PRODUCTION OF DEBITTERING ENZYME USING AGRO WASTES UNDER SOLID STATE FERMENTATION BY *Aspergillus niger*

By

NILANEE K

(Reg. No. 1120203007)

A PROJECT REPORT
Submitted to the
FACULTY OF TECHNOLOGY

In partial fulfillment of the requirements
for the award of the degree
of
MASTER OF TECHNOLOGY
IN
BIOTECHNOLOGY

KUMARAGURU COLLEGE OF TECHNOLOGY
COIMBATORE -641 049
(An Autonomous Institution Affiliated to Anna University, Chennai)

MAY – 2013

BONA FIDE CERTIFICATE

Certified that this project work titled '**PRODUCTION OF DEBITTERING ENZYME USING AGRO WASTES UNDER SOLID STATE FERMENTATION BY *Aspergillus niger*'** is a bona fide work of **Ms. NILANEE, K. (Reg. No.1120203007)** who carried out the research under my supervision. Certified further that to the best of my knowledge, the work reported herein does not form part of any other thesis or dissertation, on the basis of which, a degree or award was conferred on an earlier occasion on this or any other students.

Mr. M.SHANMUGAPRAKASH	**Dr. A. MANICKAM**
SUPERVISOR	
Assistant Professor(SRG)	Professor and Head
Department of Biotechnology	Department of Biotechnology
Kumaraguru College of Technology	Kumaraguru College of Technology
Coimbatore - 641 049	Coimbatore - 641 049

ABSTRACT

Aspergillus niger was used to produce extracellular naringinase by solid state fermentation. The agro-industrial wastes like cassava waste, rice bran, sugarcane bagasse, coconut and groundnut oilcakes were used as solid substrate to produced naringinase (complex debittering enzyme). Production conditions and media were optimized. From the study cassava waste was found to be the best substrate for naringinase production. Maximum yield was found after 5 days of incubation with 10% (v/v) of inoculum size using 4 days grown culture(inoculum age) at 27°C and at a substrate-to-moisture ratio of 1:1.5 (w/v). The production was enhanced by reducing the particle size from 3.35mm to 0.85mm and by reducing concentration of naringin from 0.1% to 0.001%. 12 Different factors(carbon, nitrogen, metal ion) were studied by Placket-Burman design, and 3 factors were screened for Response Surface Methodology to optimize the media composition. Maximum yield was found for the media containing Maltose-15g/l, Calcium Chloride-15mM, Ferric Chloride-15mM and Naringin-0.001%.

Key words: Naringinase, Naringin, *Aspergillus niger*, Inoculum age,Moisture.

ACKNOWLEDGEMENT

I express my heartful gratitude to my guide **Mr. M. Shanmugaprakash,** Assistant Professor(SRG), Department of Biotechnology, Kumaraguru College of Technology, Coimbatore, for his continuous encouragement and valuable guidance given to me throughout the study.

I wish to express my deep sense of gratitude to **Dr. S. Ramachandran**, Principal, Kumaraguru College of Technology, Coimbatore, for providing the facilities to conduct this study.

I express my humble gratitude to **Dr. A. Manickam,** Head of the Department, Department of Biotechnology, Kumaraguru College of Technology, Coimbatore, for facilitating conditions for carrying out the research work smoothly.

I solemnly submit my humble thanks to **Dr. N. Saraswathy**, Project coordinator, Associate Professor Department of Biotechnology, Kumaraguru College of Technology, Coimbatore, for giving me kind permission to carry out my project.

I would like to express my thanks to **Dr. K. Kumaresan,** Associate professor Department of Biotechnology, Kumaraguru College of Technology, Coimbatore, for helping me.

Lastly I would thank all the **teaching** and **non- teaching staff members**, my friends and family for being supportive and understanding.

(NILANEE K)

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVATIONS

U/mg	Units/ milli gram
g/l	gram/litre
°C	Degree Celsius
mm	millimeter
w/v	weight/ volume
v/v	volume/volume
g	gram
Gds	gram of dry substrate
Rpm	revolutions per minute
%	Percentage
ml	milli litre
OD	Optical Density
M	Molar
H	hours
Min	Minute
U/ml	Units/milli litre
L	Litre
mM	milli Molar
mg	milli gram
SSF	Solid state fermentation
smf	Submerged state fermentation

CHAPTER 1

INTRODUCTION

1.1 GENERAL

The enzyme naringinase is used in citrus fruits processing to improve pulp washing, increase the recovery yield of essential oils, debitter and to clarify the juice (Grassin and Fauquembergue., 1996). The most bitter compounds present in citrus juices are naringin, limonin, and neohesperidin (Kefford, 1959; Marwaha *et al*., 1994). These bitter compounds are found in all parts of grapefruit and sour oranges. Naringin is abundant in immature fruit but its concentration decreases as fruit ripens (Yusof *et al*., 1990). The naringin level can be reduced by technologies such as adsorptive debittering (Wethern., 1992., Hernandez *et al.,*1992), supercritical Carbon dioxide method (Kimball, 1987), treatment with polystyrene divinyl benzene styrene (DVB) resins(Maeda *et al.,* 1984, Mitchell and Pearce., 1985), cyclodextrin(Tatum and Berry., 1973, Puri., 1984), Ion excahange resins(Couture and Rouseff., 1982). Because of the various drawbacks such as preparative steps on the juices, unfavorable changes on the sensory properties, nutritional value (Del Nobile et al.,2003) and reducing the level of Vitamin C, antioxidant, Bioactive flavonoids (Carla *et al.,*2013) the non enzymatic debittering technologies are limited (Puri and Banerjee., 2000). In comparison, the enzymatic debittering technology is regarded as the most promising method with the advantages of high specificity and efficiency, and a convenient operation for removing the bitterness in large-scale commercial production. Enzymatic hydrolysis has a possibility to overcome the bitterness. (Thammawat *et al*., 2008)

1.2 MECHANISM OF NARINGINASE ACTION

Naringinase is an enzyme complex consisting of α-L-rhamnosidase (EC 3.2.1.40) and flavonoid β-D-glucosidase (EC 3.2.1.21). In typical processing, naringinase converts naringin to naringenin in a two-step process . The substrate naringin, (4'-5,7' -trihydroxyflavonone- 7-rhamnoglucoside), is hydrolyzed by the rhamnosidase component to produce prunin (4' -5,7' -trihydroxyflavonone-7-glucoside), which is then converted by the flavonoid β-D-glucosidase to naringenin (4'-5,7' -trihydronyflavonone) (Chandler and Nicol., 1975; Habelt and Pittner., 1983).

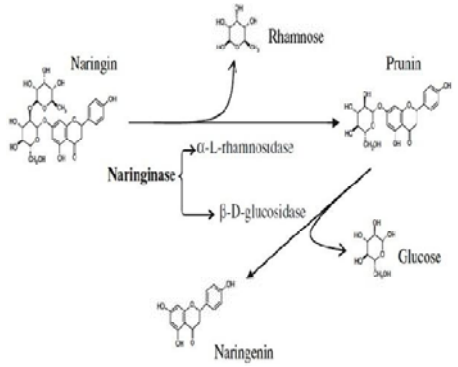


Figure 1.1: Stepwise degradation of naringin by the action of naringinase expressing α-L rhamnosidase and β-D-glucosidase activities (Puri., 2012)

1.3 APPLICATIONS OF NARINGINASE

Naringinase plays a major role in the debittering processes. The other applications of Naringinase are:

1.3.1 Preparation of the antibiotic chloropolysporin C:

The deglycosylation of novel glycopeptide antibiotic, chloropolysporin from *Faenia interjecta*, was achieved successfully with rhamnosidase activity of naringinase. Chloropolysporins A, B, and C could be enzymatically converted to deglycosylated derivatives (Sankyo., 1988).

1.3.2 Preparation of rhamnose:

Commercially available rhamnose is produced from rutin or citrus fruits which contain 10-30% rhamnose(by weight). Naringinases (α-L-rhamnosidases) hydrolyze naringin to produce L-rhamnose (Daniels *et al*., 1990). Rhamnose is a raw material for the chemical synthesis of furaneol, a strawberry flavour. Rhamnose is a chiral intermediate in organic synthesis and it is used as a pharmaceutical and a plant protective agent (Puri and Banergee., 2000).

1.3.3 Preparation of prunin:

The flavonoid prunin produced from naringin using immobilized naringinase pretreated with an alkaline buffer. Prunin has variable antiviral activity against DNA /RNA viruses.

The flavonoid possesses anti-inflammatory activity and may be used as sweetening agent for diabetics (Roitner *et al*., 1984).

1.4 PRODUCTION OF NARINGINASE

Production of naringinase from *Penicillium sp.* was originally suggested, but restricted for its industrial application in concern of high production cost, and particularly, the incomplete hydrolysis of naringin in citrus juice processing (Puri *et al*., 2000). In contrast, *Aspergillus niger* fermentation has shown more potential for industrial production of naringinase because the microorganism has been listed in the FDA's approved microbial category and proven safe for food and medicinal uses.

Also, the process is easy to be scaled up for industrial application, and easy to increase the naringinase yield by optimizing the cultivation parameters (Puri *et al*., 2005).Therefore, it was considered as the most important source of naringinase for food use (debittering citrus juice) regarding the safety characteristics and the feasible culture process of *Aspergillus niger.*

Naringinase is an inducer-dependent and continuous addition or stepwise addition of an inducer increases naringinase production. Whereas, replacement of the inducer with other carbon sources supports the growth of the organism but no enzyme is produced. Microbial naringinase may be produced by both submerged culture and solid-state fermentation. Compared to studies with submerged culture, solid-state fermentation has been barely investigated for the production of naringinase (Chisti, 1999).

1.5 SOLID STATE FERMENTATION

Solid state fermentation defined as any fermentation process occurring in the absence or near-absence of free water, employing a natural substrate or an inert substrate used as solid support (Pandey *et al*., 1999).

The selection of a substrate for SSF process depends upon several factors mainly related with cost and availability and thus may involve screening of several agro-industrial residues. In the SSF process, the solid which may interfere with microbial respiration: aeration, and thus may result in poor growth. At the same time, larger particles provide better respiration: aeration efficiency (due to increased inter-particle space) but provide limited surface for microbial attack. Thus, it would be necessary to arrive at a compromised particle size for a particular process (Pandy *et al*., 2000, Membrillo *et al.,* 2011).

SSF offers numerous opportunities in processing of agro-industrial residues. This is partly because solid-state processes have lower energy requirements, produce lesser wastewater and are environmental-friendly as they resolve the problem of solid wastes disposal (Ashok pandey ., 2003).

There are several important aspects, which should be considered in general for the development of any bioprocess in SSF. These include selection of suitable micro-organism and substrate, optimization of process parameters and isolation and purification of the product. Based on water activity, only fungi and yeast were termed as suitable micro-organisms for SSF (Sarrette *et al.,*1992, Kar *et al., 1998*).

The moisture level plays a vital role in SSF, Low moisture content is known to decrease the solubility of the nutrients, lead to low substrate swelling and higher water tension. Moisture levels higher than certain critical level cause low Oxygen transfer, decreasing porosity, decreasing microbial growth, and lower metabolic activities in SSF. Substrate to moisture ratio should be based on the clump forming capacity of the substrate (Patrick *et al.,* 2003).

1.5.1 Factors influencing enzyme production (Sarrette *et al.,*1992, Kar *et al., 1998*)

- Selection of a suitable substrate and microorganism
- Pre-treatment of the substrate
- Water content of the substrate
- Period of cultivation
- Size and age of the inoculum
- Particle size of the substrate
- Control of temperature of fermenting matter/removal of metabolic heat
- Maintenance of uniformity in the environment of SSF system
- The gaseous atmosphere, i.e. oxygen consumption rate and carbon dioxide evolution rate.

Table 1.1: Comparision of solid state fermentation and submerged state fermentation (Subramaniyam and Vimala., 2012**):**

S.N	solid state fermentation	submerged state fermentatiom
1	Utilizes solid substrates, like bran, bagasse, and paper pulp.	Utilizes free flowing liquid substrates, such as molasses and broths.
2	The main advantage of using these substrates is that nutrient-rich waste materials can be easily recycled as substrates. In this fermentation technique, the substrates are utilized very slowly and steadily, so the same substrate can be used for long fermentation periods. Hence, this technique supports controlled release of nutrients	The bioactive compounds are secreted into the fermentation broth. The substrates are utilized quite rapidly; hence need to be constantly replaced/supplemented with nutrients.
3	SSF is best suited for fermentation techniques involving fungi and microorganisms that require less moisture content. It cannot be used in fermentation processes involving organisms that require high aw (water activity), such as bacteria. (Babu and Satyanarayana, 1996).	This fermentation technique is best suited for microorganisms such as bacteria that require high moisture.
4	**Easier downstream processing than submerged state fermentation.**	Easier product purification, but not than Solid state fermentation.

1.6 AGRO WASTES

Agro-industrial wastes are mainly composed of complex polysaccharides that might serve as nutrients for microbial growth and production of enzymes. The utilization of such materials as substrates for microbial cultivation intended to produce cellular proteins, organic acids, mushrooms, biologically important secondary metabolites, enzymes, prebiotic oligosaccharides, and as sources of fermentable sugars in the second generation ethanol production has been reported (Sanchez, 2009).

Agro-industrial wastes are valuable sources of lignocellulosic materials. The lignocellulose is the main structural constituent of plants and represents the primary source of renewable organic matter on earth. It can be found at the cellular wall, and is composed of cellulose, hemicellulose and lignin, plus organic acids, salts and minerals (Pandey *et al*., 2000; Hamelinck *et al*., 2005). Therefore, such residues are superior substrates for the growth of filamentous fungi, which produce cellulolytic, hemicellulolytic and ligninolytic enzymes by solid state fermentation (SSF). These fungi are considered the better adapted organisms for SSF, since their hyphae can grow on the surface of particles and are also able to penetrate through the inter particle spaces (Santos *et al*., 2004). Filamentous fungi are the most distinguished producers of enzymes involved in the degradation of lignocellulosic material. Several agro industrial wastes are commonly used for this purpose, such as sugarcane bagasse, wheat bran, corn cob and straw, rice straw and husk, soy bran, barley and coffee husk (Sanchez, 2009).

1.7 MEDIUM OPTIMIZATION:

Medium optimization by the classical method of changing one variable while fixing the others at a certain level is laborious and time-consuming, especially when the number of variables are large. Thus the statistical methodology was adopted to optimize the nutrients for the medium to achieve the maximum enzyme production.

1.7.1 Plackett–Burman design

Plackett–Burman designs are experimental designs presented in 1946 by Robin L. Plackett and J. P. Burman while working in the British Ministry of Supply. This Plackett–Burman design allows testing of the largest number of factor effects with the least number of observations, and allows random error variability estimation and testing of the statistical significance of the parameters.

Full factorial designs are the simplest form of factorial designs in which all possible factor-level combinations are tested. The advantage of this design is that maximum information regarding the factors can be obtained. It is also possible to identify interactions between separate experimental factors and the effect that such interactions have on the experimental response. The main disadvantage of this design is that as the number of factors increases, the number of experiments required also increases. As a result, this type of design becomes increasingly impractical. In the case of Placket-Burman design which requires less experimental runs for more number of factors. And full factorial Design can be used only when we need to screen more than four factors. But Placket-Burman design is used to screen even small number of factors. So in this study Placket-Burman Design was selected for media optimization (http://www.sepscience.com).

1.7.2 Response Surface Methodology

Response surface method (RSM) is a collection of mathematical and statistical techniques that are useful for modeling and analysis in applications where a response of interest is influenced by several variables. The first step in RSM is finding a suitable approximation for the true relationship between the response and independent variables. Usually a low-order polynomial in some region of the independent variables is employed for modeling. If the response is well modeled by a linear function of the independent variables, then the approximating function is the first-order. If there is curvature in the system, then a polynomial of higher degree must be used, such as the second-order model (Myers and Montgomery., 2002). Response Surface Methodology (RSM) is a commonly used method to assess the optimal fermentation conditions and also an efficient statistical technique for optimization of multiple variables with minimum number of experiments. This method has been successfully applied to optimize alcoholic fermentation (Laluce *et al*., 2009; Palukurty *et al*., 2008; Ratnam *et al*., 2005; Yu *et al*., 2009). RSM helps identify the effective factors, study interactions, select optimum conditions and quantify the relationships between one or more measured responses and the vital input factors in limited number of experiments (Ahuja *et al.,* 2005, Bandaru *et al.,*2006, Puri *et al.,*2002).

The Box-Behnken is a three-level factorial design, which allows estimating and interpreting interactions between various variables at a time during the optimization process. It is suitable for exploration of quadratic responses and constructs a second-order polynomial model with very few runs (Ferreira *et al*., 2007). The Box-Behnken design is rotatable (or nearly so) But it contains region of poor prediction qulity. Its "missing corners" may be usefull when the experimenter should avoid combined factors extremes. This property prevents potential loss of data in this case. And Box-Bhenken design always requires less number of Experimental Run than other Response Surface Designs(http://www.itl.nist.gov).

1.5 OBJECTIVES

To screen the various agro- industrial wastes like cassava Waste, rice bran, sugarcane bagasse, coconut oilcake and groundnut oilcake for the production of enzyme.

To optimize the different factors such as inoculum size and age, incubation period, particle size, volume of moisture content influencing the enzyme production.

To screeen the media composition using Placket-Burman Design and Response Surface Methodology.

To identify the significant variabels influencing naringinase production using *A.niger* Under Solid State Fermentation by Placket-Burman Design.

To optimize the level of significant variables identified from Placket-Burman Design Using Response Surface Methodology.

CHAPTER 2

REVIEW OF LITERATURE

Puri and Banerjee ., (2000), discussed about naringinase and production, purification, characterization of the debittering enzyme naringinase and its essential role in the commercial processing of citrus fruit juice. Naringinase is an enzyme which hydrolyses naringin into prunin which is then converted by the flavonoid β-D-glucosidase to naringenin(4,5,7-trihydroxyflavonone) (Habelt and Pittner., 1983). Puri and Banerjee produced naringinase enzyme by both submerged state (Smf) and solid state fermentation (SSF) and characterized after purification. In solid state fermentation they used soybean cake as solid substrate and grown the microorganism at 28°C and for 8 days. Then they extracted the enzyme to obtain a crude preparation of naringinase. The crude enzyme was further purified to remove the contaminating pectinase. The optimum pH and temperature for the naringinase were pH 4.2 and 60–65, 8°C, respectively.

Puri *et al* .,(2005), used *Aspergillus niger* MTCC 1344 to produce extracellular naringinase . *A. niger* MTCC 1344, was maintained on Czapeck-Dox medium. Puri *et al.,* discussed that *A. niger* MTCC 1344 is the best naringinase producing strain, Media constituents were examined for the production of enzyme. They studied the effect of different carbon, nitrogen(yeast extract, beef extract, malt extract, meat extract and groundnut seed flour) and metal ions(Co^{2+}, Cu^{2+}, Fe^{3+}, Mg^{2+}, Ni^{2+}, Mn^{2+} and Zn^{2+}) on enzyme production.

However, the optimum temperature of naringinase and α-L-rhamnosidase was 60° C, whereas that of β-D-glucosidase activity was in the range of 60° to 70°C. Optimization of the medium and conditions for enzyme production in submerged fermentation found that the suitable inoculum concentration and medium were IDS spores/mi and Czapek-Dox medium, pH 4.0, containing 0.1 % naringin, respectively. The maximum naringinase production of this fungus (117.77 U/mg protein) could be obtained by supplement of the medium with 3.75 g/1 rhamnose as another carbon source and using 2.5 g/1 $NaNO_3$ as its nitrogen source. For high production of α-L-rhamnosidase (303.20 U/mg protein), 2.5 g/1 soya peptone should be used.

Mendoza-Cal *et al*., (2010), studied the production of naringinase using twelve filamentous fungi in solid-state fermentation (SSF) using orange and grapefruit rind as substrates; these agro industrial residues contain naringin, an important inductor for this enzyme. The cultures were carried out in a 500 ml Erlenmeyer flask with 10 g of orange or grapefruit rind (particle size: 0.86, 1.19, 1.69 and 2.38 mm) and 10 ml of mineral salt solution (KH_2PO_4- 13.77 g/l; K_2HPO_4- 17.629 g/l; NH_4Cl-0.2 g/l; $MgSO_4.7H_2O$- 0.2 g/l ; $FeCl_3$- 0.001 g /l). The spores were inoculated on orange or grapefruit rind and thoroughly mixed. Flasks were incubated at 30°C, without agitation. Samples were withdrawn (flask/sample) aseptically at regular time intervals and analyzed for extracellular protein content, naringinase activity and residual substrate. The extract was recovered using 75 ml of sodium acetate buffer 0.1 M, pH 4.0, and stirring with a magnetic bar for 30 min at 4°C. Subsequently, the suspension was filtered and centrifuged (14000 rpm, 30 min, 4°C). Then assay for enzyme activity was carried out. The percentage of naringin hydrolyzed from grapefruit rind by *Aspergillus foetidus, Aspergillus niger* and *Aspergillus niger* HPD-2 was 81, 80 and 79% respectively. The volumetric and specific naringinase activity of each strain was influenced by temperature, pH and water activity (Aw).

And the main parameters like Temperature, pH, inoculum age and inoculum size also studied in the shake flask level. Among various carbon and organic nitrogen sources used, molasses and peptone were the most effective for enzyme yield. The rate of enzyme production was enhanced when metal ions were added to the medium.

Emrah *et al.,* (2005), Investigated the effect of medium composition on the *Taq*I endonuclease production, by recombinant *Escherichia coli* cells carrying a plasmid encoding *Taq*I endonuclease, using response surface methodology. The concentration of glucose, di-ammonium hydrogen phosphate, potassium di-hydrogen and magnesium sulphate in media were changed according to a central composite rotatable design consisting of 29 experiments and enzyme yields were determined. The results were fitted to a second order polynomial with an R^2 of 0.828. The optimum medium composition was found to be 6 g L^{-1} glucose, 1.5 g L^{-1} $(NH_4)_2HPO_4$, 8 g L^{-1} KH_2PO_4, and 0.8 g L^{-1} $MgSO_4 \cdot 7H_2O$. The model prediction of 179×10^6 U g DCW^{-1} enzyme yield at optimum conditions were experimentally verified. The response surface methodology was found to be useful in improving the production of recombinant *Taq*I endonuclease in *E. coli*.

Thammawat *et al.,* (2008) screened forty fungal isolates from 348 fungi. Then performed Secondary screening by measurement of both enzyme activities, α-L-rhamnosidase and β-D-glucosidase at 40°C, pH 4.0 and naringinase activity at the temperatures of 50,55 and 60°C and at both pH 3.0 and 4.0 of the culture filtrates from all 40 fungal isolates. *Aspergillus niger* BCC 25166 was selected , genetically identified and which was found to be the suitable organism for the production of naringinase. The optimum pH and temperature of the enzyme in crude extract was investigated. The result showed that all naringinase, α-L-rhamnosidase and β-D-glucosidase activities had identical optimum pH of 4.0.

The culture conditions optimal for *A. foetidus, A. niger* and *A. niger* HPD_2 were as follows: pH 5.4, Temp-35°C, Water activity- 0.5222 ; pH 5.4, Temp -35°C, Water activity -0.7533; pH 5.4, Temp- 40°C, Water activity - 0.7533; respectively. The highest volumetric activity was obtained using *A. foetidus* with 2.58 U/ml, when grew on grapefruit rind. Among the strains used, optimal naringinase production was with *A. foetidus*. The use of grapefruit rind as a substrate gave a higher yield of naringinase production than using orange rind.

The nutrient requirements for extracellular naringinase production by *Aspergillus oryzae* JMU316 was studied by Dong-xio *et al*.,(2010). The fermentation was started by inoculating 3% (v/v) of the inocula in 300 mL flasks containing 30 mL medium at 28°C on a rotary shaker incubator at 160 rpm for 5 days. The base medium contained K_2HPO_4- 1.0 g, $MgSO_4 \cdot 7H_2O$ -1.0 g, NaCl -0.2 g, $CaCl_2$- 0.2 g, $(NH_4)_2SO_4$ -0.2 g, yeast extract- 1.0 g, naringin- 5.0 g and distilled water up to 1 L (initial pH 6.0).

The impact of different carbon and nitrogen sources on naringinase production was determined by one-factor-at-a-time method. Naringin exhibited the highest naringinase activity compared to all other carbon sources and pomelo pericarp powder produced comparable naringinase activity. Pomelo pericarp powder was selected as carbon source because it is a waste of fruit process, which means that it is a cheap resource and has additional environmental benefits. Peptone proved to be the most suitable nitrogen source for naringinase production. To optimize the concentration of pomelo pericarp powder, peptone, and minerals, the orthogonal matrix method was used. The optimal concentration of the components were 15 g pomelo pericarp powder, 12 g peptone, 0.2 g $CaCl_2$, 0.4 g NaCl, 2 g $MgSO_4 \cdot 7H_2O$ and 1 g K_2HPO_4 in 1 L distilled water for producing 408.28 IU/mL naringinase activity.

Response surface methodology was used to optimize the fermentation medium for enhancing naringinase production by *Staphylococcus xylosus* by Puri *et al.*,(2010). The first step of this process involved the individual adjustment and optimization of various medium components at shake flask level. Sources of carbon (sucrose) and nitrogen (sodium nitrate), as well as an inducer (naringin) and pH levels were all found to be the important factors significantly affecting naringinase production. In the second step, a 22 full factorial central composite design was applied to determine the optimal levels of each of the significant variables. Using this methodology, the optimum values for the critical components were obtained as follows: sucrose, 10%; sodium nitrate, 10%; pH 5.6; biomass concentration, 1.58%; and naringin, 0.50% (w/v), respectively. Under optimal conditions, the experimental naringinase production was 8.45 U/mL

VinothKumar *et al.,* (2010), isolated *Aspergillus niger* VB07 from the soil of citrus fruit market and used this culture to produce extracellular naringinase in a liquid medium. Naringin, naringenin, hesperidin, rutin (0.05%), and amino acids (10 mM) were filter sterilized and added to the basal medium to study the effect on enzyme production. Different carbon sources (1%, w/v), organic (1%, w/v), and inorganic nitrogen sources (0.5%, w/v) were added to the medium to determine their effect on naringinase production. The effect of temperature and initial pH on growth and enzyme production was checked in 500-mL Erlenmeyer flask containing 100 mL
medium. Enzyme activity was observed in all medium, maximum production was observed in naringin (7.48 IU/mL) followed by rutin (3.71 IU/mL), naringenin (3.36 IU/mL), and hesperidin (2.11 IU/mL). The optimum concentration of naringin is 0.1% and their respective enzyme activity was 9.45 IU/mL observed on the 7th day of fermentation. Various parameters of fermentation have been examined in order to improve overall enzyme yield. Naringinase yield of 17.28 IU/mL in an optimized medium containing naringin (0.1%), rhamnose (0.5%), peptone (0.25%), glycine (10 mM), and pH 4.5 at 28°C for 7 days has been obtained at shake flask level.

Vinoth Kumar V., (2010), studied the effect of different inducers (hesperidin, naringenin, naringin, rhamnose and rutin) for naringinase production by *Aspergillus niger* MTCC 1344. The medium components employed for naringinase production were (g/l) molasses- 15; peptone -5; $NaNO_3$- 2.0; KH_2PO_4- 1.0; KCl- 0.5; $MgSO_4{\cdot}7H_2O$-0.5; and $FeCl_3$- 0.1 (pH 4.5). 0.5 g/l of naringin, naringenin, rutin and hesperidin were added to different media. Instead of molasses, 5 g/l rhamnose was used in the production medium. A 72 h age and 15% size was used for inoculation. Flasks were incubated (27°C, 200 rpm) in a rotary shaker for 14 days. Samples were withdrawn aseptically at regular time intervals and analyzed for naringinase activity. The naringinase activity was observed in all five medium, maximum naringinase was produced in naringin (7.48 IU/ml) followed by rhamnose (6.72 IU/ml), rutin (3.71 IU/ml), naringenin (3.36 IU/ml) and hesperidin (2.11 IU/ml). Among the substrates, naringin act as a selective inducer, because it induces faster and maximized naringinase
production (7^{th} day). The optimized concentration (%) of naringin, rhamnose, naringenin, rutin and hesperidin used are 0.1, 0.375, 0.01, 0.2 and 0.2, respectively, and their corresponding enzyme productions (IU/ml) are 9.68, 7.12, 4.76, 4.19 and 3.44.

Mariya *et al.,* (2010), used response surface methodology (RSM) in order to optimize the effect of removal of the bitter taste from juices by naringinase, immobilised in Ca-alginate beads, under high pressure, A central composite rotatable design (CCRD) was employed involving two variables (pressure and temperature) at five levels ($-\sqrt{2}$, -1, 0, $+1$, $+\sqrt{2}$). The second-order polynomial equations with R^2 values above 0.9 showed good agreement between experimental and predicted. The higher naringin conversion of 81% was obtained using 205 MPa and 60 °C, during a process time of 30 min.

Gonzalez *et al.*,(2011) worked to study the effect of naringin, molasses, and rhamnose, as well as calcium (Ca_2+) and magnesium (Mg_2+) salts on the production of naringinase by *Aspergillus niger* ATCC1015. The composition of the basal medium (BM) taken was $NaNO_3$-2.0 g/L; KH_2PO_4-1.0 g/L; KCl-0.5 g/L; $MgSO_4.7H_2O$-0.05 g/L; $FeCl_3$-0.1 g/L and peptone-10 g/L. In order to study the effect of the different carbon sources naringin, rhamnose and molasses (0.5% w/v) and the effect of $CaCO_3$ and/or $MgCO_3$ (0.01 mM) on enzyme production, both were added to the basal medium. The mycelial weight, protein production, and naringinase activity were quantified. The constants Vmax and Km were determined by Lineweaver-Burk. It was found that molasses compared with the other carbon sources and the addition of calcium carbonate favored the growth of the fungus but not the production of protein or the enzyme activity. Additionally, it was found that naringin promoted 8-fold more production of protein than the other carbon sources studied if Ca_2+ and Mg_2+ were added. On the other hand, They found rhamnose with no added salts produced 2-fold more enzyme activity than other treatments.

Puri *et al.,* (2011), optimized the medium and process parameters to increase naringinase production from the newly isolated strain Staphylococcus xylosus MAK2, in a batch bioreactor. In addition, nariginase production has been further improved by optimizing parameters in the presence of CPP (comprised of peel, membranes, and juice vesicles), a waste generated from the citrus processing industry, through technological combination experiments. Important factors like carbon, nitrogen sources, metal ions, pH, temperature, inoculum age and volume, concentration of CPP
were studied to increase the enzyme production. The maximum naringinase production (8.23 IU/ml) was obtained when the production medium contained the following (gl/1): sucrose, 10; soybean meal, 10; CPP, 0.5; and pH 5.5. The process optimization using technological combinations allowed rapid optimization of large number of variables, which significantly improved enzyme production in a 5L reactor in 34 h. An increase in sugar concentration (15 g/l) in the fermentation medium further increased naringinase production (8.9 IU/ml) in the bioreactor.

Shanmugaprakash *et al.,* (2011), produced naringinase using easily available, inexpensive industrial waste residues such as rice bran, wheat bran, sugar cane bagasse, citrus peel, and press mud under solid-state fermentation (SSF) by *Aspergillus niger* MTCC 1344. They found rice bran was the best substrate among all substrates. Naringinase production was highest after 96 h of incubation at 27°C and at a substrate-to-moisture ratio of 1:1 w/v. Supplementation of the medium with 10% naringin caused maximum induction. Inoculum age of 72 h and Inoculum level of 15% resulted in maximum production of naringinase. Enzyme production was stimulated by the addition of nutrients such as naringin and peptone. For studying the effect of the initial moisture level, the quantity of substrate was kept constant and the moisture effect was studied by adding nutrient solution in the ratio of 1:0.5 (5 gds/2.5 mL) to 1:5 (5 gds/25 mL) (gds, gram of dry substrate). For studying the effect of inoculum size, petri dishes each containing RB (5 g) moistened with 5mL nutrient solution were autoclaved and inoculated at a level of 5, 10, 15, and 20% v/w of RB, using 6-day-old *A. niger* spores. the optimum inoculum level was 15% for naringinase production by *A. niger* under SSF.

Hui Ni *et al*., (2012), produced Naringinase from *Aspergillus niger* and characterized to evaluate its effectiveness in debittering citrus juice. The spore suspension of *A. niger* was inoculated into an NBS Bioflo-110 7-L fermentor that contained 5 L of fermental medium with composition (g/L) of: naringin 10, $MgSO_4{\cdot}7H_2O$ -0.5, KH_2PO_4-1.5, $(NH_4)2SO_4$-4.0, $ZnSO_4{\cdot}7H_2O$-0.09, $CaCl_2$- 0.1, yeast extract 1.0, soybean powder 2.0, and peptone 2.0 (Patil and Dayanand 2006), followed by cultivation at 28 ◦C, pH 6.0, and 300 rpm for 7 days to produce the naringinase.

Ilamathi *et al.,* (2012) used standard optimization procedures like Response Surface Methodology to optimize the fermentation medium for enhancing naringinase activity by *Aspergillus flavus.* In the first step of optimization, with plackett-Burman design, starch, peptone and naringenin were found to be the important factors affecting the naringinase activity significantly. In the second step, a 23 full factorial central composite design and RSM was applied to determine the optimal concentration of each significant variable. The optimum values for the critical components were obtained as follows: starch 1.5 (15 g/L), peptone 0.5 (5 g /L) and 0.005 (0.055 g/L) naringenin. Under

the optimal conditions, the practical naringinase enzyme activity was increased up to 3606.6U/lit an approximate 6.4 fold improvement over the previous enzyme activity (560U/lit) with un-optimized medium.

Table 2.1: Naringinase production by Solid State Fermentation:

ORGANISMS	SUBSTRATES	REFERENCES
Aspergillus niger MTCC 1344	From various agro wastes	Shanmugaprakash *et al.*,(2011)
Aspergillus foetidus, Aspergillus niger and *Aspergillus niger*	Grapefruit	Mendoza *et al.*,(2010)
Coniothrium diplodiella	soybean cake	Puri and Banerjee, (2000)

Table 2.2: Naringinase production by Submerged State Fermentation:

ORGANISMS	REFERENCES
Coniothrium diplodiella	Puri and Banerjee(2000)
Aspergillus niger MTCC 1344	Puri *et al* .,(2005)
Aspergillus niger BCC 25166	Thammawat *et al.,* (2008)
Aspergillus oryzae JMU316	Chen *et al*.,(2010)
Staphylococcus xylosus	Puri *et al*.,(2010)
Aspergillus niger VB07	VinothKumar *et al.,* (2010)
Aspergillus flavus	Ilamathi *et al.,* (2012)

Table2.3: Various source of naringinase production (Puri., 2012)

SOURCE	MICROORGANISM	REFERENCES
Plant	Celery seeds (Apium graveolens)	Hall., 1938
	Rhamnus daurica	Suzuki., 1962
	Buckwheat (Fagopyrum Esculentum)	Bourbouze *et al.*. 1976
	Grapefruit leaves	Ting., 1958
	Grapefruit leaves	Thomas *et al*., 1958
Gastropod	Turbo cornutus	Kurosawa *et al*., 1973
Mammal	Pig liver	Qian *et al*., 2005
Fungi	*Aspergillus niger*	Bram and Solomons, 1965
	Penicillium sp.	Young *et al*., 1989
	Aspergillus niger	Manzanares *et al*., 1997
	A. aculeatus	Manzanares *et al*., 2001
	A. kawachii	Koseki *et al*., 2008
	A. nidulans	Orejas et al., 1999
	Aspergillus terrus	Gallego *et al*., 2001
	Rhizopus nigricans	Shanmugam and Yadav, 1995
	*Aspergillus niger (*MTCC1344*)*	Puri *et al*., 2005
	Penicillium decumbens	Mamma *et al*., 2004
	*Aspergillus niger (*BCC 25166*)*	Thammawat *et al*., 2008
	Aspergillus	kawachii Koseki *et al*., 2008
	Penicillium ulaiense	Rajal *et al*., 2009
	Aspergillus sojae	Chang *et al*., 2011
	Aspergillus, Circinella, Eurotium, Fusarium, Penicillium, Rhizopus, and Trichoderma	Scaroni *et al*., 2002
	Aspergillus terreus	Gallego *et al*., 2001
Yeast	*Hanshula anomala, Debaryomyces Polymorphus*	McMahon *et al*., 1999
	Pichia angusta X349	Yanai and Sato, 2000
	Cryptococcus laurentii	Lei *et al*., 2011
Bacteria	*Bacteriodes distasonis,* JY-1	Jang and Kim, 1996
	Thermomicrobium roseum	Jang and Kim, 1996
	Pseudomonas Paucimobilis	Miake et al., 2000
	Clostridium stercorarium	Zverlov *et al*., 2000
	Bacillus sp. GL1	Hashimoto *et al*., 2003
	Geothermus vaporicell	Birgisson *et al*., 2004
Bacteria	*Sphingomonas Paucimobilis*	Hashimoto and Murata, 1998; Miyata *et al*., 2005
	Burkholderia cenocepacia	Cardona *et al*., 2006
	Ralstonia pickettii	Gaston Orillo *et al*., 2007
	Pseudoalteromonas sp.	Mazzaferro *et al*., 2008
	Lactobacillus ulaiense	Rajal *et al*., 2009
	Lactobacillus acidophilus	Beekwilder *et al*., 2009
	Lactobacillus plantarum	Avila *et al*., 2009
	Staphylococcus xylosus	Puri *et al*., 2010b; Puri *et al*., 2011a
	Pediococcus acidilactici	Michlmayr *et al*., 2011

CHAPTER 3

MATERIALS AND METHODS

3.1 COLLECTION OF SUBSTRATES:

Five different agro-industrial residues such as cassava waste, rice bran, sugarcane baggasse, coconut oilcake and groundnut oilcake were purchased from local market.

Figure 3.1: Five different substrates

3.2 PRETREATMENT:

Collected substrates were pretreated by mechanical methods. Pretreatment was carried out to get different particle sizes of the substrate. Mechanical methods include crushing and seiving. Substrates were sieved with five different sizes of seivers and collected in polethene bags. The final sizes of the particles were 3.35mm, 2.56mm, 1.4mm, 0.85mm, 0.25mm.

3.3 PREPARATION OF MOTHER CULTURE:

Pure *Aspergillus niger* culture was teken and it was maintained on Czapek dox broth and incubated at 28°C in shaker (kubota, model no-CD1499- G000, Tokyo) at 120 rpm for five days. The culture was isolated from banana pith and identified earlier.

3.4 MOISTURE PREPARATION:

Modified Czapek Dox broth was used as moisture. The mineral medium contained (g/l) $NaNO_3$-3; K_2HPO_4-1; $MgSO_4$-0.5; KCl-0.5; FeSO4-0.01. Final pH was 7.5 then it was adjusted to pH 5. With moisture 0.001% inducer narigin was added. Because naringinase is an inducer dependent enzyme so it is necessary to add inducer for naringinase production (Chisti., 1999).

3.5 UNOPTIMIZED SOLID STATE FERMENTATION CONDITIONS:

Solid state fermentation was carried out in petridishes having diameter of 8cm. 3g of solid substrates were moistened with moisture. The substrate moisture ratio were varied based on the absorbing capacity and clump forming capacity of the substrate. The initial conditions taken for the solid state fermentation were, substrate-3g, particle size-0.85mm, moisture content- added based on clump forming capacity, inoculum size-10%(v/w) (Puri *et al.,* 2005), incubation period-5days, Naringin(Inducer) concentration-0.001%, Inoculum age-5 days old culture(Shanmugaprakash *et al.,* 2011). These are theimportant parameters for the production of Enzyme on Solid State Fermentation. The influence of these parameters were studied by one-factor at a time method.

3.6 EXTRACTION OF ENZYME:

0.2M sodium acetate buffer (pH 4) was added in the growth medium(substrate:buffer-1:10(w/v)), and it was kept in shaker for 1 hour at 120rpm (kubota, model no-CD1499- G000, Tokyo). Then the enzyme was extracted in centrifuge tubes by centrifuging at 10000rpm for 10 min.

3.7 NARINGINASE ASSAY:

The assay for naringinase activity was carried out by the Thammawat method using naringin as substrate (Thammawat *et al.*, 2008).

3.8 ESTIMATION OF PROTEIN CONCENTRATION:

Determination of protein concentration was carried out by lowry's method (Lowry *et al.*, 1951).

3.9 SELECTION OF SUBSTRATE:

To select a suitable substrate, 5 different substrates were taken at a particle size of 0.85mm. And the effect of substrates on enzyme production was studied by one factor at a time method. i.e. 5g of different substrates were taken and all other parameters which influence on enzyme production were kept constantly.

3.10 EFFECT OF PARTICLE SIZE ON ENZYME PRODUCTION:

An optimized suitable substrate were taken with different particle size. The substrate particle sizes are 3.35mm, 2.50mm, 1.4mm, 0.85mm, 0.25mm. Solid state fermentation were carried out by not changing other parameters except particle sizes.

Figure 3.2 Effect of particle size on enzyme

3.11 EFFECT OF MOISTURE VOLUME ON ENZYME PRODUCTION:

The substrate, moisture ratio was varied from 1:1 to 1:2.5(w/v) to study the effect of moisture volume. The above optimized conditions and other parameters were kept constantly.

3.12 EFFECT OF INOCULUM SIZE AND INCUBATION TIME ON ENZYME PRODUCTION:

Above optimized suitable substrate, particle size and substrate:moisture content were taken for further studies. A 120 h old seed culture was used as inoculum. The inoculum size of 5–20% (w/v) was inoculated in the petri dishes containing moistened solid substrates and which was kept for different days of incubation(3^{rd} day to 9^{th} day)and other parameters were kept constantly to find the effect of inoculum size and incubation period on the enzyme production.

3.13 EFFECT OF INDUCER CONCENTRATION ON ENZYME PRODUCTION:

The effect of the inducer concentration were studied by adding naringin at different concentration in the moisture(0.1%, 0.05%, 0.001%). Other optimized and unoptimized parameters were kept constantly.

3.14 EFFECT OF INOCULUM AGE ON ENZYME PRODUCTION:

A loop full of culture was inoculated in 500ml flask containing 100ml sterilized czapek Dox broth and kept in rotary shaker at 120rpm (kubota, model no-CD1499- G000, Tokyo). Then the culture was taken aseptically at a regular interval and it was inoculated on moistened substrate, and other optimized conditions were used for solid state fermentation. The study effect of inoculum age was started with one day grown culture. And the study was continued upto tenth day.

3.15 MEDIA OPTIMIZATION:

3.15.1 Placket-Burman design:

The culture conditions were studied by one-factor at a time method. After that, an experimental design was adopted to find the influence of nutrients like Carbon, Nitrogen sources and mineral salts. Experimental designs were carried out using Minitab 16 software(full version). Experimental design was used for the optimization of media for naringinase production by *A. niger*. This method allows for the improvement in the medium composition. Twenty experiments were preformed for 12 components. Experiments were carried out for four times. The growth medium contained carbon sources (Maltose , Rhamnose, glucose, Sucrose), inorganic and organic nitrogen sources (Peptone , Sodium Nitrite, Urea), and various trace metals (Zinc Sulphate, Magnesium Sulphate, Calcium Chloride , Ferric chloride , manganese Sulphate). Moisture solution were prepared using all those carbon, nitrogen sources and metal ions based on the experimental runs. And naringin also added with moisture at 0.001% which was already optimized by one factor at a time method. In Table 3.1, the rows represent 20 different experiments and each column represents a different component. For each component, high (H) and low (L) concentrations were tested.

Table 3.1: Different levels of constituents used in the first optimization step of medium for Naringinase production by *A. niger* in solid state fermentation

VARIABLES	NUTRIENTS	LOW(-1)	HIGH(+1)
A	Maltose(g/L)	1	5
B	Rhamnose(g/L)	1	5
C	Glucose(g/L)	1	5
D	Sucrose(g/L)	1	5
E	Peptone(g/L)	1	3
F	Sodium Nitrite(g/L)	1	3
G	Urea(g/L)	1	3
H	Zinc Sulphate(mM)	1	5
J	Magnesium Sulphate(mM)	1	5
K	Calcium chloride(mM)	1	5
L	Ferric chloride(mM)	1	5
M	Manganese Sulphate(Mm)	1	5

Table 3.2: Placket-Burman design of experimental runs used for Naringinase production by *Aspergillus niger*

Run	A	B	C	D	E	F	G	H	J	K	L	M
1	+	+	+	-	-	+	+	-	+	+	-	-
2	+	+	-	-	-	-	+	-	+	-	+	+
3	-	+	-	+	-	+	+	+	+	-	-	+
4	+	-	-	+	+	-	+	+	-	-	-	-
5	-	-	+	+	-	+	+	-	-	-	-	+
6	-	-	-	-	-	-	-	-	-	-	-	-
7	+	+	-	-	+	+	-	+	+	-	-	-
8	-	+	+	+	+	-	-	+	+	-	+	+
9	+	-	+	-	+	+	+	+	-	-	+	+
10	-	-	-	+	-	+	-	+	+	+	+	-
11	+	-	-	-	-	+	-	+	-	+	+	+
12	+	-	+	+	-	-	-	-	+	-	+	-
13	+	+	+	+	-	-	+	+	-	+	+	-
14	-	+	+	-	-	-	-	+	-	+	-	+
15	-	-	+	-	+	-	+	+	+	+	-	-
16	+	-	+	+	+	+	-	-	+	+	-	+
17	-	+	+	-	+	+	-	-	-	-	+	-
18	-	+	-	+	+	+	+	-	-	+	+	-
19	+	+	-	+	+	-	-	-	-	+	-	+
20	-	-	-	-	+	-	+	-	+	+	+	+

3.15.2 Response Surface Methodology:

In order to characterize how the significant factors affect the enzyme production, we attempted to improve the composition of the medium by comparing different levels of several factors that were found to have more influence on the production of naringinase. Based on the results obtained in preliminary experiments, maltose, Calcium chloride, Ferric chloride were found to be the major variables in the Naringinase production . Moisture solution were prepared Using maltose, Calcium chloride, Ferric chloride and naringin (0.001% naringin , which was already optimized by one factor at a time method) were added to the substrate. Box-Behnken method was used to find the optimal concentrations of these three factors. In this regard, a set of 15 experiments were carried out. Each variable was studied at two different levels (−1, +1) and center point (0) which is the midpoint of each factor range. The minimum and maximum range of variables investigated. All experiments were carried out in duplicates.

Table 3.3: Experimental range of the three variables studied using Box-Bhenken design

VARIABLES	SYMBOLS	CODED VALUES		
		Low(-1)	Mid(0)	High(+1)
Maltose(g/L)	A	5	15	25
Calcium Chloride(mM)	B	5	15	25
Ferric Chloride(mM)	C	5	15	25

Maltose-X_1, Calcium chloride-X_2, Ferric chloride-X_3

The second-order polynomial equation that defines predicted response (Y) in terms of the independent variables ($X1$, $X2$, and $X3$) was obtained:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_1^2 + b_{12}X_2^2 + b_{33}X_3^2 + b_{12}X_1X_2 + b_{23}X_2X_3 + b_{13}X_1X_3$$

Where bo is intercept term, $b1$, $b2$, $b3$ linear coefficients, $b11$, $b22$, $b33$ squared coefficients and $b12$, $b23$, $b13$ are interaction coefficients. Combinations of factors (such as $X1X2$) represent an interaction between the individual factors in that term.

Table 3.4: Response Surface design of experimental runs used for Naringinase production by *Aspergillus niger*

Run	A	B	C
1	0	-1	-1
2	1	0	-1
3	-1	0	1
4	-1	1	0
5	0	-1	1
6	0	0	0
7	-1	-1	0
8	0	0	0
9	0	1	1
10	1	-1	0
11	1	0	1
12	0	0	0
13	-1	0	-1
14	1	1	0
15	0	1	-1

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 SELECTION OF SUBSTRATE:

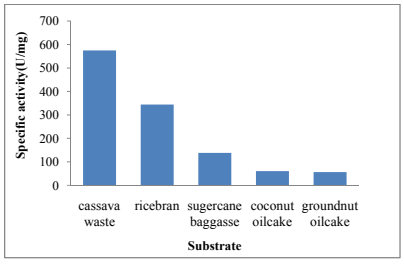


Figure 4.1 Selection of substrate

Above figure shows the production of naringinase using five different agro wastes as substrates. Among all the substrates, maximum specific activity was found for cassava waste than other substrates. The specific activity of each substrate is given.

Substrate	Specific Activity(U/mg)
Cassava waste	654.92
Ricebran	344.03
Sugarcane baggasse	137.98
Coconut oilcake	60.64
Groundnut oilcake	56.176

Figure 4.2 Cassava waste

4.2 SELECTION OF PARTICLE SIZE:

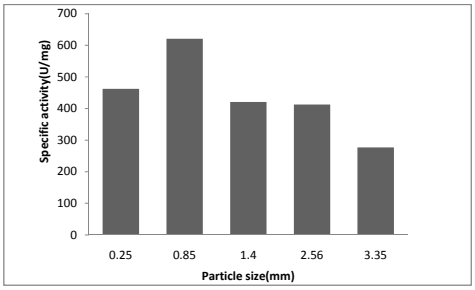


Figure 4.3 Selection of particle size

Another important factor in enzyme production by solid state fermentation is the selection of particle size. Particle size of the solid substrate also affects the enzyme production.

For this study five different sizes of particles used are 3.35mm, 2.56mm, 1.4mm, 0.85mm and 0.25. The specific activities found for different particle sizes 0.25mm, 0.85mm, 1.4mm , 2.56mm and 3.35 are 462.43(U/mg), 621(U/mg), 421(U/mg), 412.6(U/mg), and 276.68(U/mg) respectively. greater activity was found for the particle size 0.85mm.

However, too small substrate particles may result in substrate agglomeration in most cases, which may interfere with aeration and may thus result in poor growth. At the same time, larger particles provide better aeration efficiency (due to increased inter-particle space) but provide limited surface for microbial attack. Thus it may be necessary to provide a compromised particle size (pandy *et al*., 2000, Membrillo *et al.,* 2011).

4.3 EFFECT OF MOISTURE CONTENT ON ENZYME PRODUCTION:

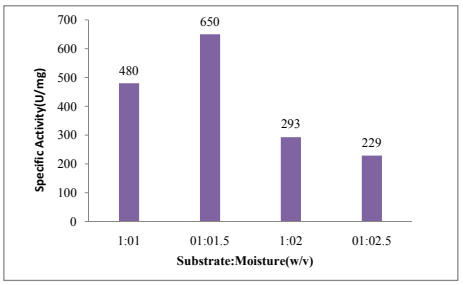


Figure 4.4 Effect of moisture content on enzyme production

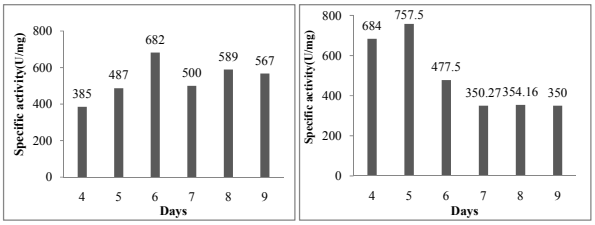
Water content is a parameter that represents the diffusional properties of a slightly hydrated medium(Meste *et al.,*1998). In SSFs, aeration has essentially two functions (1) oxygen supply for aerobic metabolism, and (2) removal of CO_2, heat, water vapour, and volatile components produced during the metabolism (Chahal., 1983, Tabak and Cooke., 1968).

The moisture level plays a vital role in SSF, Low moisture content is known to decrease the solubility of the nutrients, lead to low substrate swelling and higher water tension. Moisture levels higher than certain critical level cause low Oxygen transfer, decreasing porosity, decreasing microbial growth, and lower metabolic activities in SSF(Hamidi *et al.,2004*).

To find the effect of moisture content the volume of moisture was varied (from 1:1 to 1:2.5(Substrate:moisture volume(w/v)). Maximum activity was found for the substrate: moisture ratio 1:1.5(w/v) and the activity is 650(U/mg).

4.4 EFFECT OF INCUBATION TIME AND INOCULUM SIZE ON ENZYME PRODUCTION:

Inoculum size 5%: **Inoculum size 10%:**



Inoculum size 15%: **Inoculum size 20%:**

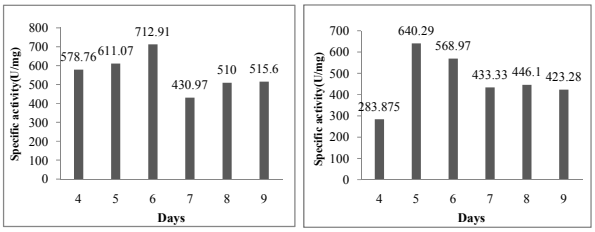


Figure 4.5 Effect of Incubation time and Inoculum size on enzyme production

Among all these, Inoculum size 10% given maximum yield on 5th day when compare to others. Inoculum size and Incubation time plays a major role in the production of enzyme in solid state fermentation. The reason for this is discussed by Mudgetti, 1986. A low inoculum density may give insufficient biomass causing reduced product formation, whereas a higher inoculum may produce too much biomass and deplete the substrate of nutrients necessary for product formation. So inoculum size and incubation time are the major factors, which affects the enzyme production.

The incubation period is directly related with the production of enzyme and other metabolic process up to a certain extent. It might be due to the depletion of nutrients in the medium which stressed the fungal physiology resulting in the inactivation of secretary machinery of the enzymes.(Nochur *et al.,*1993). The specific activity for Inoculum size 10% on 5th day incubation is 757.5(U/mg).

4.5 EFFECT OF INDUCER CONCENTRATION ON ENZYME PRODUCTION:

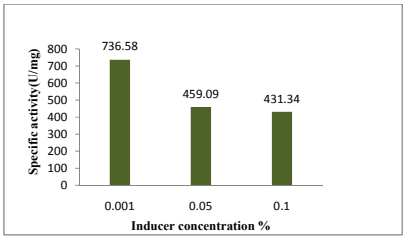


Figure 4.6 Effect of Inducer concentration on enzyme production:

Naringinase is an inducer dependent enzyme. Effect of inducer concentration was studied by varying the concentration of inducer. The above optimized conditions were taken for the study of different concentration of naringin. The studied

concentrations were 0.001%, 0.05%, 0.1%. Maximum activity was found for 0.001% of inducer concentration. And the activity is 736.58(U/mg).

4.6 EFFECT OF INOCULUM AGE ON ENZYME PRODUCTION:

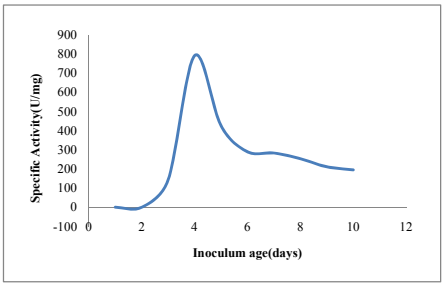


Figure 4.7 Effect of inoculum Age on enzyme production

The moistened substrate was inoculated with the inocula *Aspergillus niger*. Age of the inoculam ranging from 1-10 days, was used to optimize the effect of inoculum age. It was observed that maximum Naringinase production of 790.71 U/mg was obtained with 4^{th} day old inoculum of *A.niger*.

Further increase in the inoculum age, resulted in decrease of enzyme production, which may be due to the occurrence of microbial death phase (Suresh and Jaya Raju., 2013).

4.7 SCREENING OF SIGNIFICANT NUTRIENTS USING A PLACKETT–BURMAN DESIGN:

Plackett–Burman design was used as a screening method to determine which of the 12 factors significantly affect enzymatic hydrolysis procedure; this is achieved by simultaneously shifting variables from a low value to a high value.

Table 5.1: Plackett–Burman design- Experimental runs and Specific activities:

Run	1	2	3	4	5	6	7	8	9	10
Specific activity(U/mg)	600	669	341	431	359	74.50	600	720	450.4	701.01
Standard Deviation	7.07	0	4.94	2.12	34.6	14.14	13.0	14.14	13.03	27.57

Run	11	12	13	14	15	16	17	18	19	20
Specific activity(U/mg)	900	501	675	445	270	600	247.15	700	928.57	526.50
Standard Deviation	14.14	6.36	3.53	6.91	7.60	7.07	26.16	27.47	20.20	20.15

In Plackett–Burman design, the activity of the enzyme Nariginase varied between 74.50 to 928.57 (U/mg). The experimental results were interpreted based on the estimation of how each of the factors affected the response. Those effects were estimated by statistical analysis.

Table 5.2: Plackett–Burman design – effects of nutrient sources on the final naringinase enzyme production.

Term	Effect	Coef	SE Coef	T	P
Constant		536.96	28.46	18.87	0.000
Maltose(g/L)	197.09	98.54	28.46	3.46	0.011
Rhamnose(g/L)	111.23	55.61	28.46	1.95	0.092
Glucose(g/L)	-100.40	-50.20	28.46	-1.76	0.121
Sucrose(g/L)	117.40	58.70	28.46	2.06	0.078
Peptone(g/L)	20.82	10.41	28.46	0.37	0.725
Sodium Nitrite(g/L)	25.80	12.90	28.46	0.45	0.664
Urea(g/L)	-69.53	-34.76	28.46	-1.22	0.261
Zinc Sulphate(mM)	3.77	16.39	28.46	0.58	0.583
Magnesium Sulphate(mM)	31.78	15.89	28.46	0.56	0.594
Calcium chloride(mM)	195.30	97.65	28.46	3.43	0.011
Ferric chloride(mM)	144.10	72.05	28.46	2.53	0.039
Manganese Sulphate(Mm)	113.99	56.99	28.46	2.00	0.085

R-Sq = 87.24% , R-Sq(adj) = 65.36%

The R^2 was determined to be 0.8724, which showed the model was quite a good fit.

Table 5.2 shows the standardized effects of each variable on the Naringinase production. These effects were determined by the difference between the average of measurements made at the high level and the low level of the factor.

The negative sign shows that the shift of the variable from the low level to the high level produced a decrease in released glucose, while the positive sign means that this change increased such response (Haaland, 1989).

The analysis of variance (ANOVA) for the experimental designs was calculated, and the significant levels of each medium variable were determined by *t*-test (Table 5.1).

The analysis showed that ten variables had significant influence on the Enzyme production.

From the table 5.2 maltose, Calcium chloride and Ferric chloride had highly significantly influence on the Naringinase production. These three parameters showed maximum activity in the earlier studies also (Puri *et al.*,2005). The other insignificant variables like Urea and Glucose were neglected and optimum combinations of these three were further analyzed by a Box–Behnken design.

4.8 FURTHER OPTIMIZATION OF THE NUTRIENTS USING A BOX–BEHNKEN DESIGN:

Experiments were carried out in duplicates to find an optimum combination of the three nutrients using a Box–Behnken design. Based on the results of Plackett–Burman experiments, maltose, Calcium chloride and Ferric chloride had significantly positive effect on the naringinase production and their concentrations were increased above high levels in Box–Behnken design experiments. The results obtained were submitted to ANOVA on Minitab 16.

The goodness of fit of the model was checked by determination coefficient (R^2). In this case, the value of the determination coefficient ($R^2 = 0.972$).The value of the adjusted determination coefficient (Adj$R^2 = 0.923$) was also very high to advocate for a high significance of the model.

Table 5.3: Response surface methodology- Experimental runs and Specific activities:

Run	Specific Activity(U/mg)	Standard Deviation
1	282.96	2.02
2	231.22	1.64
3	77.58	0.65
4	339.01	0.70
5	529.20	1.41
6	860.30	4.73
7	502.25	2.29
8	868.20	5.79
9	90.01	3.52
10	426.52	2.48
11	322.22	1.25
12	857.80	1.55
13	220.00	2.12
14	231.58	1.82
15	475.77	5.49

Table 5.4: Response Surface Methodology – effects of nutrient sources on the final Naringinase enzyme production:

Term	Coef	T	P
Constant	862.1	20.44	0.000
A	9.088	0.352	0.739
B	-75.570	-2.926	0.033
C	-23.868	-0.924	0.398
A*A	-309.495	-8.141	0.000
B*B	-177.765	-4.676	0.005
C*C	-339.850	-8.939	0.000
A*B	-7.925	-0.217	0.837
A*C	58.355	1.598	0.171
B*C	-158	-4.326	0.008

R-Sq = 97.28% R-Sq(adj) = 92.38%

The second-order polynomial equation that defines predicted response (Y) in terms of the independent variables ($X1$, $X2$, and $X3$):

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_1^2 + b_{12}X_2^2 + b_{33}X_3^2 + b_{12}X_1X_2 + b_{23}X_2X_3 + b_{13}X_1X_3$$

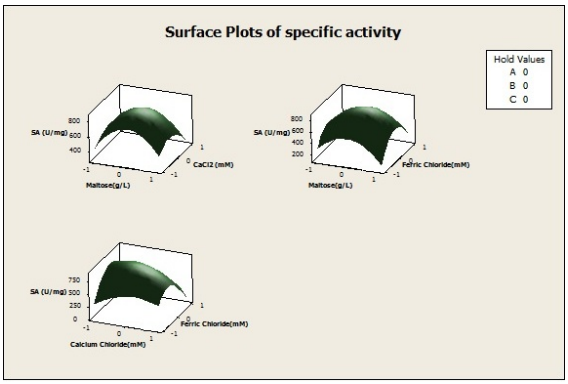
$$Y = 862.1 + X_1 9.088 + X_2(-75.570) + X_3(-23.868) + X_1^2(-309.495)^2 + X_2^2(-177.765)^2 + X_3^2(-399.850)^2 + X_1X_2(-7.925) + X_2X_3(-158) + X_1X_3(58.355)$$

Y is the predicted response corresponding to the enzyme production at the end of the fermentation process. X1, X2, X3 are independent variables, b_0 is an offset term, b_1, b_2, b_3 are linear effects and b_{12}, b_{13}, b_{23} are interaction terms.

For a good statistical model the R^2 value should be close to 1.0 where a value of 0.75 indicates the aptness of the model (Niladevi *et al*., 2009). The regression analysis of the data showed a good aptness of the proposed model.

The fitted response for the above regression model is plotted in Figure 5.1. Three-dimensional graphs were generated for the pair-wise combination of the three factors, while keeping the other one at their optimum levels for naringinase production.

Figure 5.1:Effect of each variable on Naringinase Production.



The Enzyme activity in the optimized medium from three replications (i.e., 860.30, 868.20, 857.80(U/mg)) was coincident with the predicted value and the model was proven again to be adequate. It is the highest yield reported in literature to date. The final concentration of medium optimized with RSM was Maltose-15g/L, Calcium Chloride15mM and Ferric chloride-15mM. The same results were found by Puri *et al.*,(2005), using one factor at a time method.

CONCLUSION

The present study indicated that, Cassava waste could be effectively used as substrate for the production of enzyme naringinase by solid state fermentation. The effect of incubation time, inoculum size, inoculum age, particle size of the substrate, subsrate:moisture ratio, concentration of inducer was studied and optimized by one factor at time method. Then fermentation media was optimized by standard statistical procedures like Placket Burman Design and Response Surface Methodology. The maximum activity was found for the media containing maltose-15g/l, Calcium chloride-15mM, Ferric chloride-15mM and naringin-0.001%. From the results obtained it is evident that cassava waste can be used for naringinase enzyme production by *A.niger* under optimized conditions.

REFERENCES

Ahuja, S.K., Ferreira, G.M., Moreira, A.R. " Application of Plackett–Burman design and response surface methodology to achieve exponential growth for aggregated shipworm bacterium", Biotechnol. Bioeng, Vol.85, pp.666–675, 2005.

Ashok Pandey. " Solid-state fermentation", Biochemical Engineering Journal, Vol.13, pp. 81–84. 2003.

Avila, M., Jaquet, M., Moine, D., Requena, T., Palaez, C., Arigoni, F. "Physiological and biochemical characterization of the two rhamnosidase of Lactobacillus plantarum", Microbiology, Vol.155, pp.2739–2749, 2009.

Bandaru, V.V.R. , Somalanka, S.R., Menduc, D.R., Madicherla, N.R., Chityala, A. "Optimization of fermentation conditions for the production of ethanol from sago starch by co-immobilized amyloglucosidase and cells of *Zymomonas mobilis* using response surface methodology", Enzyme Microb. Technol, Vol.38, pp.209–214, 2006.

Beekwilder, J., Marcozzi, D., Vecchi, S., deVos, R., Janssen, P., Francke, C., Hall, R.D. "Characterization of rhamnosidases from Lactobacillus plantarum and Lactobacillus acidophilus", App Environ Microbiol, Vol.75, pp.3447–3454, 2009.

Carla M. Stinco, Rocio Fernandez-Vazquez, Dolores Hernanz, Francisco J. Heredia, Antonio J. Melendez-Martinez, Isabel M. "Vicario Industrial orange juice debittering: Impact on bioactive compounds and nutritional value", Journal of Food Engineering Vol. 6, pp.155–161, 2013.

Chahal, D.S. "Growth characteristics of microorganisms in solid state fermentation for upgrading of protein values of lignocelluloses and cellulases production" ,Am. Chem. Soc. Symp. Ser. 207, pp.421–442, 1983.

Chandler, B.V., Nicol, K.J. "Some relationships of naringin: their importance in orange juice bitterness" ,CSIRO Food Res Quart, Vol. 35, pp. 79-88, 1975.

Chang, H.Y., Lee, Y.B., Bae, H.A., Huh, J.Y., Nam, S.H., Sohn, H.S., Lee, H.J., Lee, S.B. "Purification and characterization of Aspergillus sojae naringinase: the production of pruning exhibiting markedly enhanced solubility with in vitro inhibition of HMG-CoA reductase", Food Chem, Vol.124, pp.234–241, 2011.

Chisti, Y. "Solid substrate fermentations, enzyme production, food enrichment. In: Flickinger MC, Drew SW, editors. Encyclopedia of Bioprocess Technology: Fermentation, Biocatalysis, and Bioseparation", New York: Wiley, Vol. 5, pp. 2446-2462, 1999.

Couture, R., Rouseff, R., "Debittering and deacidifying sour orange (Citrus aurantium) juice using neutral and anion exchange resins". Journal of Food Science. Vol.57, pp.380-384, 1982.

Daniels, L., Linhardt, R.J., Bryan, B.A., Mayerl, F., Pickenhagen, M. "Methods for producing rhamnose", US Patent Vol. 4,933, pp.281, 1990.

Del Nobile M.A., Piergiovanni, L., Buonocore, G.G., Fava, P., Puglisi, M.L., Nicolais, L. "Naringinase immobilization in polymeric lms intended for food packaging Applications". Journal of Food Science, Vol.68, pp.2046-2049, 2003.

Dong-xiao Chen1, Tian-gui Niu and Hui-nong Cai. "Optimizing culture medium for debittering constitutive enzyme naringinase production by *Aspergillus oryzae* JMU316", African Journal of Biotechnology, Vol. 9(31), pp. 4970-4978, 2010.

Emrah Nikerel, I., Ebru Toksoy, Betul Kırdar, Ramazan Yıldırım "Optimizing medium composition for *TaqI* endonuclease production by recombinant *Escherichia coli* cells using response surface methodology" Process Biochemistry, Vol.40, pp.1633-1639.

Ferreira, S.L.C., Bruns, R.E., Ferreira, H.S., Matos, G.D., David, J.M., Brandao, G.C., da Silva, E.G.P., Portugal, L.A., Reis, P.S., Souza, A.S., dos Santos, W.N.L. " Box Behnken design: an alternative for the optimization of analytical methods", Anal. Chim. Acta, Vol.597, pp.179–186, 2007.

Gallego, M.V., Pinaga, F., Ramon, D., Valles, S. "Production and characterization of an Aspergillus terreus α-L-rhamnosidase of oenological interest", J Food Sci ,Vol.66, pp.204–209, 2001.

Gaston Orillo, A., Ledesmaa, P., Delgadoa, O.D., Spagna, G., Breccia, J.D. "Cold-active α-l-rhamnosidase from psychrotolerant bacteria isolated from a sub-Antarctic ecosystem" Enz Microbiol Technol, Vol.40, pp.236–241, 2000.

Grassin, C., Fauquembergue, P. "Fruit juices. In: Godfrey T, West S, editors. Industrial Enzymology", 2nd edition. New York: Macmillan Press, pp. 225-264, 1996.

Gonzalez Vazquez, R., Azaola Espinosa, A., Osorio-Revilla, G., Gallardo Velazquez, T.,

Cruz Victoria1, T., Arana Errasquin, R., and Rivera Espinoza, Y., "The effect of different carbon sources and salts in the production of naringinase by *aspegillus niger* ATCC1015", Revista Mexicana de Ingenieria Quimica, Vol. 10, pp. 1-8, 2011.

Haaland, P.D. "Experimental Design in Biotechnology", Marcel Dekker, Inc., New York, 1989.

Habelt, K., Pittner, F. "A rapid method for the determination of naringin, prunin, and naringin applied to the assay of Naringinase", Analytical Biochemistry, Vol. 134, pp.393–397, 1983.

Hall, D. " A new enzyme of the glycosidase type", Chem Ind Vol.57, pp.473, 1938.

Hamelinck, C.N., Hooijdonk, G., Faaij, A.P.C., "Ethanol from Lignocellulosic Biomass: Techno-Economic Performance in Short-, Midlle- and Long-Term", Biomass Bioenergy, Vol. 28, pp.384-410, 2005.

Hamidi-Esfahania, Z., Shojaosadatia, S.A., Rinzemab, A. " Modelling of simultaneous effect of moisture and temperature on *A. niger* growth in solid-state fermentation", Biochemical Engineering Journal, Vol.21, pp. 265–272, 2004.

Hashimoto, W., Murata, K. "Alpha-L-rhamnosidase of Sphingomonas sp. R1 producing an unusual exopolysaccharide of sphingan", Biosci Biotechnol Biochem, Vol.62, pp.1068–1074, 1998.

Hernandez, E., Couture, R., Rouseff, R., Chen, C.S., Barros, S., "Evaluation of ultrafiltration and adsorption to debitter grapefruit juice and grapefruit pulp wash" Journal of Food Science, Vol.57, pp.664-670, 1992.

Hui Ni, Feng Chen, Huinong Cai, Anfeng Xiao, Qi You and Yunzhen Lu. "Characterization and Preparation of *Aspergillus niger* Naringinase for Debittering Citrus Juice", Journal of Food Science , Vol. 71, Nr.1, 2012.

http://www.greatvistachemicals.com/proteins-sugars-nucleotides/l-rhamnose.html

http://www.itl.nist.gov/div898/handbook/pri/section3/pri3363.htm

http://www.sepscience.com/Sectors/Miscellaneous/Articles/161-/Optimization-of-Analytical-Methods-Using-Factorial-Designs----Part-2-A-Deeper-Look-at-Factorial-Designs

Ilamathi Radhakrishnan, Shanmugam Sampath & Satishkumar T. "Optimization of medium composition for improving naringinase activity using response surface methodology"International Journal of Bio-Technology, Vol.2, pp.29-36, 2012.

Jang, I.S., Kim, D.H. "Purification and characterization of alpha-Lrhamnosidase from Bacteroides JY-6, a human intestinal bacterium", Biol Pharm Bull, Vol.19, pp.1546–1549, 1996.

Kar, B., Banerjee, R., Bhattacharyya, B.C. "Biotransformation of tannic acid to gallic acid by solid state fermentation. In:Advances in Biotechnology", New Delhi: Educational Publishers, pp.67–74, 1998.

Kefford, J.F., "The chemical constituents of citrus fruits", Advanced Food Res; Vol.9, pp.285, 1959.

Kimball, D.A. "A research note on debittering of citrus juice using supercritical carbon Dioxide", Journal of Food Science, Vol.52, pp. 481-482, 1987.

Kurosawa, Y., Ikeda, K., Egami, F. "α-L-rhamnosidase of the liver of Turbo cornutus and Aspergillus niger", J Biochem, Vol.73,pp.31–37, 1973.

Koseki, T., Mese, Y., Nishibori, N., Masaki, K., Fuhii, T., Handa, T., Yamane, Y., Shiono, Y., Murayama, T., iefuji, H., "Characterization of an α-L-rhamnosidase from Aspergillus kawachii", Appl Microbiol Biotechnol, Vol.80, pp.1007–1013, 2008.

Laluce, C., Tognolli, J.O., Oliveira, K.F., Souza, C.S., Morais, M.R. "Optimization of temperature, sugar concentration, and inoculum size to maximize ethanol production without significant decrease in yeast cell viability", Appl. Microbiol. Biotechnol, Vol.83, 6pp.27–637, 2009.

Lei, S., Xu, Y., Fan, G., Xiao, M., Pan, S. "Immobilization of naringinase on mesoporous molecular sieve MCM-41 and its application to debittering of white grapefruit", Appl Surface Sci, Vol.257, pp.4096–4099, 2011.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, J. R.,Protein measurement with the Folin phenol reagent. J. Biol. Chem, Vol.193, pp.265–275, 1951.

Maeda, H., Takahashi, Y., Miyake, M., Ifuku, Y. “Studies on the quality improvement of citrus juices and utilization of peels with ion exchange resins 1ms Removal of bitterness and reduction of acidity in Hassaku (c. Hassaku hort. Ex. Tanaka) juice with ion exchange resins and adsorbents”, Nippon Shokuhin Kogyo Gak, Vol.31, pp.413-420, 1984.

Mamma, D., Kalogeris, E., Kekos, D., Macris, B.J., Christakopoulus, P. “Biochemical characterization of the multi-enzyme system produced by *P. decumbens* grown on ruitn” Food Biotechnol Vol.18.pp.1–18, 2004.

Manzanares, P., de Graaff L.H., Visser, J. “Production and characterization of an α-L-rhamnosidase from Aspergillus niger” FEMS Microbiol Lett, Vol.157,pp.279–283, 1997.

Manzanares, P., van den Broeck, H., de Graaff, L.H., Visser, J. “Purification and characterization of two different α-L-rhamnosidase, RhaA and RhaB from Aspergillus aculeatus”, Appl Environ Microbiol, Vol.67,pp.2230–2234, 2001.

Maria H.L. Ribeiro, Cristina Afonso, Helder J. Vila-Real, Antonio J. Alfaia, Luis Ferreira . “Contribution of response surface methodology to the modeling of naringin hydrolysis by naringinase Ca-alginate beads under high pressure”, Food Science and Technology, Vol.43, pp.482-487, 2010.

Marwaha, S.S., Puri, M., Bhular, M., Kothari, R.M., “Optimization of parameters for the hydrolysis of limonin for debittering Of kinnow mandarin juice by *Rhodococcus fascians*”, Enz Microb Technol, Vol.16: pp. 723- 725, 1994.

Mazzaferro, L.S., Orrillo, G.A., Ledesma, P., Breccia, J.D. “Dosedependent significance of monosaccharides on intracellular α-Lrhamnosidase activity from Pseudoalteromonas sp”, Biotech Letts, Vol.30, pp.2147–2150, 2008.

McMahon, H., Zoecklein, B.W., Fugelsang, K., Jasinski, Y. “Quantification of glycosidase activity in selected yeasts and lactic acid bacteria”, J Ind Microbiol Biotechnol, Vol.23, pp.198–203, 1999.

Membrillo, I., Sanchez, C., Meneses, M., Favela, E., Loera, O., ‘Particle geometry affects differentially substrate composition and enzyme profiles by Pleurotus ostreatus growing on sugar cane bagasse’, Bioresour. Technol. Vol.102, pp.1581–1586, 2011.

Mendoza-Cal, A., Cuevas-Glory, L., Lizama-Uc, G., and Elizabeth Ortiz-Vazquez. “Naringinase production from filamentous fungi using grapefruit rind in solid state fermentation”, African Journal of Microbiology Research, Vol.4(19), pp.1964-1969, 2010.

Miake, F., Satho, T., Takesue, H., Yanagida, F., Kashige, N., Watanabe, K. “Purification and characterization of intracellular alpha-Lrhamnosidase from Pseudomonas paucimobilis FP2001”, Arch Microbiol, Vol.173, pp.65–70, 2000.

Michlmayr, H., Brandes, W., Eder, R., Schumann, C., Hierro, A.M., Kulbe, K.D. “Characterization of two distinct GH family 78a-lrhmanosidases from *Pedicococcus acidilactici*”, Appl Environ Microbiol (in press; doi:10.1128/AEM.05317-11), 2011.

Mitchell, D.H., Pearce, R.M. “Removal of bitter naringin and limonin from citrus juice containing the same” U.S. patent 4,514,427, 1985.

Miyata, T., Kashige, N., Satho, T., Yamaguchi, T., Aso, Y., Miake, F. “Cloning, sequence analysis and expression of the gene encoding Sphingomonas paucimobilis FP 2001 rhamnosidase”, Current Microbiol, Vol.51, pp.105–109, 2005.

Munish Puri and Uttam Chand Banerjee. “Production, purification, and characterization of the debittering enzyme naringinase”, Biotechnology Advances, Vol.18, pp. 207–217, 2000.

Munish Puri , Anirban Banerjee , Banerjee, U.C. “Optimization of process parameters for the production of naringinase by *Aspergillus niger* MTCC 1344”, Process Biochemistry , Vol.40, pp. 195–201, 2005.

Munish Puri, Aneet Kaur, Ram Sarup Singh, Anubhav Singh. “Response Surface Optimization of Medium Components for Naringinase Production from *Staphylococcus xylosus* MAK2” , Appl Biochem Biotechnol, Vol.162, pp.181–191, 2010.

Munish Puri, Aneet Kaur, Colin J. Barrow and Ram Sarup Singh “Citrus peel influences the production of an extracellular naringinase by Staphylococcus xylosus MAK2 in a stirred tank reactor”, Appl Microbiol Biotechnol, Vol. 89, pp. 715–722, 2011.

Munish Puri. “Updates on naringinase: structural and biotechnological aspects” Appl Microbiol Biotechnol, Vol.93, pp.49-60, 2012.

Mutuswamy Shanmugaprakash, Vaidyanathan Vinoth Kumar, Manickavassham Hemalatha, Vargese Melbia, Pothiyappan Karthik. “Solid-state fermentation for the production of debittering enzyme naringinase using Aspergillus niger MTCC 1344”, Engineering Life Science, Vol.11, pp. 322–325, 2011.

Myers, R.H., Montgomery, D.C. “Response Surface Methodology: Process and Product Optimization Using Designed Experiments”, second ed., John Wiley & Sons, New York, 2002.

Niladevi, K.N., Sukumaran, R.K., Jacob, N., Anisha, G.S., Prema, P. “Optimization of laccase production from a novel strain-Streptomyces psammoticus using response surface methodology”, Microbiol. Res, Vol.164, pp.105–113, 2009.

Nochur, S.V., Roberts, M.F., and Demain, A.L., “True cellulase production by clostridium thermocellum grown on different carbon sources”, Biotechnology Letters, vol. 15, pp. 641–646, 1993.

Orejas, M., Ib anez, E., Ramon, D. “The filamentous fungus Aspergillus nidulans produces an α-L-rhamnosidase of potential oenological interest” ,Lett Appl Microbiol,Vol.28, pp.383–388, 1999.

Pandey, A., Azmi, W., Singh, J., Banerjee, U.C. “Types of fermentation and factors affecting it” Biotechnology: Food Fermentation. New Delhi: Educational Publishers, pp.383–426, 1999.

Pandey A. Soccol C.R. Mitchell D.A. “New developments in solid-state fermentation. I. Bioprocesses and products”, Process Biochem, Vol.35 (10), pp.1153-1169, 2000.

Pandey, A., Soccol, C.R., Leo, J.A.R., Nigam, P. “Solid-state Fermentation in Biotechnology”, Asiatech Publishers, Inc., New Delhi, pp. 221, 2001.

Palukurty, M.A., Telgana, N.K., Bora, H.S.R., Mulampaka, S.N. “Screening and optimization of metal ions to enhance ethanol production using statistical experimental designs”, Afr. J. Microbiol. Res, Vol.2, pp.87–94, 2008.

Patrick Gervais, Paul Molin. “The role of water in solid-state fermentation”, Biochemical Engineering Journal, Vol.13, pp. 85–101, 2003.

Puri, S., Beg, Q.K., Gupta, R., “Optimization of alkaline protease production from *Bacillus* sp. by response surface methodology”, Curr. Microbiol. Vol.44, pp.286–290, 2002.

Qian, S., Yu, H., Zhang, C., Lu, M., Wang, H., Jin, F. “Purification and characterization of dioscin-α-L-rhamnosidase from pig liver”, Chem Pharm Bull, Vol.53, pp.911–914, 2005.

Rajal, V.B., Cid, A.G., Ellenrieder, G., Cuevas, C. “Production, partial purification and characterization of rhamnosidase from Penicillium ulaiense”, W J Microbiol Biotechnol, Vol.25, pp.1025–1033, 2009.

Ratnam, B.V.V., Rao, S.S., Rao, M.D., Rao, M.N., Ayyanna, C. “Optimization of medium constituents and fermentation conditions for the production of ethanol from palmyra jaggery using response surface methodology”, World J. Microbiol. Biotechnol. Vol.21, pp.399–404, 2005.

Roitner, M., Schalkhammer, T., Pittner, F. “Characterization of naringinase from *Aspergillus niger*”, Monatsh Cheme, Vol.115, pp.1255- 1267, 1984.

Sanchez, C. “Lignocellulosic Residues: Biodegradation and Bioconversion by Fungi”, Biotechnology Advances, Vol.27, pp. 185-194, 2009.

Sankyo. “Preparation of antibiotic chloropolysporin-C”, Japanese Patent 63, Vol.146, pp.797, 1988.

Santos, M.M., Rosa, A.S., Dal boit, S., Mitchell, D.A., and Kriger, N. “Thermal Denaturation: Is Solid-State Fermentation Really a Good Technology for the Production of Enzymes?”, Bioresource Technology, Vol.93, pp. 261- 268, 2004.

Sarrette, M., Nout, M.J.R., Gervais, P., Rombouts, F.M. “Effect of water activity on production and activity of *Rhizopusoligosporus* polysaccharides”, Appl Microbiol Biotechnol, Vol.37(4), pp.420–425, 1992.

Scaron,i E., Cuevas, C., Carrrillo, L., Ellenrieder, G. “Hydrolytic properties of crude α-L-rhamnosidase produced by several wild strains of mesophillic fungi”, Lett Appl Microbiol Vol.34, pp.461–465, 2002.

Shanmugam, V., Yadav, K.D.S. “Extracellular production of alpharhamnosidase by *Rhizopus nigricans*”, Ind J Exp Biol 33:705–707, 1995.

Subramaniyam, R., and Vimala, R., “Solid State and Submerged fermentation for the production of bioactive substances: a comparative study”, International journal of science and nature, Vol.3, pp.480-486, 2012.

Suresh, J.V., and Jaya Raju, K. “Studies on the Production of L-Asparaginase by *Aspergillus terreus* MTCC 1782 using Agro-Residues under Mixed Substrate Solid State

Fermentation" , Journal of Chemical, Biological and Physical Sciences ,Vol.3, pp. 314-325, 2013.

Suzuki, H. "Hydrolysis of flavonoid glycosides by enzymes from Rhamnus and other sources", Arch Biochem Biophys, Vol.99, pp.476–483,1962.

Tabak, H.H.,, Cooke, W.M. "The effects of gaseous environment on the growth and metabolism of fungi", Bot. Rev, Vol.34, pp.126-252, 1968.

Tatum, J. H., Berry, R.E."Method for estimating limonin content of citrus juices", Journal of Food Sci, Vol.38, pp.1244-1246, 1973.

Thammawat, K., Pongtanya, P., Juntharasri, V., Wongvithoonyaporn, P., "Isolation, preliminary enzyme characterization, and optimization of culture parameters for production of naringinase isolated from Aspergillus niger BCC 25166", Kasetsart J. Nat. Sci, Vol.42, pp.61–72, 2008.

Thomas, D.W., Smythe, C.V., Labbee M.D., "Enzymatic hydrolysis of naringin, the bitter principle of grapefruit", Food Res, Vol.23, pp.591–598, 1958.

Ting, S.V. "Enzymatic hydrolysis of naringin in grapefruit", J Agric Food Chem, Vol.6, pp.546–549,1958.

Vaidyanathan VinothKumar and Periasamy Kayambu Subash RevathiBabu. "Optimization of Fermentation Parameters for Enhanced Production of Naringinase by Soil Isolate Aspergillus niger VB07", Food Science Biotechnology, Vol.19(3), pp.827-829, 2010.

Vinoth Kumar, V. "Comparative studies on inducers in the production of naringinase from *Aspergillus niger* MTCC 1344", African Journal of Biotechnology, Vol. 9(45), pp. 7683-7686, 2010.

Wethern, M. "Citrus debittering with ultrafiltration/adsorption combined technology", pp. 48-46. In: 37th Annual Citrus Engineering Conference, March 21, Lake Alfred, FL, USA, 1991.

Yanai, T., Sato, M., "Purification and characterization of an rhamnosidase from Pichia angusta X349", Biosci Biotechnol Biochem, Vol.64, pp.2179–2185, 2000.

Young, N.M., Johnston, R.A.Z., Richards, J.C. "Purification of the α-vL-rhamnosidase of Penicillium decumbens and characterisation of two glycopeptide components", Carbohydr Res, Vol.191,pp.53–62, 1989.

Yu, J.L., Zhang, X., Tan, T.W. "Optimization of media conditions for the production of ethanol from sweet sorghum juice by immobilized Saccharomyces cerevisiae", Biomass Bioenergy, Vol.33, pp.521–526, 2009.

Yusof, S., Gazali, H.M., King, G.S. " Naringin content in local citrus fruit", Food Chem, Vol.37, pp.113-121, 1990.

Zverlov, V.V., Hertel, C., Bronnernmeier, K., Hroch, A., Kellermann, J., Schwarz, W.H. "The thermostable alpha-L-rhamnosidaseRamA of Clostridium stercorarium: biochemical characterization and primary structure of bacterial alpha-L-rhamnose hydrolase, a new type of inverting glycoside hydrolase", Mol Microbiol, Vol.35,pp.173–179, 2000.