEGCSDGGREGMSQVQRWGEEYDGAITGAPAFRFAQQQVH 230
pA.oryzae        GKYITKGFYGQSSDSKVYTYYEGCSDGGREGMSQVQRWGEEYDGAITGAPAFRFAQQQVH 212
A.fumigatus      GKTLTRNFYGLSSDAKIYTYYEGCSDGGREGMSQVQRYGDLYDGAITGAPAFRYAQQQVH 237
A.niger          GKPLTQGFYGLSSDQKIYTYYEGCSDGGREGMSQVHRWGDEYDGVIAGARPSALAQQQGG 230
A.awamori        AKPLTRGFYGLSSDNKTTLQLGVVPMVVVRLFS--LALGNEYD-VFPWCPGLPFAHTGPQ 172
                 .*  :*:.*** *** *        .   . :*     *: ** .:. .     *:
```

```
A.flavus        HVFSSEVEQTLDYYPPPCELKKIVNATIAACDPLDGRTDGVVSRTDLCKLNFNLTSIIGE 290
A.oryzae        HVFSSEVEQTLDYYPPPCELKKIVNATIAACDPLDGRTDGVVSRTDLCKLNFNLTSIIGE 290
pA.oryzae       HVFSSEVEQTLDYYPPPCELKKIVNATIAACDPLDGRTDGVVSRTDLCKLHFNLTSIIGE 272
A.fumigatus     HVFSSVVEKTLDYYPPPCELAKIVNATIEACDPLDGRTDGVVSRTDLCKLHFDLSKIIGE 297
A.niger         PRLPGTIGHTMDYYPPPCELEKIVTETISACDPLDGRTDGVVSRTDLCMLNFNLTSLIGT 290
A.awamori       VFLPPLTPHFYSLHLQKFEKYQPSFQALDPPHPLP----FVAPQMTPGIPKFNLPPLMGK 228

A.flavus        PYYCAAGTSTSLGFGFSNGKRSNVKRQAEGSTTSYQPAQNGTVTARGVAVAQAIYDGLHN 350
A.oryzae        PYYCAAGTSTSLGFGFSNGKRSNVKRQAEGSTTSYQPAQNGTVTARGVAVAQAIYDGLHN 350
pA.oryzae       PYYCDAETSTSLGFGFSNGKRSNVKRQAEGSTTSYQPAQNGTVTARGVAVAQAIYDGLHN 332
A.fumigatus     PYYCAAKTSTSLGFGFS-------KRQAAGSTTSYQPAQNGTVTKEGVAVAKAIYDGLHN 350
A.niger         PYYCAAQNYTSLGFGLR-------KRKAKGSTTSYQPAQNGTITRRXVALAQAXYDGLHD 343
A.awamori       PPIGCVKKPPPGDFGLS----ASQLALFKGGTTYYKFFHTSSPPKKASCRQEDTYLTKDG 284

A.flavus        SKGERAYLSWQIASELSDAETEYNSDTGKWELNIPSTGGEYVTKFIQLLNLDNLSDLNNV 410
A.oryzae        SKGERAYLSWQIASELSDAETEYNSDTGKWELNIPSTGGEYVTKFIQLLNLDNLSDLNNV 410
pA.oryzae       SKGERAYLSWQIASELSDAETEYNSDTGKWELNIPSTGGEYVTKFIQLLDLDNLSDLNNV 392
A.fumigatus     TQGQRAYLSWQIASEFSDATTEWNNDTGSWELSIPSTGGEFVTKFVQLLDLDNLSTLDNV 410
A.niger         SEGKRAYLSWQIAAELFDADTTYDSTTDSWTLDIPSTGGEYVTKFVQLLNIDNLENLDNV 403
A.awamori       VPNNTQRRERGGPSHPPQRTTPPWASASASALKAALLATSPPRMAPSPPKVSPSPRPSTT 344

A.flavus        TYDTLVDWMNTGMVRYMDSLQTTLPDLTPFQSSGGKLLHYHGESDPSIPAASSVHYWQAV 470
A.oryzae        TYDTLVDWMNTGMVRYMDSLQTTLPDLTPFQSSGGKLLHYHGESDPSIPAASSVHYWQAV 470
pA.oryzae       TYDTLVDWMNTGMVRYMDSLQTTLPDLTPFQSSGGKLLHYHGESDPSIPAASSVHYWQAV 452
A.fumigatus     TYDTLVEWMNTAMVRYMDSLQTTVPDLTTFKSSGGKLLHYHGESDPSIPAASSVHYWQSV 470
A.niger         TYDTLVDWMNTGMIRYIDSLQTTVVDLTDFQKSGGKMIHYHGESDPSIPTASSVHYWQAV 463
A.awamori       VSTTPTASAPTSRGRSPPSCLTVTPSMTPPLTPGLASPPLAASTPSSCSFLTSTTWRTSI 404

A.flavus        RSVMYGDKTEEEALEALEDWYQFYLIPGAAHCGTNSLQPGPYPENNMEIMIDWVENGNKP 530
A.oryzae        RSVMYGDKTEEEALEALEDWYQFYLIPGAAHCGTNSLQPGPYPENNMEIMIDWVENGNKP 530
pA.oryzae       RSIMYPGVSAAKSLKELQEWYQFYLIPGAAHCGANSLQPGPYPQNNMDIMIDWVENGVQP 530
A.niger         RQAMYPNVTYTDSLKRMADWYQLYLIPGAAHCGTNSLQPGPYPEDNMEIMINWVENGVKP 523
A.awamori       MSPTTP------------------WSSGTSVSATSTVSRPPFPTS-------------PP 433

A.flavus        SRLNATVSSGTYAGETQMLCQWPKRPLWRGNSS-----------FDCVNDEKSIDS---- 575
A.oryzae        SRLNATVSSGTYAGETQMLCQWPKRPLWRGNSS-----------FDCVNDEKSIDS---- 575
pA.oryzae       SRLNATVSSGTYAGETQMLCQWPKRPLWRGNST-----------FDCVNDEKSIGS---- 557
A.fumigatus     SRLNTTVSSGDYAGETQMLCQWPTRPLWKDNST-----------FDCVNDEKSIES---- 575
A.niger         SRLNATVSSGTYEGETQMLCQCPLVPLWTSASTPPSPPVPMLVRPRCSASGLPVPSGPAT 583
A.awamori       SRSPVVRSTTTVN----LTIEFP-RPPWR---------------PGACDVGPS------- 466

A.flavus        --------------WTYEFPAFKVPVY- 588
A.oryzae        --------------WTYEFPAFKVPVY- 588
pA.oryzae       --------------WTYEFPAFKVPVY- 570
A.fumigatus     --------------WTYTFPAFKVPVY- 588
A.niger         PASLVSMTPSLLLLGTTLLTLSRCPFFK 611
A.awamori       ---------------------------
```

CLUSTAL 2.0.11 multiple sequence alignment of nucleotide sequence of TAN1

```
A.flavus        CCCTCTCTACAACCAGTC---------------GGCTGGCATGGGTAGCACCA------C 197
A.Oryzae        ACACTTTCCGTGAGACGC---------------CGGAGAAGCTACCTGAGCCG------G 143
A.oryzae        CCCTCTCTACAACCAGTC---------------GGCTGGCATGGGTAGCACCA------C 143
A.fumigatus     TGCAGTCTACAACAGTACCTCCAGCGGCGGCATGGGCGGCATGGGCGGCTCCAACAGTGC 218
A.niger         TACTGTCACCGACGCCAC---------------CGCCGGCATGGGCAGCACCA------C 2019
A.awamori       ---TGTTTCGAACCTTGC---------------CA--GGCGGCCGCGAATTCA------C 34
```



```
A.flavus        TACCTAT-GACTACTGCAATGTGACTGTCGCCTACACGCATACCGG-CAAGGGTGATAAA 255
A.Oryzae        CTGATTT-TTAAAGAAATGAATCTGTACAGTCTAGACTCGATGTCT-AGAGCCGCATATG 201
A.oryzae        AACCTAT-GACTACTGCAATGTGACTGTCGCCTACACGCATACCGG-CAAGGGCGATAAA 201
A.fumigatus     CAACTAC-CCCTACTGCAATGTGACGGTCACCTATACCCACCCCGG-TAAGGGCGACAAG 276
A.niger         CACCTAC-GACTACTGCAACGTCACCGTCACCTACACCCACACCGG-CAAGGGTGACCAG 2077
A.awamori       TAGTGATTGACTACTGCAATGTGACTGTCACCTACACCCACACCGGGCAAGGGTGACAAG 94

A.flavus        GTGGTCATCAAGTACGCATTCCCCAAGCCCTCCGACTACGAGAACCGTTTCTACGTTGCT 315
A.Oryzae        ACCATGATGACGATATAATTATGACAATAACCGTTTCAATTAAATGAGTCTAGGCAGCCA 261
A.oryzae        GTGGTCATCAAGTATGCATTCCCCAAGCCCTCCGACTACGAGAACCGTTTCTACGTTGCT 261
A.fumigatus     GTGGTCGTTAAGTATGCCTTCCCCCAGCCTTCCGACTTCAAGAACCGCTTCTACGTTGCC 336
A.niger         GTCGTCGTCAAGTACGCCTTCCCCGCTCCCTCCGACTTCGAGAACCGCTTCTACGTCGCC 2137
A.awamori       GTGGTCGTAAAGTACGCCCTGCCCGCTCCTTCTGATTTCAAGAACCGTTTCTACGTCGCC 154

A.flavus        GGTGGTGGTGGCTT-TTCCCTCTCTAGCGATGCTACCGGAGGTCTCGCCTATGGCGCTGT 374
A.Oryzae        CGAAGGTTTTGCCT-TTGTGTGAAAATTCTCTATGCCCATCTAGGAAGCATTACACTAGA 320
A.oryzae        GGTGGTGGTGGCTT-CTCCCTCTCCAGCGATGCTACCGGAGGTCTCGCCTATGGCGCTGT 320
A.fumigatus     GGGGGTGGCGGTTA-CTCCCTCTCCAGCGATGCCACTGGCGGTCTAGAGTATGGTGCGGC 395
A.niger         GGTGGTGGTGGCTT-CTCCCTCTCCAGCGATGCCACCGGTGGTCTCGAGTACGGTGCCGC 2196
A.awamori       GGTGGTGGTGGTTTTCTCTCTCTCCAGCGATGCTACTGGCGGTCTCGAGTACGGTGCTGC 214

A.flavus        GGGAGGTGCCACCGATGCTGGATACGACGCATTCGATAACAGCTACGACGAGGTAGTCCT 434
A.Oryzae        AAATTGTAAAATTGGCTCAGCCTCCACTGCCTTGAAGTCTAGTCAGGGGGAGCCGGGTAT 380
A.oryzae        GGGAGGCGCCACCGATGCTGGATACGACGCATTCGATAACAGCTACGACGAGGTAGTCCT 380
A.fumigatus     GTCTGGTGCCACCGATGCGGGTTACGATGCCTTCAGCTACAGCTACGACGAGGTGGTTCT 455
A.niger         CTCCGGTGCCACCGACGCCGGCTACGACGCCTTCTCCTACAGCTACGATGAAGTCGTCCT 2256
A.awamori       CTCGGGTGCCACCGATGCCGGCTACGACGCCTTCTCCTACAGCTACGACAAAGTCGTCCT 274

A.flavus        CTACGGAAACGGAACCATTAACTGGGACGCCACATACATGTTCGCATACCAGGCACTGGG 494
A.Oryzae        CTACGGAAACGGAACCATTAACTGGGACGCCACGTACATGTTCGCATACCAGGCGCTGGG 440
A.fumigatus     TTACGGCAACGGATCGATCAACTGGGACGCCACGTACATGTTCGCCTACCAGGCCCTGGG 515
A.niger         CTACGGCAACGGCTTCATCAACTGGGATGCCACTAACATGTTCGGCTACCAGGCTCTCGG 2316
A.awamori       CTATGGCAACGGCTCGATCAACTGGGATGCCACTTACATGTTTGGCTACCAGGCTCTGGG 334

A.flavus        -AGAGATGACCCGGATCGGAAAGTACATCACCAAGGGCTTTTATGGCCAGTCCAGCGACA 553
A.Oryzae        -TGTTCAAATGTTGAACGACCTTCGTTAACAGTCCATAATCGGTTGCCCTGTTCTGTAAA 499
A.oryzae        -TGAGATGACCCAGATCGGAAAGTACATCACCAAAGGCTTTTATGGCCAGTCCAGCGACA 499
A.fumigatus     -CGAGATGACCACTCTCGGCAAGACATTGACCCGAAACTTCTACGGTCTGTCTAGCGATG 574
A.niger         -TGAGATGACCAAGATCGGCAAGCCCCTCACCCAGGGCTTCTACGGTCTCTCCAGCGACC 2375
A.awamori       GTGAAATGACCAAGATCGGCCAAGCCCCTGACCCGTGGCTTCTACGGCCTCTCCAGCGACA 394

A.flavus        GCAAGGTCTACACCTACTACGAGGG-TTGCTCCGATGGAGGACGTGAGGGTATGAGTCAA 612
A.Oryzae        CGTATTTGGGAGCCGCTCAGCATTT-TCCGCCTTGGTATAGGTCTTCTTGTTGTAGGTAT 558
A.oryzae        GCAAGGTCTACACCTACTACGAGGG-TTGCTCCGATGGAGGACGTGAGGGTATGAGTCAA 558
A.fumigatus     CCAAGATCTACACCTACTACGAAGG-ATGCTCCGACGGTGGACGTGAAGGCATGAGCCAG 633
A.niger         AGAAGATCTACACCTACTACGAAGG-TTGCTCCGACGGTGGTCGTGAAGGTATGAGCCAG 2434
A.awamori       ATAAAA-CTACAC-TACAACTAGGGGTTGTTCCAATGGTGGTCGTGAGGTTATAATTCAG 452

A.flavus        GTCCAGCGCTGGGGTGAGGAGTATGACGGTGCGATTACTGGTGCCCCGGCTTTCCGTT-- 670
A.Oryzae        ACAATGCTACGATGTTGAGGCTGATGGTACCTGATGCTCGGAGATAAAAAATTAAACA-- 616
A.oryzae        GTCCAGCGCTGGGGTGAAGAGTATGACGGTGCGATTACTGGTGCCCCGGCTTTCCGTT-- 616
A.fumigatus     GTTCAGCGCTACGGCGATCTGTACGACGGTGCCATCACCGGTGCCCCGGCTTTCCGCT-- 691
A.niger         GTTCACCGCTGGGGAGATGAATACGACGGGGTCATTGCCGGTGCCC-GGCCTTCCGCT-- 2491
A.awamori       -TTTAGCGTTGGGGA-ATGAATATGACGT--TTTTCCCTGGTGCCCTGGCCTTCCGTTTG 508

A.flavus        -TCGCTCAG--CAACAGGTTC---------------ACCATGTGTT-----CTCGTCCGA 707
A.Oryzae        -CAACACGT--TAGGTAACGT---------------TTGATGCAAT-----TTGCCCCTG 653
A.oryzae        -TCGCTCAG--CAACAGGTTC---------------ACCATGTGTT-----CTCGTCCGA 653
A.fumigatus     -ATGCCCAG--CAGCAGGTCC---------------ACCACGTCTT-----TTCGTCGGT 728
```

4.8.2 Propyl gallate

4.8.2.1 Fourier-Transform Infra red Spectroscopy (FT-IR Spectroscopy)

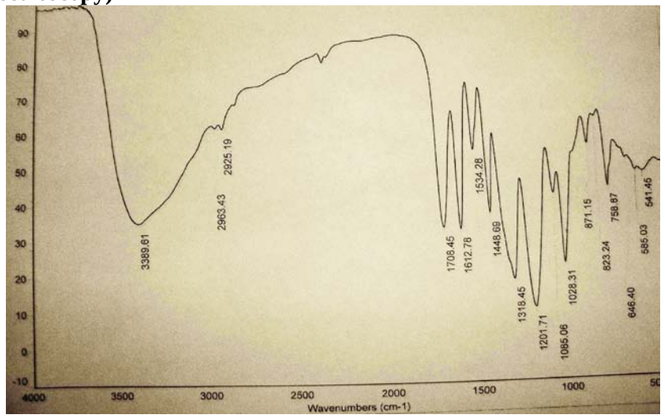


Fig.4.76 FT-IR analysis of propyl gallate extracted

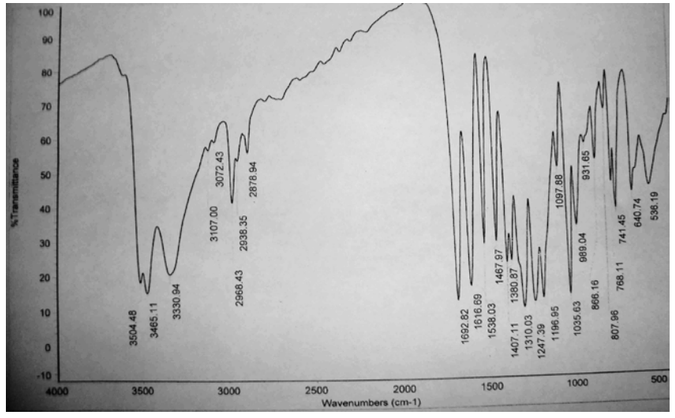


Fig.4.77 FT-IR analysis of propyl gallate standard


```
A.flavus        CGCCCAGGCCATCTACGATGGTCTCCACAACAGCAAGGGCGAGCGCGCGTACCTCTCCTG 1079
A.Oryzae        TCAGATAACAAAAATTCATCGTCGGACGACCGGAGACTACTACTACTACTAGTATCAACT 1025
A.oryzae        CGCCCAGGCCATCTACGATGGTCTCCACAACAGCAAGGGCGAGCGCGCGTACCTCTCCTG 1025
A.fumigatus     CGCCAAGGCCATTTACGACGGTCTGCACAACACCCAGGGCCAGCGCGCCTACCTCTCCTG 1079
A.niger         GGCCCAGGCCKTGTACGATGGTYTGCACGACTCCGAGGGMAAGCGCGCCTACCTGTCATG 2880
A.awamori       CGCCCAGGCCATCTACGACGGTCTCCACGACTCCAACGGCAAGCGCGCCTACCTCTCGTG 1084
                          *       *  **       *    *      *    *   **

A.flavus        GCAGATTGCCTCTGAGCTGAGCGATGCTGAGACCGAGTACAACTCTGACACTGGCAAGTG 1139
A.Oryzae        CCGCGGGTCGAGCCTCGAGGAAGACCTTTTGACTTGGCATCTTGCCACGCAACCCGGTGA 1085
A.oryzae        GCAGATTGCCTCCGAGCTGAGCGATGCTGAGACCGAGTACAACTCTGACACTGGCAAGTG 1085
A.fumigatus     GCAGATCGCCTCGGAATTCTCCGATGCCACCACCGAGTGGAACAATGACACCGGCTCCTG 1139
A.niger         GCAGATCGCCGCGGAGCTTTTCGATGCTGACACCACCTATGACTCGACCACCGACTCCTG 2940
A.awamori       GCAGATCGCCGCCGAGCTGTCTGACGGTGACACCGAGTATGACTCCACCACTGACTCCTG 1144
                  *     *                **       **                *

A.flavus        GGAGCTCAACATCCCGTCGACCGGTGGTGAGTACGTCACCAAGTTCATTCAGCTCCTGAA 1199
A.Oryzae        CGACAGCCTGAGTAGAATTAAGGATGGCAAAGCGTTGATCTGCCGTTTGGTCCACAAGCT 1145
A.oryzae        GGAGCTTAACATCCCGTCGACCGGTGGTGAATACGTTACCAAGTTCATTCAACTCCTGGA 1145
A.fumigatus     GGAGCTGAGCATCCCGTCCACCGGCGGCGAATTCGTGACCAAGTTCGTCCAACTCCTGGA 1199
A.niger         GACCCTCGATATCCCCTCTACCGGTGGCGAATATGTGACCAAGTTCGTCCAGCTCCTCAA 3000
A.awamori       GACTTTGAGCATCCCCTCCACTGGCGGCGAGTACGTGACCAAGTTCGTGCAGCTTCTTAA 1204
                          *     *  *  **  *    *  *  *        *    *

A.flavus        CCTCGACAACCTTTCGGATCTGAACAACGTGACCTACGACACCCTGGTCGACTGGATGAA 1259
A.Oryzae        TGTTACGAATCCCGAACCTTATGATGCCGAAGACGGTGGTCTCTCAGCCCTAGCCTTGCA 1205
A.oryzae        CCTCGACAACCTTTCGGATCTGAACAACGTCACCTACGATACCCTGGTGGACTGGATGAA 1205
A.fumigatus     TCTCGATAACCTGTCCACTCTCGACAACGTCACCTACGACACCCTCGTCGAATGGATGAA 1259
A.niger         CATCGACAACCTCGAGAACCTCGACAACGTCACCTACGACACCCTCGTTGACTGGATGAA 3060
A.awamori       CATCGACAACCTGGAGAACCTCGATAATGTCGCCTACGACACCCTGGTCGAGTGGATGAA 1264
                  *   **  *                *    *    *    *    *        ** *

A.flavus        CACTGGTATGGTGCGCTACATGGACAGCCTTCAGACCACCCTTCCCGATCTGACTCCCTT 1319
A.Oryzae        ATAAATAGGACGATAGTTTCCCTATGGCTCCTCCTAGATACGACCTCATCATTCGTTTAT 1265
A.oryzae        CACTGGTATGGTGCGCTACATGGACAGCCTTCAGACCACTCTTCCCGACCTGACTCCCTT 1265
A.fumigatus     CACCGCCATGGTGCGCTACATGGACAGCCTGCAGACCACCGTCCCGGACCTGACGACTTT 1319
A.niger         CACCGGCATGATCCGCTACATCGACAGTCTGCAGACCACCGTGGTCGATTTGACCGACTT 3120
A.awamori       CATCGGTATGATCCGCTACATCGACAGTCTCCAGACCACCATTCCCGACCTCACCACCTT 1324
                          *    *  *          *          *    *    *

A.flavus        CCAATCGTCCGGCGGAAAGCTGCTGCACTACCACGGTGAATCTGACCCCAGTATCCCCGC 1379
A.Oryzae        TCCTTTCGTATCCTTTGAACACTCCTTGACCTCTGCCATTCTTTTGGTTCGAAAGATGCG 1325
A.oryzae        CCAATCGTCCGGCGGAAAGCTGCTGCACTACCACGGTGAATCTGATCCTAGTATCCCCGC 1325
A.fumigatus     CAAATCCTCCGGCGGCAAGCTGCTGCATTACCACGGTGAATCCGACCCCAGTATCCCCGC 1379
A.niger         CCAGAAGTCTGGCGGCAAGATGATTCACTACCACGGCGAATCCGACCCCAGTATCCCCAC 3180
A.awamori       CCAGAAGTCCGGTGGTAAGATGATCCACTACCACGGTGAATCTGACAATCGAATTCCCGC 1384
                          *          *   *              * *

A.flavus        TGCCTCCTCGGTCCACTACTGGCAGGCGGTTCGTTCCGTCATGTACGGCGACAAGACGGA 1439
A.Oryzae        CCAACACTCGCGCATGGCCGTTGCTGCTTTGGCAGCAGGAGCGAACGCAGCTTCTTTTAC 1385
A.oryzae        TGCCTCCTCGGTCCACTACTGGCAGGCGGTTCGTTCCGTCATGTACAGCGACAAGACGGA 1385
A.fumigatus     CGCGTCCTCGGTGCACTACTGGCAGTCTGTGCGCTCGATCATGTACCCCGGTGTGTCGGC 1439
A.niger         CGCGTCCTCGGTGCACTACTGGCAGGCCGTCCGTCAGGCCATGTACCCCAACGTGACCTA 3240
A.awamori       GGCCGCCATGGCG-GCCGGGAGCATGCGA--CGTCGGCCCATC---------------- 1424
```

Motif scan of deduced amino acid sequence revealed presence of tannase family profile extending from 1to 357th residue. Secondary structure prediction of the sequences showed the proportion of different structures such as Alpha helix (Hh): 94 is

24.87% Extended strand (Ee):98 is 25.93% and Random coil (Cc):186 is 49.21%.

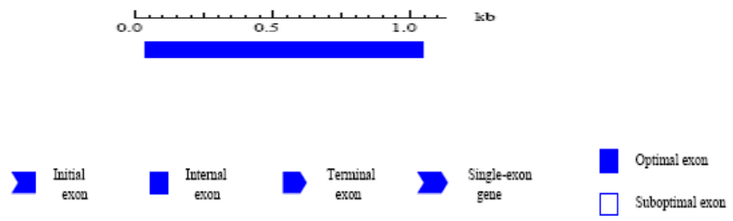


Fig.4.75 Exon sequences present in the longest ORF of Tan1. The length of the exon region indicated in the scale.

The exon analysis of the sequence using 'Genscan' tool revealed the presence of single internal exons and ORF sequence has a single exon (Fig.4.74) No terminal or initial exons could be detected.

4.8. Application studies

4.8.1 Estimation of solid content

The tea extract was prepared and the total solid content was estimated and from that cream content was determined. From this % of cream solubilised was calculated using the formula. 60.01 % of cream was solubilised by 1ml of partially purified enzyme having an activity of 699 U/ml (Table 4.22).

Table 4.22 Estimation of solid content

Total solids	0.258g
Total solids precipitated	0.174g
Cream content in control	32.558%
Cream content in sample treated with enzyme	12.98%
% cream solubilised by enzyme	60.01%

(Van Diepeningen, 2004; Cruz-Hernández *et al*., 2006). Hence, marine *Aspergillus awamori* was tested for enzyme production using tannic acid as substrate and its tolerance to higher concentrations of tannic acid, up to15 % (w/v), in the production medium prepared with 50 % (v/v) seawater and 50 % (v/v) Czapeks Dox minimal media. From the results obtained it was inferred that tannic acid concentration up to 7.5 % (w/v) supported significant tannase production while 4-5 % tannic acid seems to be optimal for enhanced enzyme production (423 U/ml), although the fungus could survive up to 12.5 % (w/v) concentration of tannic acid in the medium.

5.1 Selection of natural substrates as carbon source

The production of enzymes by bioprocesses is a good alternative to add value to agro industry residues. Tannins are polyphenolic secondary metabolites of plants, which form hydrogen bonds in solutions, resulting in the formation of tannin-protein complexes (Sharma *et al*., 1999). They are found in a large array of herbaceous and woody plants and their molecular weights range from 500 to 3000 g $mole^{-1}$ (Chamkha *et al*., 2002). Hydrolysable tannins are composed of esters of gallic acid (gallotannins) or ellagic acid (ellagitannins) with a sugar core which is usually glucose (Bhat *et al*., 1998). Hydrolysable tannins are readily hydrolyzed chemically by acidification or biologically by tannase. Different types of leaves were tried for tannase production like jawar leaves, amla leaves, jamun leaves and ber leaves (Kumar *et al*., 2007). *Pseudomonas aeruginosa* IIIB 8914 was able to produce high yield of tannase under SmF in the medium containing cost effective agro residues as sole source of crude tannin and carbon source and the results suggested that plant leaves, mainly amla and keekar leaves, could be used as suitable source of tannin and as cost-effective alternative to expensive pure tannic acid for commercial production of the enzyme (Selwal *et al*., 2009). In the present study garcinia *(Garcinia gummi gutta)* was evaluated as substrate for gallic acid

FT-IR absorption of propyl gallate showed a band at 2963.43 cm^{-1} and 2925.19 cm^{-1} present in the sample which represents the alkane group presence having CH_2 (Fig.4.77). This correlates with the bonding pattern of commercially available propyl gallate standard. All other peaks responsible for C-O, C-H, C=C, O-H are visible at their appropriate range confirming the presence of propyl gallate (Fig.4.78). Hence it is suggested that the enzyme is suitable for the production of propyl gallate by transesterification. However the method needs to be standardized for obtaining maximum propyl gallate yield from minimum tannic acid.

4.8.2.2 Thin layer Chromatography

1. Standard propyl gallate
2. Sample

Fig. 4.78 Thin layer chromatogram of propyl gallate

The thin layer chromatogram represents the standard and sample loaded and the sample has propyl gallate eventhough not pure which has an R_f value of 0.86 (Fig.4.78).

Chapter 5

DISCUSSION

Aspergillus sp. is the predominant source of tannase for industrial production and application though other microbes are also being exploited for tannase production. Most of the tannase producing fungi are of terrestrial origin and till date no reports are available on utilization of marine fungi for tannase production. In this context the present study led to the recognition of *Aspergillus awamori*, isolated from marine environment as potential source for tannase. The preliminary screening studies conducted on agar plates showed clear halo zones due to hydrolysis of tannic acid around the fungal colony after 48 h of incubation confirming tannase activity. Tannase is an inducible enzyme and the results of the preliminary studies evidence that pre induced inoculum support enhanced enzyme production compared to uninduced inoculum.

Czapek Dox medium which is being generally used for tannase production by investigators was used here as the basal medium for tannase production. Since the fungus was originally isolated from marine environment, sea water was also evaluated as enzyme production medium. Both of the basal media when used for fermentation with 1 % tannic acid as sole carbon source, it was found that sea water supported enhanced tannase production. Further, medium prepared with a combination of seawater and Czapek Dox minimal media incorporated with different tannic acid concentration could support enhanced enzyme production. The ability of *Aspergillus niger* to grow on tannic acid medium may be linked to its saprophytic lifestyle. Inspite of the fact that *Aspergillus* sp. produces tannase in the absence of tannic acid, *A. niger* tolerated tannic acid concentrations as high as 20 % without having a deleterious effect on both growth and enzyme production

response surface methodology. The five parameters optimized included garcinia leaf (26 %), glucose (3.2 mM), tannic acid (1 %), temperature (40ºC) and inoculum concentration (3 %). Time course studies using *A. awamori* under final optimized conditions revealed that tannase production increased rapidly during initial stages of fermentation and maximum enzyme activity was recorded at 24 h with an activity of 75.23 U/ml. However with further progress of fermentation the enzyme activity declined. In an earlier study tannic acid concentration, agitation speed, and pH were identified as important process parameters effecting cell growth and enzyme synthesis by *A. awamori* and these parameters were optimized in a laboratory bioreactor by response surface methodology using Box and Behnken factorial design to determine the optimum conditions for enzyme production and gallic acid accumulation (Seth and Chand, 2000). Under optimum process conditions for enzyme synthesis, the fermentation run lasted 60 h with an initial tannic acid concentration of 35.0 g l^{-1}, yielding biomass concentration of 7.13 g l^{-1} containing 771 IU of intracellular tannase per gram dry cell weight and 19 g l^{-1} of gallic acid. However, maximum gallic acid accumulation (40.3 g l^{-1}) was obtained in 24 h with an initial substrate concentration of 45 g l^{-1} (Seth and Chand, 2000). Jamun leaves and amla leaves were used for tannase production (Kumar *et al*., 2007) where a maximum yield of enzyme activity 69U/g dry substrate was obtained after 96 h of incubation. At the same time palm kernel cake and tamarind seed powder as substrate supported maximal enzyme activity of 13.03U/g dry substrate and a 6.44 U/g dry substrate respectively (Sabu *et al*., 2005b). Whereas wheat bran enriched with 0.8 % tannic acid supported higher enzyme activity of 67.5U/g dry substrate (Gustavo *et al*., 2001).

Natural substrates often serve as source of nutrients to microorganism and in turn they may utilize them and produce several byproducts which hold economic utility. In the present study the tannase produced by the fungus during the course of utilization of the tested natural substrates as a result of induction, could have acted

production and was found that the same could be a best source. As per Folin-denins method 5mg tannic acid equivalents is present in 500 mg of leaf (ie. 1 % tannic acid equivalent present in garcinia leaf) and hence could have induced tannase production and subsequent gallic acid production. It may be noted that this is the first time *Garcinia gummi gutta* leaves, which has wide medicinal properties, was successfully evaluated for tannase and gallic acid production. In fact all the other natural substrates which were tried as substrate also have certain amount of tannin content and hence were tried for tannase production. But those substrates did not support enhanced enzyme synthesis and recorded tannase at very low level compared to control. However, tamarind in combination with tannic acid in sea water medium recorded maximum enzyme activity followed by grapes wastes and tannic acid in Czapek Dox medium, and garcinia leaves and tannic acid in sea water medium. In spite of the fact that tamarind and grape waste supported enhanced production of tannase when used as carbon source and inducer they were not desired as suitable substrates since they are not economical and not easily available. With respect to the natural carbon source, maximal tannase activity was obtained using garcinia leaf and seawater without an inducer (26.2090U/ml), followed by garcinia leaf and Czapek Dox medium (22.059U/ml) after 48 h of fermentation. Hence, garcinia leaf was selected as the potential substrate for tannase production.

5.2 Optimization of bioprocess variables for tannase and gallic acid production by slurry state fermentation- statistical approach

Solid-state fermentation (SSF) is generally preferred for enzyme production owing to the fact that it allows production of highly concentrated crude enzymes with low costs for extraction of pure enzymes (Tao *et al*., 1997). Recently modified solid state fermentation (MSSF) was also proposed for gallic acid production as a better alternative. However, slurry state fermentation which is believed to offer a number of advantages over solid state and submerged

fermentation for certain enzyme production has not received much attention although the production medium is very simple with certain agro residues as substrates. In fact, enzyme production in “slurry state fermentation” (SLF) has been employed only in limited cases such as pectinase and in single cell protein production by *Aspergillus niger* and *Trichoderma viride* on pulps from lemon juice clarification (De Gregorio *et al.*, 2002). Other fermentation methods such as SSF, MSSF and SmF were reported for gallic acid with natural supports. Gallic acid accumulations with natural substrates were rarely studied with a few exceptional cases of SSF in tannin-rich desert plant (*Larrea tridentata* Cov) (Trevino-cueto *et al*., 2006). However slurry state fermentation utilizing a natural substrate was not tried for gallic acid so far for reasons unknown.

In the present study an attempt was made to explore the prospects of slurry state fermentation since the marine fungus *A.awamori* BTMFW 032 selected for the study produced enormous spores even during the early stages of solid state fermentation and consequent lack of extra cellular tannase activity. Further from the available literature it was realized that no attempt was made by earlier investigators to evaluate slurry state fermentation for cultivation and tannase and gallic acid production by terrestrial *A. awamori.* In the present study efforts were made to utilize natural substrates that contained tannic acid as an inducer cum substrate for tannase production as well sea water as cultivation medium. It was for the first time, garcinia leaf, known for its medicinal value, was used as a substrate for the production of tannase under slurry state fermentation. Results obtained in this present study indicated scope for utilizing garcinia as a potential substrate for tannase production due to its content of utilizable tannin. *A. awamori* which has a marine origin was able to produce tannase enzyme in a very short time. Hence the process parameters that influence tannase and gallic acid production during fermentation were optimized. It was observed that garcinia leaves alone could induce tannase production by the fungus in Czapek Dox medium and in sea water

suggesting its potential for use as a substrate for tannase production. Probably the tannin in garcinia could have attributed to the enhanced tannase enzyme production by *A. awamori.* The data also testified the positive role played by the sea water as a medium for enhanced production of tannase. Obviously the various ions and inorganic salts present in the sea water could have influenced positively the fungus by satisfying its micro requirements for enhanced synthesis of tannase. Of course a detailed study is warranted to establish this fact. The data further indicated that in spite of the fact that natural substrates could induce tannase production; the organism required tannic acid in the medium for enhanced synthesis confirming the inducer role of tannic acid for tannase enzyme.

Plackett-Burman design offers an effective screening procedure and computes the significance of a large number of factors in one experiment, which is time saving and maintains convincing information on each component (Sharma and Satyanarayana, 2006). From the results obtained with Plackett-Burman design it was inferred that among the eighteen variables evaluated only five variables namely garcinia leaf, tannic acid, glucose, temperature and inoculum were found to be the most significant variables. Effect of individual parameters studied in Plackett-Burman design testified that tannic acid and inoculum concentration had a positive effect in enhancing enzyme production, along with the increase in their concentrations. Whereas glucose, temperature, and garcinia leaves had a negative effect on enzyme production along with the increase in the variable. Experimental data on the effect of five selected physico-chemical factors on production of tannase by *A. awamori* in a total of 46 experiments showed strong dependence on the presence and levels of selected factors as the enzyme production varied between 37.4U/ml and 76.79 U/ml under experimental conditions studied. The pair wise interactions among the factors in terms of tannase production under the optimized condition were assessed by examining the response surfaces. A total of 18 variables were checked and out of these five parameters were optimized by

reported earlier. Hadi *et al*., (1994) reported maximum enzyme production of 6.12U/ml at 2 % tannic acid by *R. oryzae,* while Bardoo *et al*., (1997), recorded a concentration of 2 % tannic acid as optimum for tannase production by *A. japonicas.* Aguilar *et al*., (2001b) reported that the tannase secretion in submerged fermentation was initially supported at a concentration of 50g/l from *A. niger* Aa-20. Earlier investigators have recorded 5 % tannic acid as the optimum (Aoki *et al*., 1976; Lekha and Lonsane 1997; Sharma *et al*., 2007).

It has also been reported that tannase is produced during the primary phase of growth and declines thereafter (Rajkumar and Nandy, 1983; Sharma *et al*., 2007). The results of the present study are in agreement with those observations particularly in attaining a maximum productivity at an incubation period of 36 h. Kar and Banerjee, (2000) also reported 48 h as optimum incubation h for tannase production. The increase in enzyme yield was seemed to be parallel with specific activity which indicates the efficiency of designed medium in industry. Thus the present work has proved that response surface methodology could be used as a valuable and dependable tool for the optimization of tannase enzyme production and simultaneous gallic acid production from *A. awamori.*

Comparative evaluation of the designed media for tannase production and gallic acid production of an industrial strain of tannase enzyme *A. oryzae* (ATCC9362*)*, a terrestrial strain of *A. awamori (*ATCC44733) and the marine *A. awamori* of present study indicated the potentiality of this marine organism and efficiency of the medium designed. Two major differences were found on comparison of submerged and solid state conditions. Tannase yield and productivity were higher in SSC than in SMC and tannase location under SSC conditions was mostly extracellular, whilst it is bounded to the mycelium under SMC conditions (Belmares *et al.*, 2004).Tannase location during its production by SMC depends on the cultivation time. It is mainly intracellular at the beginning of

upon the tannins present in the natural substrates and resulted in the production of gallic acid and glucose. But the same could not be confirmed since production of any other intermediate in shikimate pathway may delay gallic acid production. Hence in the case of production of gallic acid using natural substrates there is a need to optimize the parameters that influence the production of gallic acid individually and it cannot be taken as function of tannase production. Hence a further optimization was conducted for gallic acid production utilizing garcinia leaf as substrate.

Experimental designs with statistical approach were applied to the optimization of cultural conditions for gallic acid production, a product of great importance, from *A. awamori*. First, Plackett-Burman design was used to evaluate the effects of variables, including concentrations of substrate (*Garcinia cambogia* leaf), seawater, pH, temperature, illumination, inoculum concentration, agitation, incubation period and media components like sodium nitrate, potassium chloride, magnesium sulphate, zinc sulphate, copper sulphate, ferrous sulphate, di potassium hydrogen phosphate, calcium chloride, glucose and tannic acid on gallic acid production. The optimum value of pH, temperature and inoculum concentration thus obtained were 3.5, 32ºC and 2 % respectively. Results obtained from the Plackett-Burman study indicated that among the eighteen variables evaluated, four factors namely tannic acid, seawater, zinc sulphate, and glucose had significant effect on the system and hence RSM was conducted in order to understand the interactive effect of seawater, tannic acid, glucose and zinc sulphate. The optimum values of the parameters thus obtained from the response surface methodology were seawater (73.58 %), glucose (7.94 mM), tannic acid (0.09%) and zinc sulphate (1.25mM). The subsequent verification experiments confirmed the validity of the models. This optimization strategy led to a nearly 6-fold increase in the gallic acid production from 0.723µg/ml obtained in unoptimized media to 4.016µg/ml in Box Behnken design. From the results it was inferred that higher

concentration of tannic acid and seawater along with lesser concentrations of zinc sulphate and glucose can contribute to maximum gallic acid production. The search for gallic acid producers other than the synthetic substrate tannic acid, which is expensive and difficult to obtain in large scale, is an important aspect affecting the viability of production of bioactive substances by industrial fermentation. Among the substrates studied, garcinia leaf was recognized as a potential substrate for gallic acid production (0.723µg/ml) under unoptimized conditions.

5.3 Optimization of bioprocess variables for tannase and gallic acid production by submerged fermentation- statistical approach

From the available literature it is noted that liquid-surface and solid-state fermentation processes are mainly employed to produce the intracellular and extracellular type of tannase, respectively (Lekha and Lonsane, 1994). In order to fully exploit the potential of this enzyme for various applications, it is imperative to explore the possibility of enhancing its production by using more efficient production methods (Rana and Bhat, 2005). Hence in this study the process parameters were optimized for maximum enzyme and gallic acid production using a single media design adopting Plackett-Burman (PB) and Response Surface Methodology (RSM) with Box Behnken design. Among the 11 process variables evaluated which included tannic acid, sodium chloride, sodium nitrate, potassium chloride, magnesium sulphate, ferrous sulphate, dipotassium hydrogen phosphate, pH, inoculum, incubation period, and agitation, only tannic acid, sodium chloride, ferrous sulphate, dipotassium hydrogen phosphate, incubation h and agitation were identified as most critical parameters that influence maximal enzyme and gallic acid production. pH was not considered as a critical factor, as in RSM the lower level of pH along with higher level of tannic acid inhibited the growth of the organism and hence pH was taken as a constant factor as per the solution by software for maximum yield. The model predicted 4824.61U/ml of tannase enzyme activity and 136.206 µg/ml gallic acid after 48 h of incubation. After time course

experiment 5085U/ml tannase activity was obtained at 36 h of incubation and 372.6 µg/ml gallic acid was obtained after 84 h of incubation. The enzyme and gallic acid yield obtained under optimized conditions were nearly 15 times higher than that obtained under the unoptimized condition. It was observed that tannic acid which is used as inducer has very high influence on enzyme and gallic acid production and its higher concentration supported maximum yield. Even though the individual effect of tannic acid on the bioprocess increased along with concentration in Plackett Burman design, the interactive effect of tannic acid with other media components and process parameters like ferrous sulphate, sodium chloride, agitation and incubation showed an optimum at medium level of tannic acid concentration in response surface methodology.

In liquid submerged fermentation the organism produced maximum tannase with 2 % tannic acid (Banerjee *et al*., 2001). Results of the present study were similar to that report. According to Mahapatra and Banerjee, (2009) 2.5% tannic acid was most suitable for maximum tannase synthesis with *Hyalopus* sp. through SSF and enzyme production was depleted at higher concentration although the enzyme production was recorded only up to a concentration level of 15 % tannic acid. Whereas, Sabu *et al*., (2005b) reported that 5% tannic acid was suitable for tannase production by *A. niger* under SSF. At higher tannic acid concentration tannase activity was higher in SSF whereas it was repressed in submerged fermentation (Lekha and Lonsane, 1994).

The results of Box–Behnken experiments demonstrated that tannic acid, incubation period, ferrous sulphate, dipotassium hydrogen phosphate, agitation and sodium chloride based on Plackett –Burman design experiment had significant linear effects on the system. Tannic acid was found to have the most significant effect on tannase production. In the present study it was observed that a concentration of 2.6 % (w/v) of tannic acid in the media was found to be supportive than its lower and higher concentrations. Similar observations were

was sucrose concentrated and applied on gel chromatography. Yield was 93 % after sucrose concentration even though tannic acid was not completely removed. Gel filtration appears to be an excellent and gentle technique to avoid inactivation of enzymes during purification (Scopes, 1987). It is a non denaturing separation technique, based on hydrodynamic volume and molecular size of the protein facilitates complete recovery of enzymes (Lillehoj and Malik, 1989). Even though gel filtration resulted in enzyme separation as a single peak, some amount of tannic acid remained bound to the enzyme with a yield of 15.47 % activity, and with a16.6 fold purity. Another method of purification was proceeded in order to get rid of the tannic acid completely. The clarification of crude extract was standardized for the first time with acidic aluminium oxide having Brockmann activity leaving the enzyme colourless and transparent. Even though the yield was only 55% there was no other interference of complex formation between tannic acid and the enzyme. Ultra filtration is a pressure driven filtration of solutes through a membrane with a defined range of pore size and is used for purifying, concentrating and fractionating macromolecules (Brummer and Gunzer, 1987; Pohl, 1990). The molecules pass through the membrane and will be present in the ultrafiltrate. Larger molecules are retained and concentrated relatively to the starting solution. In the present study ultrafiltration proved very useful as a concentrating step. When 2 cut off membranes were used for concentrating the enzyme a total of 30 times concentration occurred for the sample. Even though the yield was very less in this case the enzyme was devoid of any other materials that forms complexes. This was then subjected to gel filtration chromatography with sephadex G-200, and a single peak was obtained. This purification strategy was adopted for the characterization of the enzyme as interference of any other ingredients may affect its characteristics. The molecular weight of the protein was calculated from standard graph plotted for K_{av} versus log molecular weights of standard proteins. From the K_{av} value the molecular weight of the enzyme for the fraction was found to be 232 kDa. A similar result was obtained with SDS PAGE

the culture and it is further secreted to the culture medium. However, up to 80 % of tannase remained bounded to the mycelium when the maximum overall tannase titer is attained (Rajakumar and Nandy, 1983). At higher tannic acid concentration tannase activity was higher in SSF whereas it was repressed in submerged fermentation (Lekha and Lonsane, 1994). Actually, tannic acid at higher concentration produces complexes with membrane protein of the organism thereby both growth and enzyme production may be inhibited (Banerjee *et al*., 2007). It is important to note that such higher tannase activity levels in SSC compared to SMC have been clearly associated with the concomitant production of proteolytic activities in the SMF (Aguilar *et al*., 2002; Viniegra-Gonzalez *et al*., 2003). In the present study it was observed that the enzyme is entirely extracellular in nature and a very negligible activity was seen intracellularly during submerged fermentation.

Gallic acid (3, 4, 5-trihydroxy benzoic acid) is a useful product with many applications. It is utilized in the production of trimethoxy benzaldehyde, which is used in ink industry, dye industry and most importantly pharmaceutical industry. In pharmaceutical industry 3, 4, 5 trimethoxy benzaldehyde is converted to trimethoprim, a broad spectrum antibiotic. A combination of trimethoprim and sulphonamide is effective against many otherwise resistant species of bacteria. Though technological advances have introduced a number of antibiotics in markets, trimethoprim is still very significant. In combination with sulphonamides, it is highly effective against many drug resistant species of bacteria. Of the total requirement of gallic acid of 8000 tons per year, 75 % of it is used in production of trimethoprim. It is very important to have an economical indigenous technology for its commercial production. Gallic acid can also be used as a raw material for manufacturing an intermediate for anti oxidants, preservatives like propyl gallate. Here in the present study gallic acid production from a cheap agro residue has been standardized and a marine fungus with unique characteristic property has been exploited for the production of gallic acid. At the same time gallic acid production along with tannase production has been optimized with tannic acid as sole carbon

source. Conventionally gallic acid is produced by acid hydrolysis of tannic acid. It involves acidification of tannic acid with sulphuric acid at a high temperature. This technology has several disadvantages as yield of gallic acid is very low at the same time purity is also less, later the impurities may interfere and precipitate along with the product. Gallic acid produced by that method will turn unsuitable as a raw material for the manufacturing of drug intermediates. Here in this study a method for removing impurities has been optimized by passing the mycelia free extract through a column of diatomaceous earth, and the gallic acid binds more to diatomaceous earth compared to other matrices used. NaCl at different concentrations were tried to elute the gallic acid from the column and 0.4 M NaCl was seemed to be apt for elution and the eluted gallic acid was extracted with ethyl acetate. The ethyl acetate was evaporated here to retain gallic acid in powder form but a vacuum distillation method to recover ethyl acetate could reduce the process cost. A double extraction method of gallic acid from culture filtrate using ethyl acetate and diethyl ether has been reported in which the gallic acid produced was identified by its R_f value (0.698) obtained in thin layer chromatography and melting point of gallic acid (Kar *et al*., 2003). The gallic acid produced by marine *A. awamori* was identified by its R_f value in thin layer chromatography (0.69), FT-IR, GC and HPLC. A comparative method with commercially available gallic acid was adopted in the identification methods. Presence of C=C, C-C, OH bonds identified by FT-IR represents the bonds present in the gallic acid structure. A significant peak was obtained in HPLC analysis supported by the same peak in the commercial sample of gallic acid.

5.4 Solid state fermentation

In fact solid state fermentation has been the choice of investigators whoever has attempted exo-enzyme production using fungi owing to its several advantages. In the case of tannase also the earlier investigators have only employed SSF. May be because of the fact that filamentous fungi are ideal for SSF processes and they

are best adapted for SSF. The hyphal mode of fungal growth and their good tolerance to low water activity and high osmotic pressure conditions offer fungi major advantages over unicellular microorganisms in the colonization of solid substrates and proper utilization of available nutrients (Raimbault, 1998). While in contrast, in the present study, no significant tannase production by the fungus could be observed under SSF. The main reason inferred for the lack of tannase production by the marine fungus *A. awamori* was interestingly the rapid sporulation expressed by the fungus when grown under SSF with in 48 h of incubation. This fungus is from sea water and may have a different adaptation mechanism to survive in saline environments. May be when grown under solid state fermentation, where the moisture level is very limited they could have undergone adaptation to slightly dry conditions by sporulation. Since the SSF process was a failure no further studies on optimization studies were performed. May be separate investigation is required to know the molecular mechanism involved in rapid sporulation by this marine fungus under SSF which has not been reported for any other fungi.

5.5 Purification

Partial purification studies on enzyme showed that the recovery of tannase was low when ammonium sulphate precipitation was attempted. The dialysed samples obtained after ammonium sulphate precipitation did not show any visible bands on gel electrophoresis. Similar results were reported for tannase precipitation by ammonium sulphate with low recovery (Aoki *et al*., 1976; Lekha and Lonsane, 1994 ; Naidu *et al*., 2008). Some tannases were reported to be inactivated by solvents like ethanol and acetone (Marco *et al*., 2009). In this study also it was observed that even though the protein was precipitated, enzyme activity was completely lost during solvent precipitation. So a method was standardized to remove tannic acid from the crude extract with dialysis against phosphate buffer. But whole of the tannic acid was not separated from the sample. Then the sample

Metals act as cofactors that help enzymes to express their full catalytic activities. Most of the enzymes require the presence of metal ions for the expression of their catalytic activity and consequently metal ion activation of enzyme reaction assumes importance in industrial biocatalysis towards achieving maximal catalytic efficiency. At low concentration, metal ions act as cofactors of many enzymes, thereby increasing the catalytic activity of the enzyme, whereas at high concentrations the catalytic activity is reduced. This may be due to the partial denaturation of the enzyme by the presence of excessive free ions in the enzyme extract. From the results it was evident that metal ions like Mg^{2+}, Zn^{2+}, Cu^{2+}, Hg^{2+}, Ba^{2+}, Li^{2+}, Cd^{+} and Al^{3+} inhibited the enzyme activity. The inhibitory effect of heavy metal ions is well documented in the literature (Vallieeb and Ulmer, 1972). Hg^{2+} may interact with-SH and S-S groups of proteins in a multitude of systems thereby causing conformational changes in proteins. It is known that ions of mercury react with protein thiol groups (converting them to mercaptides) and also react with histidine and tryptophan residues. Moreover, by the action of mercury, the disulfide bond could also be hydrolytically degraded. Further, the decreased activity in the presence of divalent cations could be due to the nonspecific binding or aggregation of the enzyme (Kar *et al*., 2003). Tannase from *A. oryzae* (Iibuchi *et al*., 1968) and *P. chrysogenum* (Rajkumar and Nandy, 1983) were heavily inhibited by Zn^{-2}, $Cu^{-2,}$ and $Fe^{-2.}$ Tannase from *A. niger* was strongly inhibited by Cu^{-2} and to a lesser extent by Fe^{-3} and Zn^{-2} ions at concentrations of 20mM (Barthomeuf *et al*., 1994). Ba^{-2}, Ca^{-2}, Zn^{-2}, Hg^{-2} and Ag^{-} inhibited tannase activity at 1.0 mM concentration whereas Fe^{-3}, Co^{-2} completely inhibited tannase activity at the same concentration (Kar *et al*., 2003). Whereas, in the present study tannase activity was significantly enhanced by Fe^{+3}, Na^{+} and at lower concentrations of K^{+}. Hamdy, (2008) studied the activity of *F. subglutinans* tannase in response to some metal ions, chelators or enzyme inhibitors. Ca^{2+}, K^{+}, Mg^{2+} and Mn^{+} had a stimulatory effect on tannase activity. On the other hand, the activity of *F. subglutinans* tannase was inhibited by Ag^{+} and Hg^{2+}, which are known to react with histidine

230 kDa. Even though SDSPAGE performed under non reducing condition yielded a single band with 230 kDa, under reducing condition single band was obtained with a molecular weight of 37.5 kDa. The molecular mass of native enzyme produced by *A. niger* PKL104, estimated by gelfiltration, was 160,000 Da and under reduced condition a single protein band corresponding to a molecular mass of 42,000Da was obtained indicating that subunits are held together by disulphide linkages which get disrupted on treatment with β mercaptoethanol there by resulting in different electrophoretic mobilities. It was concluded that native form of enzyme is a tertramer consisting of 4 subunits of about 42,000 Da (Lekha and Lonsane 1997). The molecular mass of tannase produced by *A.niger* in submerged fermentation was about 186,000 Da, which was made up of 2 sub units of identical molecular mass (Barthomeuf *et al*., 1994). Tannases from *A. oryzae* (Iibuchi *et al*., 1968) and *A. flavus* (Adachi *et al*., 1971) produced by submerged fermentation were reported to have a molecular weight of around 200 kDa.

Hatamoto *et al*., (1996, 1997) cloned and sequenced the gene-encoding tannase, and a structural study of the enzyme subunit from *A. oryzae* gave the possibility to manipulate the producer systems to increase and to improve the levels of tannase activity. According to them native tannase consisted of four pairs of the two subunits, forming a hetero-octamer with a molecular weight of about 300,000 Da. Most of the available literature on tannase purification also indicated that the enzyme is composed of several subunits. The zymography after SDS PAGE was not obtained as the enzyme became inactive in the presence of SDS. The zymogram of native enzyme clearly indicated the capability of the enzyme to hydrolyse tannic acid forming clearing zones. In native form the enzyme with tannic acid showed 2 bands with clear zones in the zymogram. Whereas only single band was obtained with the other purification method where whole of the tannic acid was removed, indicating the presence of certain complexes formed between tannic acid and enzyme with tannase activity. Further it was evident that tannic acid -enzyme complex has increased stability than in its separated form.

Tannase is a glycoprotein and a typical serine esterase containing a serine in the active site of the enzyme with a molecular weight of 186,000 Da, containing 43 % sugars (Rajkumar and Nandy, 1983). The secreted tannase of *F. subglutinans* was a glycoprotein with 7.18 % carbohydrate (Hamdy, 2008). The tannase obtained in the present had a pI value of 4.4. Similar pI values of 4.3. (Bathomoeuf *et al*., 1994) and 3.8 (Ramirez coronel *et al*., 2003) reported for tannase.

5.6 Characterization

The optimum temperature for the enzyme activity was 30°C, at which the enzyme activity was the highest. Similar observations were reported for tannase from *A. oryzae*, *Aspergillus* sp. and *Penicillium chrysogenum* (Iibuchi *et al*., 1968; Lekha and Lonsane, 1997). With further increase in temperature tannase activity decreased. A terrestrial *A. awamori* nakazawa tannase exhibited optimum activity at 35 °C (Mahapatra *et al*., 2005). The tannase produced by marine *A. awamori* was active over a wide range of temperature, and retained 43 % and 25 % of enzyme activity after 1 h at 80ºC and after 24 h at 60ºC, respectively.

The enzyme was active at acidic pH and one another peak was obtained in alkaline range also. The effect of pH on the enzyme activity is determined by the nature of the amino acids at the active site, which undergoes protonation and deprotonation, and by the conformational changes induced by the ionization of the amino acids. Enzymes are very sensitive to changes in pH and they function best over a very limited range, with a definite pH optimum (Sabu *et al*., 2005a). Any change in pH affects the protein structure and a decline in enzyme activity beyond the optimum pH could be due to enzyme inactivation or its instability. An earlier report concluded that tannase from the new isolate needed an acidic protein environment to be active, and fungal tannase is an acidic protein in general (Mahapatra *et al*., 2005). The acid protease produced by *R. oligosporus* was active between pH 1.5-5 with optimal activity at pH 2 (Ikasari and Mitchell, 1996). Xylanase activity in the crude culture filtrate of a marine fungal isolate showed a

pH optimum around 3.5 with a second peak of activity at pH 8.5 (Raghukumar *et al*., 2004). Earlier reports suggest that marine fungal enzymes show two optimum peaks for pH which could be attributed to its adaptive nature at high salt concentration of the sea water. Whereas the tannase enzyme of the present study showed stability only at pH 2. The activity of the enzyme at high acidic pH has potential for probable exploitation in various industrial applications such as tea cream solubilisation.

PMSF considerably inhibited tannase activity and only 4.85 % residual activity was present indicating the presence of a serine and cysteine residue in the catalytic site of *A. awamori*. A similar observation was made earlier with the PMSF inhibition of tannase activity in *A. niger* GH1 (Marco *et al*., 2009). PMSF was found to inhibit the tannase activity completely in dimorphic yeast *Arxula adeninivorans* confirming that tannase is a serine hydrolase which needs a serine residue for its catalytic function (Boer *et al*., 2009). Phenanthroline is capable of chelating an iron atom derived from the Fe-S cluster thereby distorting the structure. Generally Fe form a complex with 1, 10-O- phenanthroline, there by inhibiting the activity (Boumans *et al*., 1997). In this present study ferrous sulphate supported increased enzyme productivity considerably and phenanthroline showed inhibitory effect. These results strongly suggest the presence of a Fe group in the active site of this enzyme.

In the present study tannase was inhibited by sodium deoxy cholate. The cholates are effective cholesterol solubilizers and cholesterol enzyme activators due to the common steroidal like backbone structure of these surfactants and cholesterol. Enzyme inhibitory characteristics can vary with factors such as pH and ionic strength. This inhibition is likely to be a general phenomenon related to desorption of the tannase from its substrate occurring after a change in the interfacial quality due to the detergent.

hydrophobic substrates in water, co solvents may also serve as activators in applications. In this study among different solvents studied only DMSO has enhanced the activity to some extent. A full characterization of an enzyme should include an analysis of how variation of solvent will affect activity and specificity. It is generally agreed that enzymes are more active in very hydrophobic solvents.

5.7 Genetic characterization

In the present study an attempt was made to isolate the gene responsible for tannase production. The degenerate primers designed from the conserved regions of other *Aspergillus* sp. tannase gene sequences available in the literature were used to amplify the gene from the genomic DNA. A 1.2 kb PCR amplicon was obtained and the same was cloned in a pGEMT vector for later use as a probe for full length gene isolation. The amplicon was sequenced and a comparison of nucleotide sequence information and the deduced amino acid sequence with the known proteins from Genbank indicated that this gene could encode tannin acyl hydrolase. A total of 1.232kb of tannase gene sequence was obtained from the genomic clone and the sequence analysis revealed an open reading frame consisting of 1122bp (374 amino acids) of one stretch in -1 strand. The longest ORF shows homology to *Aspergillus* tannase. It was already reported that the gene isolated from genomic DNA of *Aspergillus* sp. was intronless (Hatamoto *et al*., 1996). Comparison of Tan 1 with other tannase in the data bank showed that it has higher similarity of 82 % identity with *Aspergillus niger* tannase (Tan Ani) mRNA complete cds, 77 % identity with *Aspergillus flavus* NRRL3357 tannase putative mRNA sequence, 77 % identity with *Aspergillus oryzae* DNA for tannase complete cds, 77 % identity with *Aspergillus oryzae* tannase partial cds and tannase partial sequences of *Aspergillus fumigates* and *Aspergillus terreus* with 76% and 74% identity respectively. This result confirms the unique nature of this tannase as it shows variability among other *Aspergillus* sp.

and tryptophan residues and convert protein thiol groups to mercaptides. Ba^{2+}, Co^{2+}, Cu^{2+}, Fe^{3+} and Zn^{+} also inhibited tannase activity while Na^{+} showed a non significant effect. The decreased activity in the presence of divalent cations could be due to the non-specific binding or aggregation of the enzyme or possibly due to the partial denaturation of the enzyme caused by excess free ions.

K_m and V_{max} were estimated by plotting the initial velocity data as function of the concentration of the substrate. K_m and V_{max} were recorded as 19 mM and 830 µmol/min respectively for methyl gallate as substrate. The K_m and V_{max} values were found to be 0.41×10^{-4} M and 11.03 µmol/min respectively (using methyl gallate as substrate) for *Aspergillus niger* GH1 tannase (Marco *et al.*, 2009). The values of kinetic constants (K_m and V_{max}) depend on the particular substrate used and the enzyme source. A wide range of values (2×10^{-5}-1.03×10^{-3}M) for K_m and V_{max} have been reported for tannases from several microorganisms (Bhardwaj *et al*., 2003). In this work, V_{max} values using methyl gallate was higher than those reported earlier by several authors (Bhardwaj *et al.*, 2003; Sabu *et al*., 2005 a ; Sharma *et al* ., 1999). Kasieczka-Burnecka *et al*., (2007*)* reported two cold-adapted extracelullar tannases, isolated from antarctic strain *Verticillium* sp. p9, which had K_m values of 3.65×10^{-3} and 2.43×10^{-3} M using methyl gallate as substrate and 5×10^{-4} and 3.88×10^{-3} M when TA was used as the substrate. Sharma *et al*., (1999) reported that the tannase from *Penicillium* had a K_m value of 3.2×10^{-2} M (using tannic acid as substrate) and 0.48×10^{-4} M (Rajakumar and Nandy, 1983). A K_m value of 1.03×10^{-3} M was obtained for *A. niger* ATCC 16620 tannase. The tannase from *Aspergillus awamori* of this study also exhibited kinetic constants similar to those reported for other tannases from microbial sources. However, its affinity to methyl gallate was high. A small K_m indicates high affinity, and a substrate with a smaller K_m will approach V_{max} more quickly. Very high substrate concentration values are required to approach V_{max}, which is reached only when substrate concentration is high enough to saturate the enzyme (Tropp

and Freifelder, 2007). At molar level the tannase enzyme used in this study seems to have more specificity towards methyl gallate used as substrate in assay through out the study compared to other known tannase substrates such as propyl gallate and tannic acid.

Surfactants are substances that can alter the conditions prevailing at interfaces, and because of their amphiphilic property, they have a tendency to accumulate at interfaces and to adsorb onto surfaces. They can modify the surface tension by dispersing the proteins to the hydrophobic extreme of the peptide and interacting with the aqueous medium by another extreme. Surfactants can denature the enzyme protein, and because of this reason, it is very important to describe the effect of surfactants on the enzyme (Marco *et al*., 2009). Tannase produced by marine *A. awamori* had an enhancement in activity in the presence of 0.4 % triton X, lower concentrations of tween 80, all concentrations of tween 20, while got inhibited with brij-35. Similar effects were reported with a xerophilic fungus *A. niger* GH1 (Marco *et al*., 2009). This inhibition may be the result of the combined effect of factors such as reduction in the hydrophobic interactions that play a crucial role in holding the protein tertiary structure and the direct interactions with the protein molecule.

Hydrogen peroxide (H_2O_2) enhances the tannase activity enormously up to 10 % (v/v) concentration as an oxidising agent, whereas the action of oxidizing agent on tannase has not been reported. An US Patent 6482450 (2003) describes a method for manufacturing cold water infusing leaf tea. The method involves fermenting tannase pre-treated dhool (macerated tea leaves) under solid-state conditions in the presence of hydrogen peroxide. The dried leaf product infuses in cold water to give good flavour and colour. The anti oxidant property has been increased and enhancement in activity was reported. The enhancement in activity of tannase in this study by addition of hydrogen peroxide is supported by the above mentioned report.

β-mercapto ethanol has significant inhibitory effect in enzyme activity suggesting it to be a serine hydrolase. DTT as a reducing agent inhibited enzyme activity indicating the presence of disulphide bonds in its structure. A similar result was obtained for *Penicillium* variable (Sharma *et al*., 2008). Thioglycolate can protect enzymes against inactivation by maintaining protein thiol groups in the reduced state. In this study tannase enzyme activity was enhanced by increasing the concentration of sodium thioglycolate as thiol groups may be maintained in a reduced state at higher concentrations.

EDTA is a potent inhibitor of metal-dependent enzymes, which is generally used as commercial inhibitor of proteases, where it acts by chelating metals like lead and zinc. More than 30% of inhibition of tannase activity was observed under standard assay conditions. A strong inhibitory effect of EDTA on *A. oryzae* tannase was reported (Iibuchi *et al*., 1972). Nonmodulatory effect of EDTA was reported on tannase from other *Aspergillus* sp. (Adachi *et al*., 1971; Barthomeuf *et al*., 1994; Bhardwaj *et al*., 2003).

The technological utility of enzymes can be enhanced greatly by using them in organic solvents rather than their natural aqueous reaction media. Studies over the past 15 years have revealed not only that this change in solvent is feasible, but also that in such seemingly hostile environments enzymes can catalyze reactions impossible in water, become more stable, and exhibit new behaviour such as 'molecular memory'. Of particular importance has been the discovery that enzymatic selectivity, including substrate, stereo, regio and chemoselectivity, can be markedly affected, and sometimes even inverted, by the solvent. Enzyme-catalysed reactions in organic solvents, and even in supercritical fluids and the gas phase, have found numerous potential applications, some of which are already commercialized.

Amithab *et al*., (2002) suggested that the activating effect of DMSO was related to small changes in the enzyme's structure resulting in an increase in its conformational flexibility. Thus, in addition to their use for solubilising

Chapter 6

SUMMARY AND CONCLUSIONS

Aspergillus awamori BTMFW 032 isolated from sea water of Cochin (Soorej, 2008), available as stock culture at Microbial Technology Lab, Department of Biotechnology, Cochin University of Science and Technology was used as the source for production of tannase enzyme. *Aspergillus oryzae* (ATCC9362), a commercial strain known for tannase production, and *Aspergillus awamori* (ATCC44733), a terrestrial strain (both obtained from NCIM, Pune, India) were also used. Several natural substrates were tried as carbon source, independently and in combination with tannic acid as inducer. Although tannic acid influenced tannase production significantly the fungus could produce tannase even in the absence of tannic acid in a medium prepared with garcinia.

Various physico-chemical parameters affecting tannase production by the fungus, under different modes of fermentations (slurry state fermentation, submerged fermentation and solid state fermentation), were optimized towards maximal enzyme production.

Among the eighteen variables evaluated for tannase production under slurry state fermentation, employing Plackett –Burman design (PB), only five variables namely garcinia leaf, tannic acid, glucose, temperature and inoculum concentration were recorded as significant variables. Further studies conducted using Response surface methodology (RSM) for optimization of tannase production under slurry state fermentation utilizing garcinia as natural substrate suggested that three linear and six quadratic terms were significant model terms for

The phylogram which evidence the phylogenetic relationship of various *Aspergillus tannases* with that of marine *Aspergillus awamori* Tan1 suggest that it belongs to the cluster of *Aspergillus niger*. *Aspergillus awamori* is a filamentous fungus, widely used for brewing Japanese traditional spirits, and is genetically very close to *Aspergillus niger* and close to *Aspergillus oryzae* (Machida *et al*., 2009). Genscan predicted a sequence of 338 amino acids, which is an internal exon as the predicted peptide with out introns. Expasy Protopram predicted the molecular weight of protein to be 37.8 kDa and theoretical pI 4.31 which correlates with the protein characteristics as evidenced from SDS PAGE and Isoelectric point. GOR secondary structure prediction indicated the presence of alpha helix, extended strand and random coil and no beta structures. Presence of beta structures in tannase from *Aspergillus awamori* was reported (Mahapatra *et al*., 2005) and the observation made in the present study suggests that tannase obtained from marine *Aspergillus awamori* is a variant. Motif scan indicated the presence of all the motifs already reported to be present in tannase genes except the presence of basophilic serine threonine group evidencing the acidic nature of the protein.

5.8 Application studies

Preparation of iced tea involves the use of instant tea and the rapid solubility of tea in cold water (Coggon *et al*., 1975). Black tea extracts when concentrated become turbid on cooling. This turbidity is caused by precipitated tea material which is referred as ‘Tea cream’ in trade (Takino, 1976). The tea cream is a hydrogen bonded complex formed between polyphenolic compounds of black tea, especially the theaflavins and thearubigins with caffeine (Wickremansinghe, 1978). Clouding or creaming has a serious obstacle in the preparation of instant tea and its acceptability for use in ice cold tea (Takino, 1976). The earlier methods of removal of tea cream involved addition of small amounts of proteins or poly vinyl pyrrolidone to tea extracts to improve tea cream precipitation which was then separated and discarded (Sanderson *et al*., 1972). In chemical process for tea cream

solubilisation, tea cream solids were treated with water soluble sulphites or with oxidizing agents under alkaline condition followed by bleaching to the desired colour intensity with hydrogen peroxide (Barch, 1964). Removal of tea cream results in loss of large amounts of black tea colour, flavor components and physiologically active principle of tea such as theaflavins, thearubigins and caffeine (Sanderson *et al*., 1972). The more elegant process is the enzymatic solubilisation of tea cream solids with tannase, which catalyses the hydrolysis of ester linkages between galloyl groups and various polyphenolic compounds known to be present in tea extract (Sanderson and Coggon, 1974). The effect of this de-esterification is to enhance the natural levels of gallic acid and epicatechin, which in turn favours the formation of large amounts of epitheaflavic acid, which is responsible for the bright reddish tea colour and has high cold water solubility (Sanderson and Coggon, 1974). The natural flavor, colour and desirable tea astringency is retained and improved (Takino, 1976). The antioxidant activity of the tea was also reported to increase after tannase treatment (Mai *et al*., 1990) Tannase treatment of tea cream has been reported to increase the iron and calcium solubility, thereby improving the mineral availability (Jackson and Lee, 1988).

Efficiency of tannase produced by *A.niger* PKL104, for solubilisation of tea cream solids was reported (Lekha and Lonsane, 1994). Partially purified enzyme with an activity of 600U could solubilise 30.4 % tea cream, and after optimization of temperature and enzyme concentration 92 % of solubilisation was obtained with 3000 U of enzyme at 35ºC with an incubation of 2 h. In this present study optimization of solubilisation was not conducted, and only the efficiency of partially purified enzyme on solubilisation was tested. It was recorded that 699 U of enzyme could give 60 % solubilisation within 1 h which could be increased by optimization of temperature, incubation time, and concentration of the enzyme. The advantages of enzymatic processing over the conventional methods are improved conversion in a short period of time without any interference of other chemicals.

Propyl gallate is used as a food addictive as it acts as an antioxidant. Gaathon *et al*., (1989) suggested that tannase catalyzed transesterification could produce propyl gallate and in recent years there have been considerable developments in the use of enzymes in low water systems for organic synthesis. Use of tannase for the synthesis of propyl gallate by direct transesterification of tannic acid using propanol itself as organic reaction media under low water conditions was reported (Sharma and Gupta, 2003). The tannase enzyme produced by marine *Aspergillus awamori* was checked for this property in this study. The identity of the product was tested with FT-IR and thin layer chromatography. A comparison was made with commercially available propyl gallate while evaluating the identification methods. As revealed by thin layer chromatography, even though not so pure some amount of propyl gallate was produced after 72 h of incubation. In FT-IR spectrum alkane (CH_2) groups were found. This observation showed correlation with the bonding pattern of commercially available propyl gallate run as standard. All other peaks responsible for C-O, C-H, C=C, O-H were visible at their appropriate range confirming the presence of propyl gallate and hence it is suggested that the enzyme is suitable for the production of propyl gallate by transesterification. However it should be stated that the method needs to be standardized for achieving maximum propyl gallate yield from minimum tannic acid.

More than 75% of the enzyme required the presence of metal ion activators to express the full catalytic activity. Results obtained suggested that tannase activity was significantly enhanced by Fe^{3+}, Na^{+} and at low concentrations of K^{+}. The oxidizing agent, hydrogen peroxide enhanced the tannase activity enormously upto10 % (v/v) concentration. Significant inhibitory effect was recorded in the presence of β-mercapto ethanol and Di thiothreitol. Sodium thioglycolate enhanced the tannase activity. All the solvents at different concentrations reduced the enzyme activity except DMSO at its lower concentration. More than 30 % of the activity was inhibited in the presence of chelator EDTA.

DNA isolation was standardized for the strain and the degenerate primers were designed for tannase. PCR amplification yielded an amplicon of 1.2 kb for tannase which was cloned in pGEMT vector and transformed with DH5α as host cell. The insert was confirmed by restriction digestion with *EcoR*1 and amplification with degenerate primers. The insert was further sequenced and confirmed as tannase gene. The comparison of nucleotide sequence information and the deduced amino acid sequence with the known proteins from Genbank indicated that this gene encode tannin acyl hydrolase. A total of 1.232kb of tannase gene sequence was obtained from the genomic clone and sequence analysis revealed an open reading frame consisting of 1122bp (374 amino acids) of one stretch in -1 strand. The longest ORF shows homology to *Aspergillus* tannase. Comparison of Tan 1 with other tannase in the data bank showed that it has higher similarity of 82% identity with *Aspergillus niger* tannase (Tan Ani) mRNA complete cds, 77% identity with *Aspergillus flavus* NRRL3357 tannase putative mRNA sequence, 77% identity with *Aspergillus oryzae* DNA for tannase complete cds, 77% identity with *Aspergillus oryzae* tannase partial cds and tannase partial sequences of *Aspergillus fumigates* and *Aspergillus terreus* with 76% and 74%

the response. A threefold increase in tannase activity (75.2U/ml) was obtained after 24 h of incubation during time course experiment.

With regard to gallic acid production during slurry state fermentation the results obtained in Plackett–Burman design indicated that among the eighteen variables screened; only four factors namely, tannic acid, seawater, zinc sulphate and glucose were found to be the most significant variables. A 6 fold increase in gallic acid (4.016µg/ml) production was obtained after 36 h of fermentation after optimization studies carried out with PB and RSM under slurry state fermentation utilizing garcinia as natural substrate.

Optimization of variables for simultaneous tannase and gallic acid production under submerged fermentation with tannic acid as sole carbon source was performed with Plackett- Burman and Response surface methodology. From the results obtained in Plackett –Burman design it was inferred that among the eleven variables six factors namely tannic acid, incubation period, ferrous sulphate, dipotassium hydrogen phosphate, agitation and sodium chloride were found to be the most significant variables. A 15 fold increase in tannase (5085 U/ml) and gallic acid (372.6µg/ml) production were obtained after 36 h and 84 h of incubation respectively. Comparative evaluation of the designed media for tannase and gallic acid production with *Aspergillus awamori*, a terrestrial strain and *Aspergillus oryzae* used in industry for tannase production, evidenced the suitability of the designed medium for tannase production.

A method for removing impurities was optimized by passing the mycelia free extract through a column of diatomaceous earth. Gallic acid got bound more easily to diatomaceous earth compared to other matrices used. Among the different concentrations of NaCl tried to elute the gallic acid from the column, 0.4 M NaCl supported maximal elution of gallic acid which was later extracted with

ethyl acetate. Gallic acid was retained as powder after evaporation of ethyl acetate. The gallic acid was identified by its R_f value in thin layer chromatography (0.69), FT-IR, GC and HPLC. A comparative analysis was also performed with commercially available gallic acid during its identification. Presence of C=C, C-C, OH bonds identified by FT-IR represented the bonds present in the gallic acid structure. Significant peaks obtained for the test sample in HPLC and GC analysis were very much same obtained with the commercial sample of gallic acid, confirming the identity of gallic acid produced during fermentation.

Enzyme purification was carried out employing ammonium sulphate precipitation followed by chromatography. Precipitation with ammonium sulphate did not give satisfactory enzyme activity. Thus even at 80 % salt concentration only 32 % yield was obtained. In solvent precipitation methods even though protein was precipitated the activity was lost and hence the method was not found to be significant. Dialysis of crude extract through dialysis membrane was standardized to get rid of the tannic acid present in the crude extract. The extract was later sucrose concentrated and then passed through sephadex G200 column. This resulted in 16.6 fold purification of the enzyme. But still certain amount of tannic acid was found to form complex with the protein and hence a new protocol was standardized for complete removal of tannic acid. In this method crude extract was treated with acidified alumina, passed through ultrafiltration membranes Amicon ultra 30 kDa cutoff first and later through 100 kDa cut off. The ultrafiltrate above 100 kDa was then passed through sephadex G200 column which yielded 6.33 fold of purification without tannic acid. But it was observed that the stability of enzyme was very less when whole of the substrate was removed. The enzyme concentrate obtained after ultrafiltration through ultra filtration membrane showed a single band of about 230 kDa in SDS PAGE, and a single smiling band in NATIVE PAGE which testified the presence of enzyme substrate complexes present in its native form since the activity staining also gave

similar results as to that of native PAGE. In reducing gel all of the protein was reduced and formed a band of 37.5 kDa size. Elution profile of tannase on sephadex G200 gave a single peak and the molecular weight was determined as 232 kDa. Glycoprotein staining gave purple band which evidenced the enzyme's conjugated protein nature. Esterase plate assay yielded a clearing zone documenting the esterase activity of the enzyme after 1 h of incubation. Phenol-sulphuric acid assay using tannase enzyme indicated that it was glycosylated to 8.02% and the pI was 4.4.

Effect of pH on purified tannase was determined in different buffers from pH 1 to pH 10. Maximum activity was observed at pH 2 (156.3365U/ml) and one another peak was obtained at pH 8 while the stability of the enzyme was retained at pH 2 up to 24 h of incubation and hence the enzyme is considered to be active at extreme acidic condition.

Effect of temperature on the activity of purified tannase was determined at various incubation temperatures (5ºC to 100ºC) and 30ºC was found to be the optimum temperature although the enzyme was active over a wide range of temperatures. Maximum temperature stability was also observed at 30ºC up to 24 h. At molar levels tannase was observed to have more specificity towards methyl gallate as substrate in assay throughout the study when compared to other known tannase substrates.

The K_m and V_{max} were estimated by plotting the initial velocity data as the function of the concentration of the substrate. K_m was obtained as 19mM and V_{max} 830µmol/min. Inhibition of enzyme activity was recorded at higher concentrations of detergents like triton X, tween 80 and brij35. Complete inhibition of tannase activity was observed in the presence of 1,10-o-phenanthroline and sodium deoxy cholate.

Aissam, H., Errachidi, F., Penninckx, M. J., Merzouki, M., and Benlemlih, M. (2005): Production of tannase by *Aspergillus niger* HA37 growing on tannic acid and Olive Mill Waste Waters. *World journal of Microbiology and Biotechnology* **21**, 609-614.

Aissam, H., Errachidi, F., Merzouki, M., and Benlemlih, M. (2002): Identification des levures isolées des margines et étude de leur activité catalase. *Cahiers de l'Association Scientifique Européenne pour l'Eau et la Santé* **7**, 23-30.

Albertse, E.K. (2002): Cloning, expression and characterization of tannase from *Aspergillus* species. M.Sc. thesis, Faculty of Natural and Agricultural Sciences, Department of Microbiology and Biotechnology,University of the Free State Bloemfontein, South Africa.

Amitabh, C.S., Richele, T., John, C., and Robert M.K., (2002): Structural and catalytic response to temperature and cosolvents of carboxylesterase EST1 from the extremely thermoacidophilic archaeon *sulfolobus solfataricus* P1. *Biotechnology and Bioengineering* **80**, 784-793.

Aoki, K., Shinke, R., and Nishira, H. (1976): Purification and some properties of yeast tannase. *Agricultural and Biological Chemistry* **40**, 79–85.

Aoki, K., Tanaka, T., Shinke, R., and Nishira, H. (1979): Detection of tannase in polyacrylamide gels. *Journal of Chromatography* **170**, 446–448.

Ayed, L., and Hamdi, M. (2002): Culture conditions of tannase production by *Lactobacillus plantarum. Biotechnology Letters* **24**, 1763-1765.

Bajpai, B., and Patil, S. (2008): A new approach to microbial production of gallic acid. *Brazilian Journal of Microbiology* **39**, 708-711.

Bajpai, B., and Patil, S. (1997): Introduction of tannin acyl hydrolase (EC 3.1.1.20) activity in some members of *fungi imperfecti*. *Enzyme Microbial Technology* **20**, 612–614.

Bajpai, B., and Patil, S. (1996): Tannin acyl hydrolase activity of *Aspergillus, Penicillium, Fusarium* and *Trichoderma. World Journal of Microbiology and Biotechnology* **12,** 217-220.

Ballou, C.E. (1982): The Molecular Biology of the Yeast *Saccharomyces cerevisiae: Metabolism and Gene Expression*. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp. 335.

Banerjee, R., Mukherjee, G., and Patra, K.C. (2005) : Microbial transformation of tannin-rich substrate to gallic acid through co-culture method. *Bioresource Technology* **96**, 949–953.

Banerjee, D., Mahapatra, S., and Pati, B. R. (2007): Gallic acid production by submerged fermentation of *Aspergillus aculeatus* DBF9. *Research Journal of Microbiology* **2**, 462-468.

Banerjee, D., Mondal, K.C., and Bikas, R. (2001): Production and characterization of extracellular and intracellular tannase from newly isolated *Aspergillus aculeatus* DBF9. *Journal of Basic Microbiology* **6**, 313–318.

identity respectively. The phylogram which indicates the phylogenetic relationship of various *Aspergillus* tannases with that of marine *Aspergillus awamori* Tan1 revealed that it belongs to the cluster of *Aspergillus niger*. Genscan predicted a sequence of 338 amino acids, which is an internal exon as the predicted peptide is without introns. Expasy Protopram predicted the molecular weight of protein to be 37.8 kDa and theoretical pI 4.31 which correlated with the protein characteristics as evidenced from SDS PAGE and Isoelectric focussing. GOR secondary structure prediction indicated the presence of alpha helix, extended strand and random coil and no beta structures. Motif scan suggested the presence of all the motifs present in already reported tannase genes except the presence of basophilic serine threonine group.

The efficiency of partially purified enzyme on tea cream solubilisation was tested and 699U of enzyme could give 60% solubilisation with in 1h which could be increased by optimization of temperature, incubation time and concentration of the enzyme.

The use of tannase for the synthesis of propyl gallate by direct transesterification of tannic acid using propanol itself as organic reaction media under low water conditions has been established. The tannase enzyme produced by marine *A. awamori* was checked for this property in this study. The identity of the product was tested with FT-IR and thin layer chromatography. A comparative analysis was performed with commercially available propyl gallate. Results obtained from thin layer chromatography, clearly evidenced production of some amount of propyl gallate even though not so pure, after 72 h of incubation. Evidence for presence of alkane (CH_2) group in FT-IR spectrum obtained for the test sample shared the same bonding pattern obtained for the commercially available propyl gallate which was run as standard. All other peaks responsible for C-O, C-H, C=C, O-H were visible at their appropriate range confirming the

presence of propyl gallate. These observations strongly indicate the suitability of the enzyme for the production of propyl gallate by transesterification.

Based on the results obtained from the present study it is concluded that the marine fungus *Aspergillus awamori* BTMFW 032 has potential for industrial production of extracellular tannase. Garcinia a natural substrate has scope for use as the ideal source of tannin for maximal tannase production. The characteristics of tannase recognized during the course of this study revealed the extreme acidic nature of the enzyme which has immense scope for exploitation in several applications. Further the stability of the enzyme observed over a wide range of temperature and other characteristics observed with the enzyme adds further evidence to its potentiality for industrial applications. Further studies on tea cream solubilisation and synthesis of propyl gallate testify the potentiality of the enzyme for exploitation in respective industries. The results of the present study strongly indicate ample scope for further research on the biochemistry of the enzyme, structure elucidation and enzyme engineering towards a wide range of applications, besides enriching the knowledge on marine enzymes. Of course there is option for cloning and expressing the gene encoding this tannase enzyme into a domesticated host and expression of the enzyme for industrial production. It may be noted that this is the first report on tannase as well as gallic acid production by a marine fungus.

Chapter 7

REFERENCES

Abdel-Naby, M.A., Sherif, A.A., El-Tanash, A.B., and Mankarios, A.T. (1999): Immobilization of *Aspergillus oryzae* tannase and properties of the immobilized enzyme. *Journal of Applied Microbiology* **87**, 108–114.

Adachi, O., Watanabe, M., and Yamada, H. (1971): Physicochemical properties of the tannase from *Aspergillus flavus*. *Agricultural Biology and Chemistry* **32**, 1079-1085.

Adachi, O., Watanabe, M., and Yamada, H. (1968): Studies on fungal tannase. Part II. Physicochemical properties of tannase of *Aspergillus flavus*. *Agricultural Biology and Chemistry* **32**, 1079–1085.

Aguilar, C. N., and Gutiérrez-Sánchez, G. (2001): Review: Sources, properties, applications and potential uses of tannin acyl hydrolase. *Food Science and Technology* **7**, 373-382.

Aguilar, C. N., Rodriguez, R., Gutierrez-Sanchez, G., Augur, C., Favela-Torres, E., Prado-Barragan, L. A., Ramirez-Coronel, A. and Contreras-Esquivel., J. C. (2007): Microbial tannases: Advances and perspectives. *Applied Microbiology and Biotechnology* **76**, 47-59.

Aguilar, C.N. (2000): Induction and Repression of Synthesis of Tannase from *Aspergillus niger* Aa/20 in Submerged and Solid State Cultures. Mexico: Metropolitan Autonomous University; Ph.D Thesis, (in Spanish).

Aguilar, C.N., Augur, C., Favela-Torres, E., and Viniegra-González, G. (2001a): Induction and repression patterns of fungal tannase in solid-state and submerged cultures. *Process Biochemistry* **36**, 565-570.

Aguilar, C.N., Augur, C., Favela-Torres, E., and Viniegra-González, G. (2001b): Production of tannase by *Aspergillus niger* Aa-20 in submerged and solid state fermentation: influence of glucose and tannic acid. *Journal of Industrial Microbiology and Biotechnology* **26**, 296–302.

Aguilar, C.N., Augur, C., Viniegra-González, G., and Favela-Torres, E. (1999): A comparison of methods to determine tannin acyl hydrolase activity. *Brazilian Archives of Biology and Technology* **42**, 355–361.

Aguilar, C.N., Favela-Torres, E., Viniegra-González, G., and Augur, C. (2002): Culture conditions dictate protease and tannase production in submerged and solid-state cultures by *Aspergillus niger* Aa-20. *Applied Biochemistry and Biotechnology* **102**, 407–414.

García-Peña, I., Augur, C., and Favela-Torres, E. (1999): Partial purification of *Aspergillus niger* tannase produced by solid state culture (in Spanish). In: Prado A, Huerta S, Rodríguez G, Sancedo G (eds) *Advances in enzyme purification and applied biotechnology*.Ediciones UAM-Iztapalapa, Mexico, DF, pp. 247–261, 2207–2212.

Giovanelli, G. (1989): Enzymatic treatment of malt polyphenols for stabilization.*Ind. Bevande* **18**, 497 –502.

Godfrey, T., and West, S. (1996): Introduction to Industrial Enzymology, In Godfrey, T, and West, S. (Eds.): *Industrial Enzymology* (2nd Edn), New York. Stockton Press. pp. 1-8.

Goel, G., Puniya, A.K., Aguilar, C.N., and Singh, K. (2005): Interaction of gut microflora with tannins in feeds. *Naturwissenschaften* **92**, 497–503.

Govardhan, C.P., and Margolin, A.L. (1995): Extremozymes for industry – from nature and by design. *Chemistry and Industry* **4**, 689-693.

Gupta, N., Mehra, G., and Gupta R. (2004): A glycerol inducible thermostable lipase from *Bacillus* sp: Medium optimization by a Plackett- Burman design and by response surface methodology. *Canadian Journal of Microbiology* **50**, 361-368.

Gupta, R., Bradoo, S., and Saxena, R.K. (1997): Rapid purification of extracellular Tannase using polyethylene glycol – tannic acid complex. *Letters in Applied Microbiology* **24**, 253 – 255.

Gustavo, A.S.P., Selma, G.F., Leite, S.C., Terzi, S.C. (2001): Selection of tannase-producing *Aspergillus niger* strains. *Brazilian Journal of Microbiology* **32**, 24–26.

Hadi, T.A., Banerjee, R., and Bhattarcharyya, B.C. (1994): Optimization of tannase biosynthesis by a newly isolated *Rhizopus oryzae*. *Bioprocess Engineering* **11**, 239–243.

Hagerman, A.E., and Butler, L.G. (1976): Protein precipitation method for the quantitative determination of tannins. *Journal of Agricultural Food Chemistry* **26**, 809–812.

Hamdy, H.S. (2008): Purification and Characterisation of a Newly Isolated Stable Long-Life Tannase produced by *F. subglutinans* (Wollenweber and Reinking) Nelson et al. *Journal of Pharmaceutical Innovations* **3**, 142–151.

Haslam, E., and Tanner, J.N.(1970): Spectrophotometric assay of tannase. *Phytochemistry* **90**, 2305–2309.

Hatamoto, O., Watarai, T., Kikuchi, M., and Sekhin, H. (1996): Cloning and sequencing of the gene encoding tannase and a structural study of the tannase subunit from *Aspergillus oryzae*. *Gene* **175**, 215–221.

Hatamoto, O., Watarai, T., Kikuchi, M., Mizusawa, K., and Sekine, H. (1997): Erratum to: 'cloning and sequencing of the gene encoding tannase and a structural study of the tannase subunit from *Aspergillus oryzae*' [*Gene* 175 (1996) 215–221], *Gene* **186**, 1–153.

Banerjee, S.D. (2005): Microbial production of tannase and gallic acid. Ph.D. Thesis, Vidyasagar University, India.

Barch, W.E. (1964): US Patent 3 163 539.

Barthomeuf, C., Regerat, F., and Pourrat, H. (1994): Production, purification and characterization of a tannase from *Aspergillus niger* LCF8. *Journal of Fermentation and Bioengineering* **77**, 320-323.

Bate-Smith, E.C., and Rasper, V. (1969): Tannins of grain sorghum: luteoforol (leucoluteolinidin), 3',4,4',5,7-pentahydroxyflavan. *Journal of Food Science* **34**, 203-209.

Batra, A., and Saxena, R.K. (2005): Potential tannase producers from the genera *Aspergillus* and *Penicillium*. *Process Biochemistry* **40**, 1553–1557.

Battestin, V., and Macedo, G.A. (2007b): Partial purification and biochemical characterization of tannase from a newly isolated strain of *Paecilomyces variotii*. *Food Biotechnology* **21,** 207-216.

Battestin, V., and Macedo, G.A. (2007a): Tannase production by *Paecilomyces variotii*. *Bioresources Technology* **98**, 1832–1837.

Belmares, R., Contreras-Esquivel, J.C., Rodriguez-Herrera, R., Coronel, A.R., and Aguilar, C.N. (2004): Microbial production of tannase: an enzyme with potential use in food industry. *Lebensmittel-Wissenschaft und-Technologie - Food Science and Technology* **37**, 857-864.

Berry, D.R., and Paterson, A. (1990): Enzymes in Food Industry. In Enzyme Chemistry, Impact and applications, 2nd ed. C.J Suckling (Ed.). pp. 306-351.

Beverini, M., and Metche, M. (1990): Identification, purification and physiochemical properties of tannase of *Aspergillus oryzae*. *Scinces des Aliments* **10**, 807–816.

Bhardwaj, R., Bhat, T.K., Singh, B. (2003): Purification and characterization of tannin acyl hydrolase from *A. niger* MTCC-2425. *Journal of Basic Microbiology* **43**, 449–461.

Bhat, T.K., Singh, B., and Sharma, O.P. (1998): Microbial degradation of tannins—a current perspective. *Biodegradation* **25**, 343–357.

Boer, E., Bode, R., Mock, H.P., Piontek, M., and Kunze, G. (2009): Atan1p-an extracellular tannase from the dimorphic yeast *Arxula adeninivorans* : molecular cloning of the *ATAN1* gene and characterization of the recombinant enzyme. *Yeast* **26**, 323-337.

Boumans, H., van Gaalen, M.C., Grivell, L.A., and Berden, J.A. (1997): Differential inhibition of the yeast bc1 complex by phenanthrolines and ferroin. Implications for structure and catalytic mechanism. *The Journal of Biological Chemistry* **272**, 16753-16760.

Box, G. E.P., Hunter, W.G., and Hunter, J. S. (1978): *Statistics for Experimenters*. John Wiley & Sons, New York, USA, pp. 653.

Bradford, M.A.(1976): Rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Analytical Biochemistry* **72**, 248–254.

Bradoo, S., Gupta, R., and Saxena, R. (1996): Screening of extracellular tannase producing fungi: development of a rapid simple plate assay. *Journal of General and Applied Microbiology* **42**, 325–329.

Bradoo, S., Gupta, R., and Saxena, R.K. (1997): Parametric optimization and biochemical regulation of extracellular tannase from *Aspergillus japonicus. Process Biochemistry* **32**, 135–139.

Brummer, W., Gunzer, G. (1987): Laboratory techniques of enzyme recovery. In *Biotechnology*; Kennedy, J. F., Ed.; VCH: Weinheim, Germany, Vol. 7a, pp. 260-264.

Cerda-Gomez, A., Contreras-Esquivel, J.C., Reyes-Valdes, H., Rodríguez, R., Aguilar, C.N. (2006): Molecular characterization of *Aspergillus* strains producers of tannase. Proceedings of the second international congress on food science and technology in developing countries, Saltillo, Coahuila, Mexico.

Cerda-Montalvo, M.L., Contreras-Esquivel, J.C., Rodríguez-Herrera, R., and Aguilar, C.N. (2005): Glucose diffusion on support for solid state fermentation and its influence on tannase production profiles. *International Journal of Chemical Reactor Engineering* **3**, 1–10.

Chae, S., and Yu, T. (1983): Experimental manufacture of a corn wine by fungal tannase. *Hanguk Sipkum Kwahakoechi* **15**,326–332.

Chamkha, M., Patel, B.K.C., Traore, A., Garcia, J.L., Labat, M.(2002): Isolation from a shea cake digester of a tannin-degrading Streptococcus gallolyticus strain that decarboxylates protocatechuic and hydroxycinnamic acids, and emendation of the species. *International Journal of systematic and Evolutionary Microbiology* **52**, 939-944.

Chandrasekaran, M. (1997): Industrial enzymes from marine microorganisms: The Indian scenario. *Journal of Marine Biotechnology* **5**, 86-89.

Chandrasekaran, M., and Rajeev Kumar, S. (2002): Marine microbial enzymes in Biotechnology. In H.Doelle, and E.DaSilva (Eds): Encyclopedia of Life support systems (EOLSS), Developed under the Auspices of the UNESCO, Eolss Publishers, Oxford, UK.

Chaterjee, R., Dutta, A., Banerjee, R., and Bhattacharyya, B.C. (1996): Production of tannase by solid state fermentation. *Bioprocess Engineering***14**, 159–162.

Chhokar, V., Sangwan, M., Beniwal, V., Nehra, K.,and Nehra, K.S.(2009): Effect of Additives on the Activity of Tannase from *Aspergillus awamori* MTCC9299. *Applied Biochemistry and Biotechnology* **9,** 8813-8817.

Chung, K.T., Wong, T.Y., Wei, C.I., Huang, Y.W., and Lin, Y. (1998): Tannins and human health: a review. *Critical Reviews in Food Science and Nutrition* **38**, 421–464.

Coggon, P., Graham, N.H., and Sanderson, G.W. (1975): UK Patent 1,380,135.

Cruz-Hernández, M., Augur, C., Rodríguez, R., Contreras-Esquivel, J., Aguilar, C.N. (2006): Evaluation of culture conditions for tannase production by *Aspergillus niger* GH1. *Food Technology and Biotechnology* **44**, 541–544.

Cruz-Hernandez, M.A., Contreras-Esquivel, J.C., Lara, F., Rodríguez-Herrera, R. and Aguilar, C.N. (2005): *Zeitschrift für Naturforschung* **60**, 844-848.

De Gregorio, A., Mandalari, G., Arena, N., Nucita, F., Tripodo, M.M., and Lo Curto, R.B. (2002): SCP and crude pectinase production by slurry-state fermentation of lemon pulps, *Bioresource Technology* **83**, 89–94.

Deschamps, A.M., Otuk, G., and Lebeault, J.M. (1983): Production of tannase and degradation of chestnut tannin by bacteria. *Journal of Fermentation Technology* **61**, 55–59.

Deschamps, A.M.,and Lebeault, J.M. (1984): Production of gallic acid from tara (*Caesalpinia spinosa*) tannin by bacterial strains. *Biotechnology Letters* **6**, 237 – 242.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith. F. (1956): Colorimetric method for determination of sugars and related substances. *Analytical Chemistry* **28**, 350-356.

Egmond, M. R., and de Vlieg, J. (2000): *Fusarium solani pisi* cutinase. *Biochimie* **82**, 1015-1021.

Englard, S., and Seifter, S. (1990): Precipitation techniques. *Methods in Enzymology* **182**, 285-300.

Farias, G.M., Elkins, J.R., and Griffin, G.J. (1992): Tannase activity associated with growth of *Cryphonectria parasitica* on American and Chinese chestnut extracts and properties of the enzyme. *European Journal of Plant Pathology* **22**, 392 – 402.

Farias, G.M., Gorbea, C., Elkins, J.R., and Griffin, G.J. (1994): Purification, characterization, and substrate relationships of the tannase from *Cryphonectria parasitica*. *Physiology and Molecular Plant Pathology* **44**, 51–63.

Gaathon, A., Gross, Z., Rozhanski, M. (1989): Propyl gallate: enzymatic synthesis in a reverse micelle system. *Enzyme Microbial Technology* **11**, 604–609.

Gander, J.E. (1984): Gel Protein stains: Glycoproteins. *Methods in Enzymology* **104**, 447-449.

Ganga, P.S., Nandy, S.C., and Santappa, M. (1977): Effect of environmental factors on the production of fungal tannase. *Leather Science* **24**, 8–16

García-Conesa, M.T., Ostergaard, P., Kauppinen, S., and Williamson, G. (2001): Hydrolysis of diethyl diferulates by a tannase from *Aspergillus oryzae:* Breaking cross-links between plant cell wall polymers. *Carbohydrate Polymers* **44**, 319–324.

Mattiason, B., and Kaul, R. (1994): 'One – pot' protein purification by process integration. *Biotechnology* **12**, 1087 – 1089.

Micheli, P.A. (1729): Nova Plantarum Genera. Juxta tournefortii Methodum disposita. Florentia. Typis Bernardi Paperinii.

Mitchell, D.A., and Lonsane, B.K. (1992) In: Doelle,H.W.,Mitchell, D.A., Rolz, C.E. (Eds.), Solid Substrate Cultivation. Elsevier Science Publishers, London, pp. 1-16.

Mohapatra, P.K.D., Pati, B.R., and Mondal K.C.(2009):Effect of amino acids on tannase biosynthesis by *Bacillus licheniformis* KBR6. *Journal of Microbiology, Immunology and Infection* **42**, 172-175.

Mondal, K.C., Banerjee, D., Banerjee, R., and Pati, B.R. (2001b): Production and characterization of tannase from Bacillus cereus KBR9. *Journal of General and Applied Microbiology* **47**, 263–267.

Mondal, K.C., Banerjee, D., Jana, M., and Pati, B.R. (2001 a): Colorimetric assay method for determination of tannin Acyl hydrolase (EC. 3.1.1.20) activity. *Analytical Biochemistry* **295**, 168–171.

Mondal, K.C., Samanda, S., Giri, S., and Pati, B.R. (2001): Distribution of tannic acid degrading microorganisms in the soil and comparative study of tannase from soil fungal strain , *Acta Microbiologica Polonica* **50**,75-82.

Mudgett, R.E. (1986): Solid state fermentation. In: Demain AL, Solomon NA (eds) *Manual of industrial microbiology and biotechnology*. American Society of Microbiology, Washington DC, pp. 66–83.

Mueller – Harvey, I., Reed, J.D., and Hartley, L.D. (1987): Characterization of phenolic compounds, including tannins of ten ethiopian browse species by high performance liquid chromatography. *Journal of the Science and Food Agriculture* **39**, 1 – 14.

Mueller- Harvey, I, (2001): Analysis of hydrolysable tannins. *Animal Feed Science and Technology* **91**, 3-20.

Mukherjee, G., and Banerjee, R. (2004): Biosynthesis of tannase and gallic acid from tannin rich substrates by *Rhizopus oryzae* and *Aspergillus foetidus. Journal of Basic Microbiology* **44**, 42–48.

Mukherjee, G., and Banerjee, R. (2005): Effects of temperature, pH and additives on the activity of tannase produced by a coculture of *Rhizopus oryzae* and *Aspergillus foetidus*. *World Journal of microbiology and Biotechnology* **22**, 207-211.

Murugan, K., Saravanababu, S., and Arunachalam, M. (2007): Screening of tannin acyl hydrolase (E.C.3.1.1.20) producing tannery effluent fungal isolates using simple agar plate and SmF process. *Bioresource Technology* **98**, 946–949.

Naessens, M., Vercauteren, R., and Vandamme, E.J. (2004): Three-factor response surface optimization of the production of intracellular dextran dextrinase by *Gluconobacter oxydans*. *Process Biochemistry* **39**, 1299-1304.

Haworth, R. D., Jones, K., and Rogers, H. J. (1985): Ion–exchange chromatography of *Aspergillus niger* extract. *Proceedings of Chemical Society*, 8 – 9.

He, Y.Q., and Tan, T.W. (2006): Use of response surface methodology to optimize culture medium for production of lipase with *Candida* sp. *Journal of Molecular Catalysis B: Enzymatic* **43**, 9-14.

Hohmann, S. (1991): *PDC6*, a weekly expressed pyruvate decarboxylase gene from yeast, is activated when fused spontaneously under the control of the *PDC1*promoter. *Current Genetics* **5**, 373 – 378.

Huang, W., Ni, J., and Borthwick, A.G.L. (2005): Biosynthesis of valonia tannin hydrolase and hydrolysis of valonia tannin to ellagic acid by *Aspergillus* SHL 6. *Process Biochemistry* **40**, 1245–1249.

Ibrahim, H.M., Yusoff, W.M.W., Hamid, A.A., Illias, R.M., Hassan, O., and Omar, O. (2005): Optimization of medium for the production of β-cyclodextrin glucanotransferase using central composite design (CCD). *Process Biochemistry* **40**, 753-758.

Iibuchi, S., Minoda, Y., and Yamad, K. (1968): Studies on tannin acyl hydrolase of microorganisms. Part III. Purification of the enzyme and some properties of it. *Agricultural Biology and Chemistry* **32**, 803–809.

Iibuchi, S., Minoda, Y., and Yamada, K. (1967): Studies on tannin acyl hydrolase of microorganisms part III. Purification of the enzyme and some properties of it. *Agricultural and Biological Chemistry* **32**, 803–809.

Iibuchi, S., Minoda, Y., and Yamada, K. (1972): Hydrolyzing Pathway, Substrate Specificity and Inhibition of Tannin Acyl Hydrolase of *Asp. oryzae* No.7. *Agricultural Biology and Chemistry* **36**, 1553 – 1562.

Ikasari, L., and Mitchell, D.A. (1996): Leaching and characterization of *Rhizopus oligosporus* acid protease from solid-state fermentation. *Enzyme and Microbial Technology* **19**, 171-175.

Inoue, K.H., and Hagerman, A.E.(1988): Determination of gallotannin with rhodanine. *Analytical Biochemistry* **169**, 363-369.

Jackson, L. S., and Lee, Y. (1988): Chemical forms of iron, calcium, magnesium and zinc in black, oolong, green and instant black tea. *Journal of Food Sciences*, ***53***, 181–184.

Jacob, N., and Prema, P. (2006): Pectinases from Actinomycetes, *Food Technology and Biotechnology* **44**, 263–267.

Jean, D., Pourrat, H., Pourrat, A., and Carnat, A. (1981): Assay of Tannase (Tannin Acyl Hydrolase E.C. 3.1.1.20) by Gas Chromatography. *Analytical Biochemistry* **110**, 369 – 372.

Kar, B., and Banerjee, R. (2000): Biosynthesis of tannin acyl hydrolase from tannin rich forest residue under different fermentation conditions. *Journal of Industrial Microbiology and Biotechnology* **25**, 29–38.

Kar, B., Banerjee, R., and Bhattacharyya, B. C. (2003): Effect of additives on the behavioural properties of tannin acyl hydrolase. *Process Biochemistry* **38**, 1285-1293.

Kar, B., Banerjee, R., and Bhattacharya, B.C. (1999): Microbial production of gallic acid by modified solid-state fermentation. *Journal of Industrial Microbiology and Biotechnology* **23**, 173–177.

Kasieczka-Burnecka, M., Kuc, K., Kalinowska, H., Knap, M., and Turkiewicz, M. (2007): Purification and characterization of two cold-adapted extracellular tannin acyl hydrolases from an Antarctic strain *Verticillium* sp. P9. *Applied Microbiology and Biotechnology***77**, 77-89.

Kawakubo, J., Nishira, H., Aoki, K., and Shinke, R. (1993): Isolation of a gallic acid-producing microorganisms with sake cake medium and production of gallic acid. *Bioscience Biotechnology and Biochemistry* **57**, 1360-1361.

Kostinek, M., Specht, I., Edward, V.A., Pinto, C., Egounlety, M., Sossa, C., Mbugua, S., Dortu, C., Thonart, P., and Taljaard, L. (2007): Characterization and biochemical properties of predominant lactic acid bacteria from fermenting cassava for selection as starter cultures. *International Journal of Food Microbiology* **114**, 342–351.

Kumar, R., Sharma, J., and Singh, R. (2007): Production of tannase from *Aspergillus ruber* under solid state fermentation using jamun (Syzygium cumini) leaves. *Microbiological Research* **162**, 384-390.

Laemmlli, U. K. (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Laurence Du-Thumm., Szeles, L.H., Sullivan, R.J., Masters, J.G., and Robinson, R.S.(2005): Chewable antiplaque confectionery dental composition, US20050008582 dated 01/13/2005.

Lee, S.B., and Taylor, J.W (1990): Isolation of DNA from fungal mycelia and single spores. In M.A Innis, D.H. Gefland, J.J. Sninsky, and T.J.White (Eds):*PCR Protocols: A guide to methods and applications.* Academic Press San Diego, California, pp. 282-287.

Lekha, P. K., Ramakrishna, M., and. Lonsane, B. K. (1993): Strategies for isolation of potent culture capable of producing tannin acyl hydrolase in higher titres. *Chem Mikrobiol Technol Lebensm* **15**, 5-10.

Lekha, P.K., and Lonsane, B.K. (1994): Comparative titres, location and properties of tannin acyl hydrolase produced by *Aspergillus niger* PKL 104 in solid-state, liquid surface and submerged fermentations. *Process Biochemistry* **29**, 497-503.

Lekha, P.K., and Lonsane, B.K. (1997): Production and application of tannin acyl hydrolase. State of the art. *Advances in Applied Microbiology* **44**, 215–260.

Lewis, J.A., and Starkey, R.L. (1969): Decomposition of plant tannins by some soil microorganisms. *Soil Science* **107**, 235 – 241.

Lillehoj, E. P., Malik, V.S.(1989): Protein purification. *Advances in biochemical engineering and biotechnology* **40**,19-71.

Liu, C.H., Lu, W.B., and Chang, J.S. (2006): Optimizing lipase of *Burkholderia* sp. by response surface methodology. *Process Biochemistry* **41**, 1940-1944.

Lokeswari, N., and Jaya-Raju, K. (2007): Optimization of gallic acid production from terminalia chebula by *Aspergillus niger*. *E- Journal of Chemistry* **4**, 287-293.

Lopez-Rios, G.F. (1984): Fitoquimica, (1st Edn). pp. 13, Universidad Autonoma de Chapingo, Estado de Mexico.

Machida, M., Sano, M., Tamano, K., Terabayashi,Y., Yamane, N., Hatamoto,O., Umitsuki,G., Takahashi,T., Toda, T., Sunagawa, M., Marui, J., Ohashi-Kunihiro, S., Nishimura, M., Abe, K., Koyama,Y., Yamada, O., , Jinno, K., Horikawa, H., Hosoyama,A., Kushida,N., Hatttori, K., Fukuda,K., Kikuzato, I., FujimoriKE., Teruya, M., Tsukahara,M., Imada,S., Wachi,Y., Satou,Y., Miwa,Y., Yano,S., Kawarabayasi,S., Yasuhara.T., Kirimura, K., Arita,M., Asai,K., Ishikawa,T., Ohashi, S., Gomi,S., Mikam,I. S., Koike,H., Hirano,T., Nakasone, N., and Fujita, N. Genomics of industrial filamentous fungi, *Aspergillus oryzae* and *Aspergillus awamori* The 17th Congress of The International Society for Human and Animal Mycology Tokyo 2009.

Madhavakrishna, W., and Bose, S.M. (1962): Chemical abstracts. *Bulletin of. Central Leather Research Institute* **56**, 11759.

Madhavakrishna, W., Bose, S.M., and Nayudamma, Y. (1960): Estimation of tannase and certain oxidizing enzymes in Indian vegetable tanstuffs. *Bulletin of Central Leather Research Institute* **7**, 1 – 11.

Mahapatra, K., Nanda, R.K., Bag, S.S., Banerjee, R., Pandey, A., and Szakacs, G. (2005): Purification, characterization and some studies on secondary structure of tannase from *Aspergillus awamori* nakazawa. *Process Biochemistry* **40**, 3251–3254.

Mahapatra, S., and Banerjee, D. (2009): Extracellular tannase production by endophytic *Hyalopus. Journal of General and Applied Microbiology* **55**, 255-259.

Mahendran, B., Raman, N., and Kim, D. (2005): Purification and characterization of tannase from *Paecilomyces variotii*: hydrolysis of tannic acid using immobilized tannase. *Applied Microbiology and Biotechnology* **70**, 445–451.

Mai, J., Chambers., Laura, J. M., Richard, E.(1990): US Patent 4891231 - Process for inhibiting liquid oxidation in food Inventors

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982): Molecular cloning. A laboratory Manual. Cold Spring Harbor Laboratory Press. Cold Spring Harbor N.Y.

Marco., Mata-Gómez., Rodríguez, L.V., Ramos, E.L., Renovato, J., Cruz-Hernández, M.A., Rodríguez,R., Contreras, J., and Aguilar, C.N.(2009): A Novel Tannase from the Xerophilic Fungus *Aspergillus niger* GH1. *Journal of Microbiology and Biotechnology* (Published online first).

Sharma, S., Bhat, T.K., and Dawra, R.K. (2000): A Spectrophotometric Method for Assay of Tannase using Rhodonine. *Analytical Biochemistry* **278**, 85-89.

Sharma, S., and Gupta, M.N. (2003): Synthesis of antioxidant propyl gallate using tannase from *Aspergillus niger* van Teighem in non aqueous media. *Bioorganic and Medicinal Chemistry Letters* **13**, 395–397.

Shi, B., He, Q., Yao, K., Huang, W., Li, Q. (2005): Production of ellagic acid from degradation of valonea tannins by *Aspergillus niger* and *Candida utilis*. *Journal of Chemical Technology and Biotechnology* **80**, 1154–1159.

Singh, R., Gupta, N., Goswami V.K., and Gupta, R.(2006): A simple activity staining protocol for lipases and esterases. *Applied Microbiology and Biotechnology* **70**, 679-682.

Singleton, V. L., and Kratzer. F. H.(1969): Toxicity and related physiological activity of phenolic substances of plant origin. *Journal of Agricultural Feed Chemistry* **17**, 497.

Soorej, M. Basheer., (2008): Ph.D. thesis of Biotechnology, Cochin University of Science and Technology, India.

Swain, T. (1965): The Tannins. *In:* Bonner, J. and J. Varner (eds.), *Plant Biochemistry*. Academic Press, New York, pp. 552–580.

Takamine, J. (1914): Enzymes of *Aspergillus oryzae* and the application of its amyloclastic enzyme to the fermentation industry. *Journal of Industrial Engineering and Chemistry* **6**, 824-829.

Takino, Y.(1976):US Patent 3 959 497.

Tao, S., Peng, L., Beihui, L., Deming, L., and Zuohu,L. (1997): Solid state fermentation of rice chaff for fibrinolytic enzyme production by *Fusarium oxysporum*. *Biotechnology Letters* **19**, 465-467.

Tieghem, P. (1867): Sur la fermentation gallique. CR Academy of Science, Paris **65**, 1091–1094.

Treviño-Cueto, B., Luis, M., Contreras-Esquivel, J. C., Rodríguez, R., Aguilera, A., Aguilar, C.N. (2007): Gallic acid and tannase accumulation during fungal solid state culture of a tannin-rich desert plant (*Larrea tridentate* Cov). *Bioresource Technology* **98**,721–724.

Treviño-Cueto, B., Luis, M., Contreras-Esquivel, J.C., Rodríguez, R., Aguilera, A., and Aguilar, C.N. (2006): Gallic acid and tannase accumulation during fungal solid state culture of a tannin-rich desert plant (*Larrea tridentata* Cov.) *Bioresource Technology* **98**, 721-724.

Tropp, and Freifelder, (2007): Molecular biology: genes to proteins - Burton E. Tropp - *Science* – pp.1000

Uma Maheswar Rao, J.L., and Satyanarayana, T. (2007): Improving production of hyperthermostable and high maltose-forming a-amylase by an extreme thermophile *Geobacillus thermoleovorans* using response surface methodology and its applications. *Bioresource Technology* **98**, 345–352.

Naidu, R. B., Saisubramanian, N., Selvakumar, D., Janardhanan S., and Puvanakrishnan, R. (2008): Partial purification of tannase from *Aspergillus foetidus* by aqueous two phase extraction and its characterization. *Current Trends in Biotechnology and Pharmacy* **2**, 201-207.

Nawani, N. N., and Kapadnis, B. P. (2005): Optimization of chitinase production using statistics based experimental designs. *Process Biochemistry* **40**, 651-660.

Niehaus, J. U., and Gross, G. G. (1997): A gallotannin degrading esterase from leaves of pedunculate oak. *Phytochemistry* **45**, 1555–1560.

Nishira, H. (1961): Studies on tannin decomposing enzyme of molds.X. Tannase fermentation by molds in liquid culture with phenolic substances. *Journal of Fermentation Technology* **39**, 137–146.

Nishitani, Y., and Osawa, R. (2003): A novel colorimetric method to quantify tannase activity of viable bacteria. *Journal of Microbiological Methods* **54**, 281–284.

Nishitani, Y., and Osawa, Ro. (2005): Deceptive Halo Formation by Tannase-Defective Bacteria on Tannin-Treated Plate Media. *Microbes and Environments* **20**, 117-119.

Nishitani, Y., Sasaki, E., Fujisawa, T., and Osawa, R, (2004): Genotypic Analyses of Lactobacilli with a range of tannase activities isolated from human feces and fermented foods. *Systematic and Applied Microbiology* **27**,109–117.

Parthasarathy, N., and Bose, S.M., (1976): Glycoprotein nature of tannase in *Aspergillus niger*. *Acta Biochimica Polonica* **23,** 293–298.

Piater, L.A. (1999): *Aspergillus* tannase: Purification, properties and application. M.Sc. Thesis. University of the Free State.

Plackett, R. L., and Burman J. P. (1946): The design of optimum multifactorial experiments. *Biometrica* **33**, 305-325.

Pohl, T. (1990): Concentration of proteins and removal of solutes. *Methods in Enzymology***182**, 68-83.

Pourrat, H., Regerat, F., Morvan, P., and Pourrat, A. (1987): Microbiological production of gallic acid from *Rhus coriaria* L. *Biotechnology Letters* **9**, 731-734.

Pourrat, H., Regerat, F., Pourrat, A., and Jean, D. (1982): Production of Tannnase (Tannin Acyl Hydrolase E.C. 3.1.1.20) by a strain of *Aspergillus niger*. *Biotechnology Letters* **4**, 583 – 588.

Pourrat, H., Regerat, F., Pourrat, A., and Jean, D. (1985): Production of gallic acid from tara tannin by a strain of *A. niger*. *Journal of Fermentation Technology* **63**, 401–403.

Purohit, J.S., Dutta, J.R., Nanda, R.K., and Banerjee, R. (2006): Strain improvement for tannase production from coculture of *Aspergillus foetidus* and *Rhizopus oryzae*. *Bioresource Technology* **97**,795–801.

Rajakumar, G.S., and Nandy, S.C, (1983): Isolation, purification, and some properties of *Penicillium chrysogenium* tannase. *Applied Environmental Microbiology* **46**, 525-527.

Raghukumar, C., Muraleedharan, U., Gaud, V.R., and Mishra, R. (2004): Xylanases of marine fungi of potential use for biobleaching of paper pulp. *Journal of Industrial Microbiology and Biotechnology* **31**, 433–441.

Raimbault, M. (1998): General and microbiological aspects of solid substrate fermentation. *Electronic Journal of Biotechnology* **1**, 1–15.

Ramírez-Coronel, A., Viniegra-González, G., and Augur, C. (1999): Purificationof a tannase produced by *Aspergillus niger* Aa-20, in solid state fermentation. Proceedings of the VIII Mexican congress and IV Latin American congress of biotechnology and bioengineering, Huatulco, Oaxaca, Mexico.

Ramirez-Coronel, M. A., Marnet, M., Kulli., V. S., Roussos, S., Guyot, S., and Augur., C.(2004): Characterization and estimation of proenthocyanidins and other phenolics in coffee pulp (Coffea arabica) by thiolysis-high performance liquid chromatography. *Journal of Agricultural Food Chemistry* **52**, 1344–1349.

Ramirez-Coronel, M.A., Viniegra-Gonzalez, G., Darvill, A., and Augur, C. (2003): A novel tannase from *Aspergillus niger* with β-glucosidase activity. *Microbiology* **149**, 2941–2946.

Rana, N., and Bhat, T. (2005): Effect of fermentation system on the production and properties of tannase of *Aspergillus niger* van Tieghem MTCC 2425. *Journal of General and Applied Microbiology* **51**, 203–212.

Regerat, F., Pourrat, H., and Pourrat, A. (1989): Hydrolysis by fermentation of tannins from gall nuts. *Journal of the American Leather Chemists Association* **84,** 323-328.

Ribereau-Gayon, M. (1973): In les tannins des vegetaux. Metche, M, & Girardin, M. (Eds.), France: Dunod, pp. 262–287.

Rodriguez Couto, S., and Sanroman, M. A. (2005): Coconut flesh: a novel raw material for laccase production by *Trametes hirsuta* under solid-state conditions: Application to Lissamine Green B decolourization, *Journal of Food Engineering* **71**, 208–213.

Rutter, P., and Stainsby, G. (1975): The solubility of tea cream. *Journal of the Science of Food and Agriculture* **26**, 455–463.

Sabu, A., Augur, C., Swati, C., and Pandey, A. (2006): Tannase production by *Lactobacillus* sp. ASR-S1 under solid-state fermentation. *Process Biochemistry* **41**, 575–580.

Sabu, A., Kiran, G.S., and Pandey, A. (2005a): Purification and characterization of tannin acyl hydrolase from *A. niger* ATCC 16620. *Food Technology and Biotechnology* **43**, 133-138.

Sabu, A., Pandey, A., Daud, M.J., and Szakacs, G. (2005b):Tamarind seed powder and palm kernel cake: two novel agro residues for production of tannase under solid state fermentation by *Aspergillus niger* ATCC16620.*Bioresources Technology* **96**,1223-1228.

Sambrook, J., Fritsch, E. F., Maniatis, T. (1989): Molecular Cloning: A Laboratory Manual, 2nd ed.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, N.Y.

Sanderson G.W., Berkowitz J.E., Co H., and Graham, H.N. (1972): Biochemistry of Tea Fermentation: Products of the Oxidation of Tea Flavanols in a Model Tea Fermentation System. *Journal of Food Science* **37**, 399-404.

Sanderson, G.W., and Coggon, P.(1974): Green tea conversion using tannase and natural tea enzyme. U.S. Patent 3,812,266.

Sasaki, E., Shimada, T., Osawa, R., Nishitani, Y., Spring, S., and Lang, E. (2005): Isolation of tannin-degrading bacteria isolated from feces of the Japanese large wood mouse, Apodemus speciosus, feeding on tannin-rich acorns. *Systematic and Applied Microbiology* **28**, 358–365.

Scalbert, A. (1991): Antimicrobial properties of tannins. *Phytochemistry* **30**, 3875-3883.

Schanderl, S.H. (1970): In: Method in Food Analysis. Academic Press, New York, pp.709.

Scopes, R. K. (1987): Protein Purification, 2nd ed.; Springer-Verlag : New York, pp 41-71.

Selwal, M.K., Yadav' A., Selwal, K.K., Aggarwal, N.K., Gupta, R., and Gautam, S. K. (2009): Optimization of cultural conditions for tannase production by *Pseudomonas aeruginosa* IIIB 8914 under submerged fermentation. *World Journal of Microbiology and Biotechnology* (published online).

Seth, M., and Chand, S., (2000): Biosynthesis of tannase and hydrolysis of tannins to gallic acid by *Aspergillus awamori*, optimization of process parameters. *Process Biochemistry* **36**, 39–44.

Sharma, D.C., and Satyanarayana, T. (2006): A marked enhancement in the production of a highly alkaline and thermostable pectinase by *Bacillus pumilus* dcsr1 in submerged fermentation by using statistical methods, *Bioresource* Technology **97**, 727–733.

Sharma, S., Agarwal, L., and Saxena, R. K. (2007): Statistical optimization for tannase production from *Aspergillus niger* under submerged fermentation. *Indian Journal of Microbiology* **47**, 132–138.

Sharma, S., Agarwal, L., and Saxena, R.K.(2008): Purification, immobilization and characterization of tannase from *Penicillium* variable. *Bioresource Technology* **99**, 2544–2551.

Sharma, S., Bhat, T.K., and Dawra, R.K. (1999): Isolation, purification and properties of tannase from *Aspergillus niger* van Tieghem. *World Journal of Microbiology and Biotechnology* **15**, 673-677.

Valleeb, L., and Ulmerd, D. (1972): Biochemical effects of mercury, cadmium and lead. *Annual Review of Biochemistry* **41**, 91-128.

Van de Lagemaat, J., and Pyle, D.L. (2001): Solid-state fermentation and bioremediation: development of a continuous process for the production of fungal tannases. *Journal of Chemical Engineering* **84**, 115–123.

Van de Lagemaat, J., and Pyle, D.L. (2005): Modelling the uptake and growth kinetics of *Penicillium glabrum* in a tannic acid-containing solid state fermentation for tannase production. *Process Biochemistry* **40**, 1773–1782.

Van de Lagemaat, J., and, Pyle, D.L. (2001): Solid-state fermentation and bioremediation: development of a continuous process for the production of fungal tannase. *Chemical Engineering Journal*, **84**, 115-123.

Van Diepeningen, A.D., Debet, A., Varga, J., Van der Gaag, M., Swart, K., and Hoekstra, R. (2004): Efficient degradation of tannic acid by black *Aspergillus* species. *Mycological Research* **108,** 919–925.

Viniegra-González, G., Favela-Torres, E., Aguilar, C.N., Romero-Gomez, S.J., Diaz-Godinez, G., and Augur C. (2003): Advantages of fungal enzyme production in solid state over liquid fermentation systems. *Biochemical Engineering Journal* **13,** 157–167.

Wickremasinghe, R.L. (1978): Tea. ln: Chichester, C.O., Mark, E.M. & Stewart, G.E., eds, *Advances in Food Research* **24**, New York, Academic Press, pp. 229-286.

Wilke, D. (1999): Chemicals from biotechnology: molecular plant genetics will challenge the chemical and fermentation industry. *Applied Microbiology and Biotechnology* **52**, 135-145.

Wiseman, A. (1985): Handbook of enzyme biotechnology. In Ellis Horwood Ltd., London England, pp. 274–379.

Yamada, D., and Tanaka, T. (1972): Wine making using tannase in fermentation process. Patent. Ger. Offen. 2.224 100 (cl. 12g). Japanese Patent Application no. 71 33, 151.

Yamada, H., Adachi, O., Watanabe, M., and Sato, N. (1968): Studies on Fungal Tannase. Part I. Formation, Purification and Catalytic Properties of Tannase of *Aspergillus flavus. Agricultural and Biological Chemistry* **32**, 1070 – 1078.

Yu, X.W., and Li, Y.Q. (2006): Kinetics and thermodynamics of synthesis of propyl gallate by mycelium-bound tannase from *Aspergillus niger* in organic solvent. *Journal of Molecular Catalysis B Enzyme* **40**, 44–50.

Zhong, W., and Cen, P. (2005): Pectinase Production by *Aspergillus Niger* P-6021 on *Citrus Changshan-huyou* Peel in Slurry-state Fermentation *Chinese Journal of Chemical Engineering* **13**, 510 - 515.

Zhong, X., Peng, L., Zheng, S., Sun, Z., Ren, Y., Dong, M., and Xu, A.(2004): Secretion, purification, and characterization of a recombinant *Aspergillus oryzae* tannase in Pichia pastoris. *Protein Expression and Purification* **36**, 165-169.

Ph.D. Thesis

Beena P.S

January 2010

Ph. D. Thesis

PRODUCTION, PURIFICATION, GENETIC CHARACTERIZATION AND APPLICATION STUDIES OF TANNASE ENZYME FROM MARINE FUNGUS ASPERGILLUS AWAMORI

by

Beena P.S

Microbial Technology Laboratory

Department of Biotechnology

Cochin University of Science and Technology

Cochin – 682022, Kerala, India

January 2010