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OPTIMIZATION AND PURIFICATION OF NARINGINASE FROM *Aspergillus flavus*

PROJECT REPORT

Submitted by

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PROJECT WORK -PHASE II

MAY 2010

This is to certify that the project entitled **OPTIMIZATION AND PURIFICATION OF NARINGINASE FROM *Aspergillus flavus*** is the bonafide record of project work done by **R.ILAMATHI Register No: 0820203002** of M.Tech during the year 2009-2010.

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DECLARATION

I affirm that the project work titled **Optimization and Purification of naringinase from *Aspergillus flavus*** being submitted in partial fulfilment for the award of **M.Tech (Biotechnology)** is the original work carried out by me. It has not formed the part of any other project work submitted for award of any degree or diploma, either in this or any other University.

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I certify that the declaration made by the above candidate is true.

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R.ILAMATHI

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ABSTRACT

Naringin is the dominant flavanoid bitter principle in citrus fruit juices. The microrganisms like *Aspergillus niger* and *Aspergillus flavus* were screened for the production of naringinase, in order to hydrolyze the naringin for removal of the bitter taste from juices. It was found that the *Aspergillus flavus* possess better activity. The main aim of this work was the modeling of medium composition for the high production of naringinase, in order to hydrolyze the naringin for removal of the bitter taste from juices, using Plackett-Burman and Response Surface Methodology (RSM). *Aspergillus flavus* was used to produce extracellular naringinase enzyme in the medium and inducers were used to increase the activity of the enzyme. The experiment involves six factors (starch, peptone, Mg^{2+}, pH, naringenin and inoculum size) and the choice of significant factor resulted from Plackett-Burman study was taken for RSM with 97% confidence level. Using the software Minitab 15, it was found that starch and peptone were the most effective for enzyme production (R-Sq=0.8949) indicated that only 4.87% of the total variations were not explained by the model. Moderate amount of starch and peptone were proven to increase the naringinase activity 3.2 times from maximum production of 1158.33 U/L using Plackett-Burman design to 3606.6 U/L using central composite design. The combination of starch and peptone on the catalytic activity of naringinase was investigated at pH 4.0 using naringin as the substrate with the maximum of 3606.6U/lit, at 1.5g and 0.5g of starch and peptone. The enzyme was purified by Ammonium sulfate precipitation, Ion exchange chromatography, and SDS-PAGE. About 8.6 fold purification was obtained.

Abstract

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LIST OF ABBREVATIONS

DNS	DinitroSalicylic
BSA	Bovine Serum Albumin
SDS-PAGE	Sodium Dodecyl sulfate- PolyAcrylamide Gel Electrophoresis
kDa	KiloDaltons
RSM	Response Surface Methodology
DEAE	Diethyl Amino Ethyl
rpm	Rotation Per Minute

1. INTRODUCTION

Naringin, a bitter flavonone glycoside which is responsible for the bitterness in citrus fruits (Akira konno *et al.*, 1982). Naringin (4,5,7-trihydroxy-flavonone 7-rhamnoglucoside) which consists of aglycone naringenin (4,5,7 - trihydroxy-flavonone) and sugar complex of α-L-rhamnose and β-D-glucose (Bailey *et al.*, 2000).

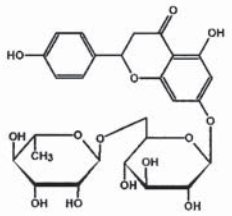


Figure: 1.1 Structure of naringin

Naringinase (E.C.3.2.1.40) is an enzyme which can hydrolyse the naringin into prunin and then into naringenin, which is non-bitter and tasteless. Hence this enzyme has two different enzyme activities on naringin. One is α-L-rhamonosidase (E.C.3.2.1.40) which can act on naringin and it releases prunin (aglycone and β-D-glucose) and α-L-rhamnose, second is β-D-glucosidase(E.C.3.2.1.21) which acts on prunin and releases aglycone naringenin and β-D-glucose(Luis Ferreira *et al., 2008*).

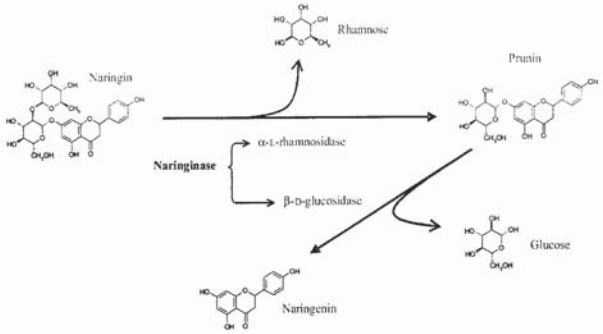


Figure: 1.2 Hydrolysis of naringin into prunin, rhamnose, glucose and naringenin by naringinase containing α-L-rhamnosidase activities and β-D-glucosidase activities

In the processing of citrus fruits the bitter compounds like limonin, naringin, neohesperidin, present in citrus fruits are the problem in clarifying the fruit juices, to maintain the organoleptic and stability of the fruit juices. This enzyme has significant application in fruit juice industry to de-bitter the citrus fruit juices during its processing, and helps to improve the properties and stability of the juices (Munish Puri *et al.*, 2000).

In recent years, Consumer awareness about the health promoting ability of bitter flavanoids such as naringin has generated keen interest in processing of indigenously available fruits such as grapefruit, not only for the export market. Some bitterness in citrus fruit products is acceptable to consumers, but excessive bitterness is one of the major consumer objections to such products. Considearable information ia available on naringin, the principal bitter substance in citrus fruit products. Debittering using the enzyme naringinase has been reported (Puri M *et al.*, 1996) For complete modification degradation of bittering components, concerted efforts were made either to develop soluble enzymes or microbes capable of metabolizing naringin (Puri M *et al.*, 1996). Suitable enzyme have been immobilized on many matrices and their application for the above purposes have been reported (Manjon A *et al.*, 2005).

The use of enzymes with significant industrial application is increasing but there are few reports available on the production of naringinase, most of them are either guarded secrets of the industry or are patented (Ito *et al.*, 1970). The debittering process could be more cost effective and economically viable if naringinase production is achieved industrially using microorganisms

The end product of naringinase on naringin is naringenin is a good pharmacological agent, as anticancer, in the treatment of diseases like atherosclerosis, with some properties like antioxidant, anti-inflammatory, anti-thrombotic and vasodilator (Isabel *et al.*, 2008).

This bitter trihydroxy flavonone naringin also interfere with the drugs like felodipine. This drug is used is a (calcium channel blocker) calcium antagonist, a drug used to control hypertension (high blood pressure). Recent findings have suggested that felodipine in combination with grapefruit juice can cause abnormal (toxic) effects. Oral administration of Felodipine is first metabolized in the gastrointestinal tract (GI) and liver by CYP3A4. Grapefruit juice is found to have an inhibiting effect over cytochrome P450 3A4 (Bailey *et al.*, 2000)

The enzyme has to be purified as it is used in the fruit juice industry. This enzyme has many applications and so the purified enzyme may easy for use. For the industrial use the naringinase can be immobilized to reduce the cost of removing the bitterness from the juice. The structure of the enzyme is not yet predicted. Hence if the sequences were known then it is easy to predict the structure using the software. Many studies were carried out in the immobilization process (Busto MD *et al.*, 2007).

The debittering process could be more cost effective and economically viable if naringinase production is achieved industrially using microorganisms (Munish Puri *et al.*, 2005). Optimization of the medium composition for naringinase production by *Aspergillus flavus* using Response surface methodology in order to predict the best performance conditions with the minimum number of experiments (Giovanni, 1983) and thereby increasing the activity of enzyme. This is a non-conventional method that has been successfully used for the optimization of enzymatic reactions conditions (Ferreira-Dias *et al.*, 2003; Ribeiro et al. 2003), medium composition (Ribeiro *et al.*, 2006; Kapat *et al.*, 1996; Montserrat *et al.*, 1993) and food preservation parameters (King, 1993).

Objective of the Study

With this background, the study was designed with the following objectives

1.1 Objectives

- To screen for naringinase producing fungi (*Aspergillus niger, Aspergillus flavus*)
- To evaluate and characterize naringinase using different parameters like pH, temperature and substrate concentration.
- To study the effect of metal ions on crude naringinase enzyme
- To study the activity of naringinase enzyme using inducers like naringin and naringenin
- To optimize the medium composition for naringinase enzyme using Plackett-Burman and Response Surface Methodology by *Aspergillus flavus*
- To purify the crude naringinase enzyme using Ion Exchange Chromatography

Review of Literature

2. REVIEW OF LITERATURE

2.1 Naringin

Naringin belongs to the group of flavonoids. The structure of naringin is similar to the hesperidin. It is a natural chemical compound known as a bioflavanoid found in grapefruit, giving it its bitter flavor. The naringin and limonin are the two compounds in citrus fruit, which is giving a bitter taste (Akira Konno *et al*., 1982). Due to these compounds the commericial acceptance of the citrus fruit juices is limited (Munish Puri *et al*., 2005). This naringin is present in tissues of many citrus fruits like *Citrus paradisi* (grapefruit), *Citrus lemon* (lemon), *Citrus limetta* (musambi), *Citrus sinensis* (oranges) and *Vitis vinifera* (Grapes) etc.,

This naringin is a potential inhibitor of the drugs including calcium channel blockers, cholesterol lowering drugs, caffeine and estrogen.

2.2 Naringinase

Naringinase is multienzyme complex, which has two enzymes which acts on the sugar complex and aglycone part of the naringin. The released glucose molecule can also be estimated by different biochemical methods for reducing sugars. DNS method for the estimation of glucose has also been followed (Prakash *et al*., 2002). This enzyme also acts on the different substrates like rutin and hesperidin. The action of enzyme on naringin is higher than rutin (Munish Puri *et al*., 2005)

2.3 Source of naringinase enzyme:

The naringinase enzyme was produced by different fungal species were reported (Munish Puri *et al*., 2000).

Table: 2.1 Microroganism used for the naringinase production

Microorganism	References
Aspergillus niger, Aspergillus oryzae	Bram and Solomns, 1965
Cochiobolus miyabeanus	Ito and Takiguchi,1970
Coniothyrium diplodiella	Nomura,1965
Penicillium decumbens	Fukumoto and Okado,1973
Phanopsis citri	Ito and Takiguchi,1970
Rhizotonia solani	Ito and Takiguchi,1970
Rhizopus nigricans	Shanmugam and Yadav,1995

2.4 Production of the enzyme

The production of enzyme from many species was commercially available under many names. So far the *Aspergillus sp.*, and *Penicillium decumbens* producing enzyme were characterized. The companies like Sigma-Aldrich are producing this enzyme from *Penicillium decumbens*.

2.5 Effect of inducers

Many carbon and nitrogen sources were used to induce the organisms like *Aspergillus niger* and *Penicillium decumbens*. Various nitrogen sources were used for *Aspergillus niger* like soya bean meal, yeast extract, whole corn meal, corn steep liquor (Bram and Solomns,1965).The different carbon sources like rhamnose, sucrose, maltose, starch were used to increase the production. Lower concentration of naringin is used for increasing the naringinase enzyme activity (Munish Puri *et al*., 2005).

2.6 Characterization of naringinase enzyme

2.6.1 Effect of pH

The acetate buffer of different values of pH had been used to find its optimum pH. The pH obtained for *Penicillium decumbens* is pH 4.5 (Nourouzian *et al*., 2004). For the mutated *Aspergillus niger* it was found as pH 4.0. But they have reported that the enzyme was still active at pH 3-5. From low pH of 2.8 to 7.0 has been reported in bacteriodes, *A.terreus* (Munish Puri *et al*., 2005). The α-L rhamnosidase from the *Aspergillus nidulans*, was optimally active at pH 4.5-6 (Manzanres *et al*., 2000). The enzyme α-L rhamnosidase had an isoelectric point at 4.9, and was optimally active at pH 6.0 (Takkaaki Yanai and Michikatsku Sato, 2000). The enzyme β-D-glucosidase has an optimal pH at 5.0 (Saha and Bothast, 1996).

2.6.2 Effect of temperature

The optimum temperature had been found by keeping the enzyme at different temperature ranges from 25-60°C. It has been reported as 55°C for *Penicillium decumbens* (Nourouzian *et al*., 2000) and for *Aspergillus niger* it has been found as 45°C (Munish Puri *et al*., 2005). The α-L rhamnosidase from the *Aspergillus nidulans* was optimally active at 60°C (Manzanres *et al*., 2000). The enzyme α-L rhamnosidase had an optimal temperature around 40°C (Takkaaki Yanai and Michikatsku Sato, 2000). The enzyme β-D-glucosidase has an optimal temperature is at 50°C (Saha and Bothast, 1996).

2.6.3 Substrate concentration

The Km Value was reported as 1.7mM in *Penicillium decumbens* (Nourouzian *et al*., 2000) and in *Aspergillus niger* the value was 1.9mM (Munish Puri *et al*., 2005). For the enzyme α-L rhamnosidase with rho-nitrophenyl-alpha-L-rhamnopyranoside as the substrate it showed Km and Vmax values are 0.27 mmol /l and 64.6 U/mg, respectively (Manzanres *et al*., 2000).

2.7 Substrate specificity

The crude enzyme naringinase hydrolysis both the substartes rutin (0.1%) and naringin (0.1%). The enzyme obtained from the *Aspergillus niger* were more specific towards the naringin than rutin (Munish Puri *et al*., 2005). In some cases they have been used hesperidin and quericitin was also used. But it has been reported as the extent of hydrolysis was higher towards the naringin and not towards the other flavonoids (Munish Puri *et al*., 2005). The α-L-rhamnosidase was highly specific for α-L-rhamnopyranoside and liberated rhamnose from naringin, rutin, hesperidin, and 3-quercitrin (Takkaaki Yanai and Michikatsku Sato, 2000).

2.8 Effect of metal ions

Different metal ions were used in the cultivation medium to determine the effects of metal ions on growth and naringinase production by *A. niger*. Cu^{2+}, Co^{2+}, Ni^{2+}, were found to be inhibitory both to growth and naringinase production. Different metal ions were used in the cultivation medium to determine the effects of metal ions on growth and naringinase production by *A. niger*. Cu^{2+}, Co^{2+}, Ni^{2+} were found to be inhibitory both to growth and naringinase production. Zn2+ at a concentration of 5mM showed 38% inhibition of enzyme activity whereas Ca^{2+}, (5–10 mM), Mg^{2+}, (5–10 mM) stimulated naringinase synthesis. In the case of Mg^{2+}, the maximum activity 8.5 IU ml−1 was observed at 10mM which accounted for a 31% increase in enzyme activity. Above 10mM enzyme activity decreased drastically. Ca^{2+} at 5–10mM also supported maximal production (7.4 IU ml−1) of naringinase whereas at higher (30 mM) concentrations, a small decrease (6 IU ml−1) in enzyme activity was observed. This suggests that Mg^{2+} and Ca^{2+} ions are required for the production of naringinase by *A. niger*. Fe^{2+} and Mn^{2+} show an inhibitory action on growth and enzyme production by *A. niger* (Munish Puri *et al*., 2005).

2.9 Optimization of medium composition

Selection of the best sources of different nutrients, such as carbon, nitrogen, minerals, trace metals, buffering agents, growth promoters and inducer/precursor of the product, is one of the most critical stage in optimization of any fermentation process, as it determines the overall economic feasibility of the process. One faces a formidable task of screening a large

number of chemicals under each of these categories and the exercise dictates the requirement of conducting a large number of experiments, an involvement of lot of time as well as resources, pressing of infrastructural facility which otherwise could have been utilized for other purposes, and the consequent delay in industrial exploitation of the process. In the traditional method, such screening for each category of the sources, is done at an arbitrarily selected level of each source, one category at a time, while keeping the other parameters or ingredients constant, again at an arbitrarily selected levels. Difficulty is also faced in selecting the levels at which to fix the ingredients of other categories and also the selection of their sources. The data thus generated are used to select a few compounds in each category, based on highest product promotion. Different levels of the selected compound are then evaluated to achieve optimum level. The interactive effects among the sources of different categories are ignored completely in such traditional methods for want of better practices or exposure to design methods (Srinivasan, 1979)

2.9.1 Plackett-Burman design and Response Surface Methodology

Different strategies can be used for the optimization of cultivation conditions (Mao *et al*., 2007; Farid *et al*., 2000). Conventional "one-variable-at-a-time" approach was used more often than other approaches, but it is time-consuming and likely leads to confusion in understanding the process parameters (Kumar and Satyanarayana, 2007). Some statistical techniques, such as Plackett-Burman design and response surface methodology (RSM), were proved to be useful for developing, improving and optimizing processes, and were extensively used in the industries and in bioprocesses including the formulation of culture medium for bacteria and fungi (Didier *et al*.,2007).

Fractional factorial, in the class of factorial designs, provides one solution to tackle the large number of sources of a category at a time. However, this method also requires a considerably large number of experiments. Moreover, the results obtained are generally not the pure effects of the sources as these are confounded with the effects of interactions among the sources of different categories used in the experiment. Plackett-Burman designs are of particular help to eliminate the problems indicated in the above methods. The number of experiments required generally are of a multiple of four, i.e., 4, 8, 12, 16, 20... etc. The number of sources/categories, which are considered in the experiments, is one less than the

number of experiments, viz., 27 sources can be screened in 28 experiments. Moreover, the design is orthogonal in nature, meaning thereby that the effects of each source/category worked out are pure in nature and not confounded with interactions among sources/categories or other terms. It allows reliable short-listing of a few sources/categories for further optimization (M. R. S. srinivas *et al*., 1994).

Response surface methodology (RSM) is a powerful and efficient mathematical approach widely applied in the optimization of fermentation process, e.g. media components on enzyme production (Adinarayana and Ellaiah, 2002; Park *et al*., 2002; Puri *et al*., 2002), production of other metabolites (Zhang *et al*., 1996) spore production (Yu *et al*., 1997) and biomass production optimization (Lhomme and Roux, 1991). It can give information about the interaction between variables, provide information necessary for design and process optimization, and give multiple responses at the same time.

Response surface methodology (RSM) is an efficient statistical technique for the modeling and optimization of multiple variables in order to predict the best performance conditions with a minimum number of experiments (Giovanni, 1983). This is a non-conventional approach that has been successfully used for the optimization of enzymatic reactions conditions (Ferreira-Dias, Correia, & da Fonseca, 2003; Ribeiro, Silveira, & Ferreira-Dias, 2003), medium composition (Ribeiro, Manha, & Brito, 2006; Kapat, Rakshit, & Panda, 1996; Montserrat, Inaki, Franc_ois, Francesco, & Carles, 1993) and food preservation parameters (King, 1993).

2.10 Purification of enzymes

As this enzyme has two subunits different methods were encountered for purification of the enzyme.

2.10.1 Purification of α-L-rhamnosidase

The enzyme α-L-rhamnosidase from the *Aspergillus nidulans* were carried out by adsorption to DEAE A-50 and the separated proteins were eluted using NaCl with Tris-Bis buffer pH 6.0. Then the proteins were separated using Sepharose FF column. The purification of the enzyme was checked; using SDS-PAGE and the single band were obtained at 102 kDa (Manzanres *et al*., 2000).

The enzyme α-L-rhamnosidase was purified by anion exchange chromatography from an *Aspergillus niger* for commercial preparation. The enzyme was shown to be N-glycosylated. and had a molecular mass of 85 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis of which approximately 12% was contributed by carbohydrate (Manzanres *et al*., 1997).

The enzyme α-L-rhamnosidase from the liver of *Turbo cornutus*, a marine gastropiod was purified 111 fold using P nitrophenyl- α-L-rhamnoside as a substrate. Column chromatography with CM- Cellulose and Sephadex G –150 was used (Yoshikazu Kurosawa., *et al*., 1973).

An intracellular α-L-rhamnosidase from *Pichia angusta* X349 was purified to homogeneity through four chromatographic steps. The α-L-rhamnosidase appeared to be a monomeric protein with a molecular mass of 90 kDa (Takkaaki Yanai and Michikatsku Sato, 2000).

2.10.2 Purification of β-D-glucosidase

Debaryomyces varijiae was found to produce high level of an extracellular β-D-glucosidase .The enzyme was purified by gel filtration chromatography, ion exchange chromatography and chromatofocusing techniques (Andrea Belanic *et al*., 2003).

Candida peltata (NRRL Y-6888) produced extracellular β-glucosidase was purified 1,800-fold to homogeneity from the culture supernatant of the yeast grown on glucose by salting out with ammonium sulfate, ion- exchange chromatography with DEAE Bio-Gel A agarose, Bio-Gel A-0.5m gel filtration, and cellobiose-Sepharose affinity chromatography. The enzyme was a monomeric protein with an apparent molecular weight of 43,000 as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and gel filtration (Saha and Bothast, 1996).

2.11 Purification of naringinase

Affinity chromatography with immobilised triazine dyes was used to separate the main enzymes present in the naringinase complex produced by *Aspergillus terreus* CECT 2663. α-L-rhamnosidase and β-D-glucosidase were separated by a simple two-step procedure involving chromatography with Red HE-3B immobilised on Sepharose 4B first at pH 5.5 and

then at pH 4.7 (Soria *et al*., 2004).

Aspergillus niger MTCC 1344 producing naringinase was purified by using different steps include ultrafiltration, ammnonium sulfate precipitation anion exchange column Q-Sepharose and gel filtration chromatography Sephadex G-200. Purity was checked by PAGE (Munish Puri *et al*., 2005).

Penicillium decumbens PTCC 5248 produced naringinase were purified with ispropanol 24-fold purification was achieved (Nourouzian *et al*., 2000).

Penicillium citrimim producing naringinase were purified using a series of chromatographic steps involving ion exchange chromatography on DEAE-methacrylate, Hydrophobic interaction chromatography on methylmethacrylate and Gel permeation chromatography through Sephacryl S-200. The purity was checked by SDS-PAGE.

2.12 Molecular weight of the two subunits

The two subunits α-L rhamnosidase and β-D-glucosidase has difference in molecular weight producing by different species.

2.12.1 α-L-rhamnosidase

In case of α-L-rhamnosidase the molecular weight reported as 102 kDa (Manzanres *et al*., 2000).

The enzyme produced by *Aspergillus niger* has molecular mass of 85 kDa on sodium dodecylsulfate-polyacrylamide gel electrophoresis of which approximately 12% was contributed by carbohydrate (Manzanres *et al*., 1997).

The yeast like *Pichia angusta* producing α-L-rhamnosidase has appeared to be a monomeric protein with a molecular mass of 90 kDa (Takkaaki Yanai and Michikatsku Sato, 2000).

2.12.2 β-D-glucosidase

The enzyme β-D-glucosidase was a monomeric protein with an apparent molecular weight of 43 kDa as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as well as gel filtration (Saha and Bothast, 1996).

2.12.3 Naringinase

The molecular weights has been reported as 168 kDa in gel filtration chromatography and in sodium dodecyl sulphate poly acrylamide gel electrophoresis showed 80 kDa and 85 kDa of two subunits were found (Munish Puri *et al*., 2005).

2.13 Application of naringinase enzyme

The major application of this enzyme is to hydrolyse the naringin in citrus fruits during its juice preparation. Besides this application there are many includes preparation of the antibiotic chloropolysporin, preparation of rhamnose, preparation of prunin, the rhamnosidase activity of naringinase, steroid transformation (Munish Puri *et al*., 2000).

2.14 Removal of naringin from fruit juices using immobilized naringinase

Before using this enzyme the radial flow chromatographic techniques have been followed to remove naringin from the juices (Munish Puri *et al*., 2000). The fruit juice industry has patented substance to remove naringin from the juices during its processing.

Now-a-days immobilized naringinase enzyme was using to remove the naringin from the juices. Immobilization biocatalysts have many advantages,

- Biocatalyst reuse
- Ease separation of biocatalyst from reaction media
- Continuous mode operation prevention of contamination.

The preparation of immobilized naringinase that may be useful to develop processes of aqueous or aqueous/organic synthesis is an important field in which two main features must be considered

a) Immobilized derivatives have to be compatible with very different reaction requirements

b) Activity/stability properties of naringinase should be improved during immobilization

The crude naringinase from *Aspergillus niger* was immobilized in poly cryogels for the debittering of grapefruit juice. Poly vinyl alcohol and NaOH were dissolved and it was autoclaved for 15 minutes and neutralized with Hcl before mixing with the crude extract from *A.niger*. The mixture was dropped into liquid nitrogen to get the uniform beads. The reduction of naringin content of about 45% and 35% was observed after 24 hrs incubation at 20°C in free and immobilized enzyme respectively. About 36% efficiency of immobilized

enzyme was observed after six batches (Busto *et al*., 2007).

Cellulose triacetate fiber was also used to remove naringin as well as limonin removal from the citrus fruit juices. Naringinase from *Penicillium sp*., was entrapped in fibers, an enzyme column were made which could remove the bitter components. The sugar components, total organic acids and turbidity were not affected. Washing with warm water can regenerate the enzyme. Such column can be used for industrial use (Hau-Yang Tsen *et al*., 2004).

The effect of pressure was studied in an enzymatic reaction with an immobilized biocatalyst. Naringinase immobilized by entrapment in calcium alginate beads was the biocatalyst used to catalyze, at high-pressure, the hydrolysis of naringin to naringenin. At high-pressure, the influence of relevant parameters on naringinase catalytic activity such as temperature, substrate concentration, and biocatalyst reuse was studied. At 160 MPa, naringinase entrapped in Ca-alginate beads displayed higher activity, namely in the range of 35–40°C, whereas the optimum, at atmospheric pressure, was 35°C. The immobilized naringinase presented a Michaelis–Menten kinetic, with a 65% higher maximum initial rate, and a 70% lower at 160 MPa, as compared to kinetic parameters, at atmospheric pressure.

Naringin hydrolysis was carried out under high pressure in grapefruit juice with naringinase enzyme from *Aspergillus niger* was immobilized in alginate beads. Certain volume of naringinase solution in 0.02M acetate buffer, pH 4.0 was added to 4% sodium alginate solution all these were put in to 4% calcium chloride solution. The gelling was allowed to proceed for 30 minutes at 4 °C. Beads were separated by filtration and it was used for further conversion. In grape fruit juice a debittering of about of 75% was occurred under a pressure of 160 mpa at 37°C for 20 minutes after naringin hydrolysis by naringinase immobilized in calcium alginate beads (Luis Ferreria *et al*., 2008).

Naringinase was immobilized on celite by simple adsorption; naringin content was determined by HPLC. The retained activity of celite-adsorbed naringinase was found to be 83% at their optimal conditions (Sekeroglu *et al*., 2006).

Naringinase from *Penicillium sp*., was covalently linked to glyophase coated controlled pore-glass. This has been used to treat the naringin containing juices (Manjon *et al*., 2005).

Naringinase from *Penicillium sp*., was immobilized by covalent binding to wood chips to improve its activity. The immobilization of naringinase on glutaraldehyde coated wood-

chips (600mg wood chips, 10U naringinase, 45°C, pH 4.0 and 12 hrs) through 1% glutaraldehyde was optimized .The immobilized naringinase was stable during storage at 4°C. The immobilized enzyme was used for seven consecutive cycles for operations. The efficiency of the immobilization was 120%, while soluble naringinase afforded 82% efficacy for the hydrolysis of standard naringin under optimal conditions (Puri *et al.*, 2005)

Purified naringinase was immobilized in various supports like Alginate, κ-carrageenan, Polyacrylamide. The calcium alginate beads had shown high efficiency of removing debitterness in Kinnow juice. After 24h incubation it shows 62 and 56% hydrolysis of naringin by soluble and entrapped naringinase (Munish Puri *et al.*, 1996).

Crude naringinase produced by *Penicillium decumbens* was immobilized in calcium alginate beads, hen egg white, gelatin open pore matrix, and covalently attaching to activated *Oscimum basilicum*. The optimum pH was found for all these supports. The stability study was carried out (Norouzian *et al.*, 1999).

2.15 Application in fruit juice industry

At different time intervals and temperature was used to remove the naringin from the grapefruit juices were reported. The debittering of concentrated grape fruit juices with commercial naringinase results in the reduction of 76% was observed after 7 hrs at 122 F and 79% reduction was observed at 80 F (Olsen and Hill, 1964).

2.16 Predicting the structure (*In-silico*)

The structure of naringinase was not yet found. The structure of α-L rhamnosidase and β-D-glucosidase has been found out in many organisms.

2.16.1 α-L rhamnosidase

In case of α-L rhamnosidase only prokaryotic structure has been found. Some of them are shown in figure.

2.16.2 β-D-glucosidase

There are many eukaryotic and prokaryotic structures of beta glucosidase have been found.

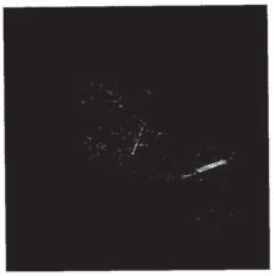


Figure 2.1: Low Temperature Structure of P22 tailspike Gh78 Family protein fragment (Becker *et al.*, 2007)

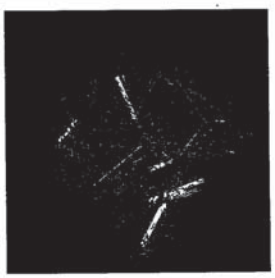


Figure 2.2: Crystal structure of rhamnosidase of *Bacillus sp.*, (Cui *et al.*, 2007)

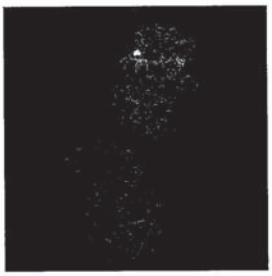


Figure 2.3: X-Ray structure of human acid-covalent beta-glucosidase (Prem kumar *et al.*, 2004)

Figure 2.4: Crystal structure of the intermediate of human cytosolic beta-glucosidase (Noguchi *et al.*, 2008)

3. MATERIALS AND METHODS

Commercial naringin was obtained from sigma, USA. the other culture media (Potato dextrose broth and Agar) used was obtained from Hi-Media laboratories, Mumbai, India and, all other chemicals were of analytical grade. *Aspergillus niger* and *Aspergillus flavus* were obtained from the soil culture of namakkal poultry farm, Tamilnadu, India.

3.1 Microorganisms and its cultivation conditions

The species of fungi were isolated from the soil culture of namakkal poultry farm includes *Aspergillus niger and Aspergillus flavus.* The isolated species were grown on appropriate culture medium and culturing conditions. Both the species were grown on Potato Dextrose Broth and it was incubated at 28°C for 8 days.

3.2 Isolation of microorganisms and enzyme extraction

The isolated organisms were screened for the production of naringinase activity. The isolated culture of different microorganisms were subcultured and it was used to grow in the media. The isolated cultures were subjected to microscopic examination and it was used. The seven-day-old culture media were centrifuged and the supernatant were collected and used for further activities. The organisms were used for characterization and application. The subculture was maintained as stock in agar slants and it was used for the whole processes.

3.3 Screening of Naringinase producing organism

Naringinase producing organism was screened using 1% ferric chloride without using assay methods. It is found that ferric chloride reacted with naringenin to give reddish brown colour(Ono *et al.*, 1977). Naringenin, the end product of naringinase action on naringin. Hence the potato dextrose agar medium containing naringin was used to grow the naringinase producing organism. If the organism produces naringinase then the naringin present in the medium will be cleaved and the end product naringenin reacts with ferric chloride and produces reddish brown colour. Hence this was tested with commercial narenginin obtained from sigma, USA.

3.4 Protein quantification

Protein concentration was measured by the method (Lowry *et al.*, 1951) using Bovine serum albumin (BSA) as standard. Protein assay mixture consisted of 0.05ml of ample and standard of 0.05mg of BSA prepared were taken as working standards of different volumes, 0.15 ml of distilled water and 0.1 ml of 1N Folin's reagent was added. Then the contents were incubated at room temperature for 30 minutes. A reaction mixture containing distilled water was used as blank. Blue colour developed in the standard and tests were measured at 660nm.

Bradford Method was followed for the estimation of protein. The protein samples were prepared in series of tubes in PBS. To the protein solution 5 ml of diluted dye binding solution was added to each tube. Mixed well and allowed to develop the colour for at least 5 minutes but no longer than 30 minutes. The red dye turns blue and the absorbance was measured at 595nm.

3.5 Assay for naringinase activity

Naringin was estimated using Davis method. To the 0.3ml of pH 4 .0 sodium acetate buffer 1 ml of 0.1% naringin were added and 0.2ml of enzyme supernatant was added and incubated at 50°C for 1 hr. From the incubated mixture 0.1 ml aliquot was taken and added in 5 ml of 90% diethylene glycol. The naringin present in the sample will give yellow colour and the intensity is measured at 420nm (Davis, 1947).

3.6 Characterization of crude enzyme

3.6.1 Effect of pH

The optimal pH of the crude enzyme was found by dissolving the naringin at various sodium acetate buffer concentrations varies from pH 2.5 to 5.5.

3.6.2 Effect of Temperature

With the optimum pH of the crude enzyme the optimum temperature was found by incubating the enzyme with the substrate at varying temperature ranges from 20 to 55°C.

3.6.3 Substrate Concentration

For the determination of Km and Vmax value for the crude naringinase different concentrations of naringin (0.02-0.2mM) was used.

3.7 Effect of metal ions

Different metal ions were used in the cultivation medium to determine the effect of metal ions on growth and naringinase production by *A.flavus*. Fe^{2+}, Mg^{2+}, Mn^{2+}, Ca^{2+}, Cu^{2+} were used at different concentrations of 5mM, 10mM and 30mM and the residual activity of the enzyme was found based on the assay condition

3.8 Effect of inducers

To the Czapeck-Dox medium, the two studies were conducted to increase the activity of naringinase.

1. Effect of naringin
2. Effect of naringenin

To the cultue medium of *A.flavus* different concentration of naringin and naringenin were used. Naringin concentration of 50mg, 100mg, 150mg and 200mg were taken to study the effect as inducer. Similarly, naringenin concentration of 0.002mg, 0.004mg, 0.006mg and 0.008mg were taken to study the increase in activity of the naringinase enzyme.

3.9 Medium optimization

3.9.1 Plackett-Burman experimental design

The medium components were evaluated using Plackett-Burman statistical design(Plackett *et al.*, 1946). This is a fraction of a two-level factorial design and allows the investigation of '*n*-1' variables with at least '*n*' experiments. Twelve factors were screened in sixteen combinations with three dummy variables which will provide an adequate estimate of the error (Stowe *et al.*, 1996) and all the trials were performed in duplicate and the average of observation was used as the response of the design. This design requires that the frequency of each level of a variable should be equal and that in each test, the number of high and low variables should be equal. Then the effect of changing the other variables was cancelled out

when determining the effect of particular variable. The main effect was calculated as the difference between the average of measurements made at the high setting (+1) and the average of measurements observed at low setting (−1) of each factor. Plackett-Burman experimental design is based on the first order model

$$Y = \beta_0 + \Sigma \beta_i X_i$$

where Y is the response (naringinase productivity), β_0 is the model intercept and β_i is the variable estimates. This model describes no interaction among factors and it is used to screen and evaluate the important factors that influence naringinase production. The factors that have confidence level above 95% are considered the most significant factors that affect the naringinase production.

In addition, the optimum concentration of these media components was determined by using the three-factor response-surface method. Statistical analyses were carried out with Minitab software (Version 15, Minitab Co., PA, USA).

A Plackett-Burman design was used to determine the most important factors influencing naringinase production and remove the dispensable ones to conclude a smaller and more manageable set of factors. The different factors were prepared in two levels, −1 for low level and +1 for high level based on Plackett-Burman design (as shown in Table 3.1).

Table 3.1 - Factors and levels in Plackett-Burman design

S.No	Factors	High level (+1)	Low level(-1)
1	Glucose (g/l)	20	10
2	Peptone (g/l)	75	25
3	Mg (g/l)	0.8	0.3
4	Naringenin (g/l)	0.08	0.03
5	pH	5.0	4.0

3.9.2 Response surface experimental Design

Best medium composition was established via Response Surface Methodology (RSM). With RSM, several variables are tested simultaneously with the minimum number of trials, according to experimental designs, which enables to find the interaction between variables (Myers & Montgomery, 2002). In addition, RSM has the advantage of being less expensive and time-consuming than the classical methods.

The best compositions for the production of naringinase with higher activity were established via RSM. The response y is described by a polynomial equation as a function of the p independent variables, xi, that is, y = f(x1, x2. . . xp) + e, where e represents the error observed in the response y; usually, the response is well modeled by a first or a second-order polynomial, representing a (p + 1) - dimensional surface, i.e. the response surface. The parameters of these equations are usually unknown and, therefore, must be estimated from the experimental data by using the statistical principle of least squares. In second-order equations, the coefficients of the squared terms influence the direction of the curvature of the response surfaces. The designs most commonly used to fit first order models are the 2p full factorial design. In addition to the 2p points, a centre point (repeated several times) is frequently added to the designs. They are used to provide an estimation of the variance of the experimental error, which is assumed to be constant along the experimental domain. The contribution to the error variation is not only due to the experimental errors alone, but also to the lack of fit of the estimated model (Giovanni, 1983). To fit second-order models, composite designs are usually followed. They consist of augmented 2p factorial designs with star points (also called axial points) and center points. In our study, the experiments were carried out with a central composite rotatable design (CCRD) (Vuataz, 1986) as a function of starch, peptone and naringenin (as shown in Table 3.2). With central composite rotatable design, five levels for each factor were used, which allowed fit of first or second-order polynomials to the experimental data points. Therefore, curved surfaces can be fitted to the experimental data. Partial differentiation of these polynomial equations is used to find the optimum points i.e. stationary points (Weisberg, 1985). However, the identification for each variable, on the regions corresponding to optimal responses, may be directly achieved by visual examination of the response surfaces and/or contour plots. In all, 20 experiments were

carried out in CCRD: three factorial points [coded levels as (+1) and (-1)] and three centre points as zero

Table 3.2

Coded and decoded levels of the experimental factors used in central composite rotatable design

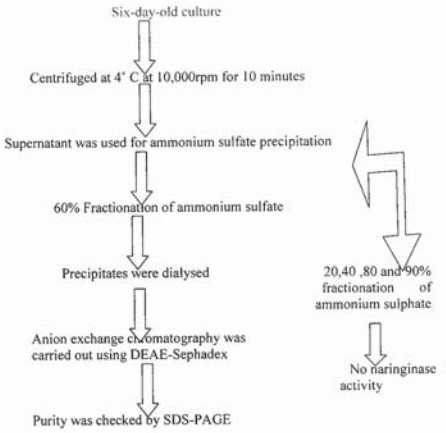
CCRD	Starch	Peptone	Naringenin
-1	1.0	0.25	0.003
0	1.5	0.50	0.005
+1	2.0	0.75	0.008

3.9.3 Data analysis

The results of each CCRD were analyzed using the software "Minitab", version 15. Both linear and quadratic effects of the three variables under study, as well as their interactions, on naringinase activity, after 1 h of reaction, were calculated. Their significance was evaluated by analysis of variance. A surface, described by a second-order polynomial equation, was fitted to each set of experimental data points. First and second-order coefficients of the polynomial equations were generated by regression analysis. The fit of the models was evaluated by the determination coefficients (R2) and adjusted R2 (R2 adj).

3.10 Purification of enzyme

With the literature obtained the enzyme was purified with the following steps



3.11 Steps in brief

Under aseptic conditions the MGYP media was inoculated with the *Aspergillus flavus*. The culture flask was kept at room temperature for five days. Then the culture was centrifuged at 10,000rpm for 10minutes at 4°C. The culture supernatant was used for Ammonium Sulphate fractionation

3.11.1 Ammonium sulfate precipitation

The retentate was saturated with ammonium sulfate fractionation of different concentration of ammonium sulfate like 20, 40, 60, 80 and 90% at 4° C. The precipitated protein was taken out by centrifuged the mixture at 10,000rpm for 10 minutes at 4°C. The precipitates were dissolved in aceatate buffer of pH 4.5 and stored at 4°C

3.11.2 Dialysis

The proteins were dialysed against 50mM acetate buffer at pH 4.5 at 4°C

3.11.3 Anion exchange chromatography

The dialysed proteins were used as analyte for the chromatography. The column was packed with DEAE-Sephadex of about 10 cm in height. The column was equilibrated with acetate buffer of pH 4.5. The sample was added to the column. The sample fraction was eluted with (0.1-1.5 M NaCl) in acetate buffer of pH 4.5. The eluted sample was assayed for naringinase activity. The sample with higher activity was pooled together and it was dialysed to remove the salts and again it was lyophilized for next step.

3.11.4 SDS –PAGE

The sodium dodecyl sulphate -polyacrylamide gel electrophoresis was used to check its purity. The lammeli method was followed and BSA, Casein was used as Standard protein marker. The stacking and separating gel mixture of 4% and 10% was used respectively. The sample was prepared with sample solubilizing buffer. The graph was plotted with the Rf values and molecular weight of the protein. With the relative mobility of the protein the molecular weight was calculated.

The sample was kept in boiling water bath for five minutes prior to load and it was mixed with dye coomassie brilliant blue. The purified sample was analysed and compared with the protein marker to find its size

Results and Discussion

4.1 Isolation of microorganism and enzyme extraction

The species of fungi were isolated from the soil culture of poultry house. The isolated species were grown on appropriate culture medium and culturing conditions. Based on morphology *Aspergillus niger & Aspergillus flavus* were isolated and subcultured on PDA and PDB and it was incubated.

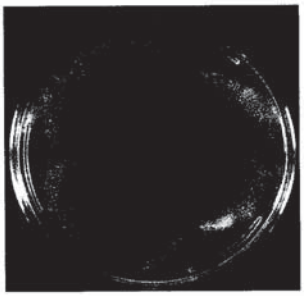


Figure :4.1 Isolation of *Aspergillus niger* from soil culture

Figure: 4.2 Isolation of *Aspergillus flavus* from soil culture

The culture supernatant from the two organisms was estimated for the protein concentration by Lowry method (Lowry *et al.*, 1951). The maximum protein concentration was observed after 6 day old culture. Hence the sixth-day-old culture supernatant was used for all the activities.

4.2 Screening of Naringinase producing organism

Naringinase producing organism was screened using 1% ferric chloride without assay methods. It is found that ferric chloride reacted with naringenin to give reddish brown colour. Naringenin, the end product of naringinase action on naringin. Hence the potato dextrose agar medium containing naringin was used to grow the naringinase producing organism. If the organism produces naringinase then the naringin present in the medium will be cleaved and the end product naringenin reacts with ferric chloride and produces reddish brown colour. Hence this was tested with commercial narenginin obtained from sigma, USA.

Figure: 4.3 Screening using ferric chloride

- 1-Control plate with ferric chloride
- 2-organism not producing naringinase
- 3-organism producing naringinase, *Aspergillus niger*
- 4- organism producing naringinase in higher level, *Aspergillus flavus*

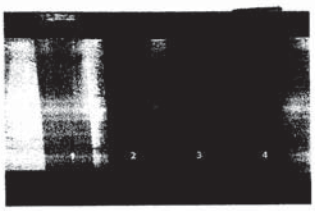


Figure : 4.4 Confirmatory test with pure naringenin

- 1-1% ferric chloride
- 2-Naringin
- 3-Naringenin
- 4-Rutin

Reddish brown colour is due to the end product naringenin is confirmed. But again the test was done with the end product glucose to prove that the colour was only due to naringenin.

Figure : 4.5 Confirmatory tests with the end product glucose

4.3 Activity of enzyme

The culture supernatant from the two organisms were taken and assayed for the production of the naringinase enzyme. In that *Aspergillus flavus* has shown a better activity which is shown in Table: 4.1.

TABLE: 4.1 Screening for the activity of naringinase

Organism	Activity (U/ml)
Aspergillus niger	0.206
Aspergillus flavus	0.209

4.4 Characterization of crude naringinase enzyme

4.4.1 Effect of pH

The optimum pH of crude soluble naringinase was found to be pH 4.5 for *Aspergillus niger* and *Aspergillus flavus* (Figure: 4.1). The activity of the enzyme remains good at pH 5.0 also. Hence this enzyme can be used in wider range from low to high pH during the processing of citrus fruits. This pH obtained is comparatively same pH as reported in *Penicillium decumbens* pH 4.5 (Nourouzian *et al*., 2000) and high in *Aspergillus niger* pH 4.0 (Munish Puri *et al*., 2005).

4.4.2 Effect of temperature

The optimum temperature was found as 50°C for *Aspergillus flavus* (Figure: 4.2) and 45°C for *Aspergillus niger*. This temperature is low as reported in *Penicillium decumbens* 55°C (Nourouzian *et al*., 2000) and same as reported in *Aspergillus niger* 45°C (Munish Puri *et al*., 2005).

4.4.3 Substrate concentration

The Km Value obtained as 0.29mM and the Vmax is 0.2 U/ml. This is the very low Km obtained for this enzyme. As reported in *Penicillium decumbens* as 1.7mM (Nourouzian *et al*., 2000) and in *Aspergillus niger* 1.9mM (Munish Puri *et al*., 2005).

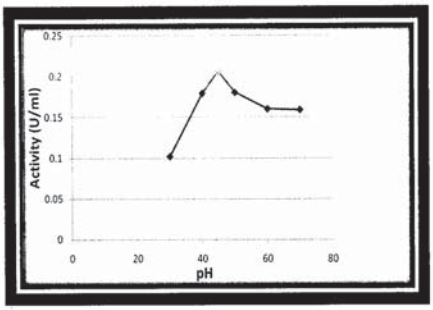


Figure : 4.6 Optimum pH for the crude Naringinase enzyme in *Aspergillus flavus*

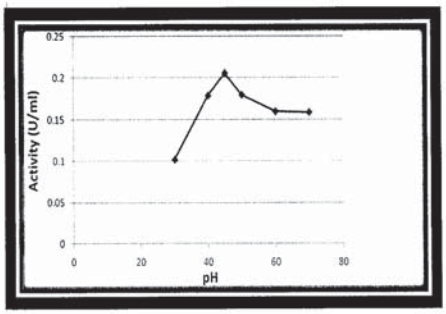


Figure : 4.7 Optimum pH for the crude Naringinase enzyme in *Aspergillus niger*

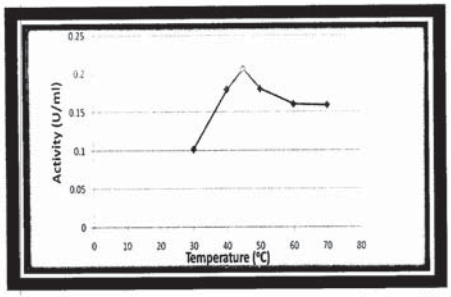


Figure : 4.8 Optimum temperature for the crude Naringinase enzyme in *Aspergillus flavus*

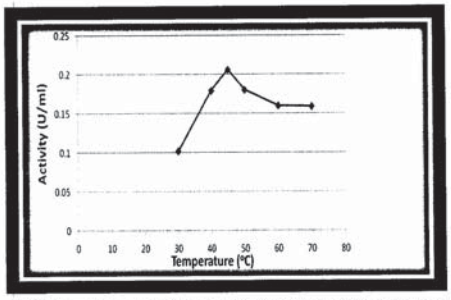


Figure : 4.9 Optimum temperature for the crude Naringinase enzyme in *Aspergillus niger*

4.5 Effect of inducers

Different concentration on naringin and naringenin were used to determine the best inducer of naringinase production from *A.flavus* (Table 4.2 & Table 4.3)

Table : 4.2 Effect of naringin as inducer in naringinase activity from *A.flavus*

Naringin (mg/L)	Activity (U/ ml)
50	1.107
100	1.002
150	0.957
200	0.988

Table : 4.3 Effect of naringenin as inducer in naringinase activity from *A.flavus*

Naringenin (mg/L)	Activity (U/ml)
0.02	0.690
0.04	1.152
0.06	1.158
0.08	1.160

4.6 Effect of metal ions

Different metal ions were used in the cultivation medium to determine the effects of metal ions on growth and naringinase production by *A. flavus*. Fe^{2+}, Cu^{2+} and Mn^{2+} show an inhibitory action on growth and enzyme production by *A. flavus*. Along with Co^{2+}, Ni^{2+}, were found to be inhibitory both to growth and naringinase production by *A. niger* (Munish Puri *et al.*, 2005). Cu^{2+} at a concentration of 5mM showed high inhibition of enzyme activity whereas Ca^{2+}, (5–10 mM), Mg^{2+}, (5–10 mM) stimulated naringinase synthesis. In the case of Mg^{2+}, the maximum activity 1.2 U ml−1 was observed at 10mM which accounted for a 24% increase in enzyme activity. Above 10mM enzyme activity decreased drastically. Ca^{2+} at 5–10mM also supported maximal production (1.1 U ml−1) of naringinase whereas at higher (30 mM) concentrations, a small decrease (0.9 U ml−1) in enzyme activity was

observed. This suggests that Mg^{2+} and Ca^{2+} ions are required for the production of naringinase by *A. flavus*. Fe^{2+} and Mn^{2+} show an inhibitory action on growth and enzyme production by *A. flavus* (Table 4.4)

Table : 4.4 Effect of metal ions on the production of naringinase by *A.flavus*

Metal ions (mM)	Enzyme activity (U/ml)	Residual activity (%)
Control	0.988	100
Fe^{2+}		
5	0.834	84
10	0.523	52
30	0	0
Cu^{2+}		
5	0	0
Mn^{2+}		
5	0.502	51
10	0.634	64
30	0.705	71
Mg^{2+}		
5	1.157	117
10	1.230	124
30	0.634	64
Ca^{2+}		
5	1.079	109
10	1.1133	114
30	0.967	97

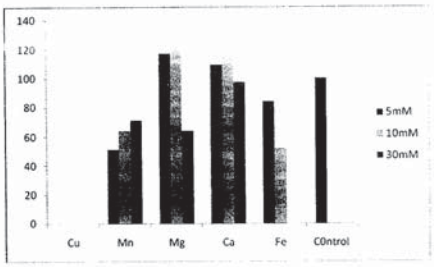


Figure : 4.10 Residual activity profile of metal ions for naringinase

4.7 OPTIMIZATION OF MEDIUM

4.7.1 Effect of culture medium components on naringinase production

Six medium components were examined using Plackett-Burman stastical experimental design. The main effect of the medium components examined in the present study is given in Table . The regression coefficient, F values and P values of the factors were calculated for naringinase production using the statistical software, Minitab, Version 15, PA, USA. The naringinase enzyme synthesized by *A.flavus* was found to vary from 0.3 U/L to 1158.33 U/L. in the twelve experiments conducted which shows the strong influence of medium componemts on naringinase enzyme production. On analysis of regression coefficient of six variables (Table 4.5), starch, peptone, naringenin, Mg^{2+} Inoculum size showed positive effect for naringinase activity, whereas pH alone showed negative effect in the tested range of concentration. The significant factors are shown in figure 4.12

Table : 4.5 Plackett-Burman design for screening variables for naringinase production

Run	Starch	Peptone	Mg^{2+}	Naringenin	pH	Inoculum size	Enzyme activity (U/L)
1	+	+	-	+	+	-	1158.33
2	+	+	-	+	-	-	1268
3	-	-	-	-	-	-	133
4	+	-	+	-	-	-	173
5	-	-	-	+	+	+	180
6	-	+	+	+	-	+	278.3
7	-	+	-	-	-	+	1.68
8	-	-	+	+	+	-	1.3
9	+	-	+	+	-	+	2.16
10	+	-	-	-	+	+	0.3
11	-	+	+	-	+	-	8.16
12	+	+	+	-	+	+	4.6

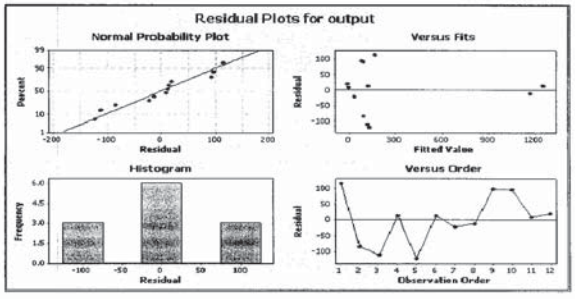


Figure : 4.11 Residual plot of naringinase resulted from Plackett-Burman design

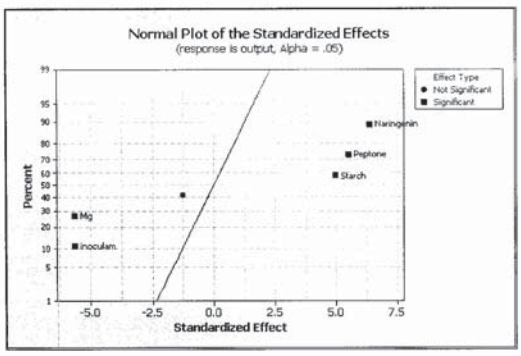


Figure : 4.12 Response plot of Plackett-Burman design for naringinase

Table : 4.6 Statistical analysis of Plackett-Burman of each variable for naringinase production by *A.flavus*

Variables	Effect	Coefficient	Standard Error	T statistic	P-Value
Constant		267.4	33.62	7.95	0.001
Starch	334.0	167.0	33.62	4.97	0.004
Peptone	371.6	185.8	33.62	5.53	0.003
Mg	-379.0	-189.5	33.62	-5.64	0.002
Naringenin	427.9	213.9	33.62	6.36	0.001
pH	-83.9	-42.0	33.62	-1.25	0.267
Inoculums size	-379.1	-189.6	33.62	-5.64	0.002

Linear Multiple regression analysis of Plakett-Burman design and main effect of each variable using Plackett-Burman design was shown in Table 4.6

Table 4.7 ANOVA test for naringinase response

Source	DF	Adj Sum of Squares	Adj Mean Square	F-Ratio	P-Value
Main Effects	6	2181250	363542	26.80	0.001
Residual Error	5	67820	13564		
Total	11	2249069			

R-Square = 96.98% R-Square (adjusted for d.f) = 93.37%

The P-value in the ANOVA in table was equal to 0.001(Table 4.7). This gave a statistically significant relationship between the variables at the 97% confidence level. The R-Squared statistic indicates that the model fitted explains 96.98% of the variability in naringinase amount. The use of linear regression analysis of Plackett-Burman experiments was important to select the most effective variables which give the highest confidence level %. This will be lead to select each of starch, peptone, Mg and naringenin as the most effective variable where they have a confidence level % higher than 95%.

These results clearly put a conclusion that the logical analysis of the data is the best for selecting the most effective variables. The linear multiple regression investigated the overall experiments. In this study, optimization of the environmental conditions has been neglected such as temperature, shaking rate etc. and should be considered in the further optimization studies.

4.8 Optimization of important medium components by RSM

A Central Composite Design(CCD) was used for screening out the combinations of the process variables that would lead the high concentration. Based on the regression analysis of the data from CCD, the effect of three variables (starch, peptone and naringenin) on naringinase production were predicted by second-order polynomial functions.

Table : 4.8 Experimental data obtained for the optimization of medium components for naringinase production

Run	Starch	Peptone	Naringenin	Enzyme activity (U/L)
1	1.5	0.5	0.001296	3535
2	1.5	0.5	0.009704	3553.2
3	1.0	0.75	0.008	2207.8
4	1.5	0.5	0.0055	3532.8
5	0.659504	0.5	0.0055	2224.4
6	1.5	0.5	0.0055	3487.2
7	2.0	0.750	0.003	2788.8
8	2.34086	0.5	0.005	2324
9	2.0	0.25	0.008	3037.8
10	1.0	0.25	0.003	3336.2
11	1.5	0.920	0.0055	2373.8
12	1.5	0.079	0.0055	2357.2
13	1.0	0.75	0.003	2473.4
14	2.0	0.75	0.008	2639.4
15	1.0	0.25	0.0055	3336.6
16	1.5	0.50	0.0055	3407.2
17	1.5	0.50	0.0055	3606.6
18	1.5	0.5	0.0055	3424
19	1.5	0.5	0.0055	3404.2
20	2.0	0.25	0.003	3115.2

In all the 20 experiments the activity of the enzyme was determined as shown in Table 4.8. The analysis of variance of the quadratic regression was a highly significant model, as was evident from the Fisher's F-test with a very low propability value [(P model)>F)=0.0001].

Table : 4.9 Estimated Regression Coefficients for Enzyme activity

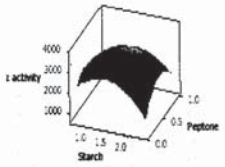
Variables	Coefficient	SE Coefficient	T stasistic	P-value
Constant	3481.53	95.62	36.411	0.000
Starch	28.90	63.44	0.456	0.658
Peptone	-196.86	63.44	-0.533	0.606
Naringenin	-33.78	63.44	-0.533	0.606
Starch*Starch	-389.92	61.76	-6.314	0.000
Peptone*Peptone	-357.64	61.76	-5.791	0.000
Naringenin*Naringenin	59.06	61.76	0.956	0.361
Starch*Peptone	158.35	82.89	1.910	0.085
Starch*Naringenin	4.80	82.89	0.058	0.955
Peptone*Naringenin	-42.25	82.89	-0.510	0.621

The model's goodness of fit was checked by determination coefficient(R-square). In this case, the value of the determination coefficient (R-Sq = 0.8949) indicated that only 4.87% of the total variations were not explained by the model. The value of the adjusted determination coefficient [Adj(R-Sq)=0.8002] was also very high in supporting the high significance of the model.

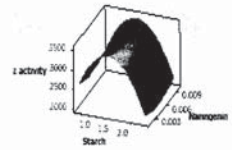
Table : 4.10 Analysis of variance for naringinase activity

Source	DF	Adj Sum of squares	Adj Mean square	F	P
Regression	9	4677866	519763	9.46	0.001
Linear	3	556249	185416	3.37	0.063
Square	3	3906554	1302185	23.69	0.000
Interaction	3	215063	71688	1.30	0.326
Residual Error	10	549646	54965		
Lack-of-Fit	5	522138	104428	18.98	0.003
Pure Error	5	27507	5501		
Total	19	5227511			

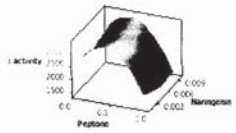
The fitted response for the above regression model (Table 4.10) was plotted in figure graphs were generated for the pair-wise combination of the three factors while in keeping the other one at its optimum levels for naringinase production. Graphs are given here to highlight the roles played by the physical constraints.



(a)



(b)



(c)

Figure : 4.13 Effects of (a) starch and peptone, (b) naringenin and starch (c) naringenin and peptone and their interactive effect on the naringinase production with other nutrient set at centre level

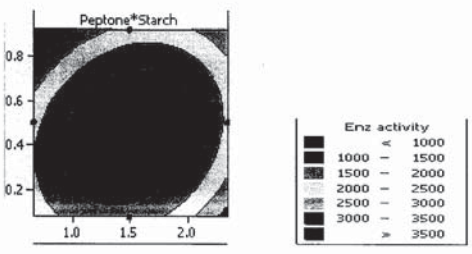


Figure : 4.14 Contour plot, fitted to the experimental data points, corresponding to naringinase activity, as a function of starch and peptone.

In response to the naringinase production, starch and peptone had shown significant effect on their linear, quadraric and interaction terms. Maximum naringinase activity was obtained when the optimum levels of starch, peptone and naringenin were 1.5g/L, 0.5g/L, 0.005g/L, respectively(Figure 4.14). Moderate amount of starch and peptone were proven to increase the naringinase activity 3.2 times from maximum production of 1158.33 U/L using

Plackett-Burman design to 3606.6 U/L using central composite design.

These experiments indicated that the initial concentrations of carbon and nitrogen sources in the culture medium were important for achieving high naringinase activity.

4.9 Enzyme Purification

The supernantant obtained from the culture medium was subjected to ammonium sulfate concentration of various saturation concentration. The 60% saturation of ammonium sulfate precipitation had shown high naringinase activity. The precipitated protein was dialyzed and used for anion exchange chromatography. The protein was eluted at 25mM acetate buffer of pH 4.5 and 0.5M NaCl. The eluted protein has high naringinase activity.

SDS-PAGE

The purified enzyme was loaded to the SDS-PAGE. The two bands were obtained. . It shows the protein is heterodimer. Their molecular weight was calculated with Rf value. It shows that their molecular weight is 70 and 39 kDa. The value obtained was closer to the molecular weight of purified α-L-rhamnosidase showed 102 kDa (Manzanares *et al.*, 2000). The purified β-D-glucosidase from *Candida peltata* showed 43 kDa (Saha and Bothast, 1996). Many reports have shown higher molecular weights and some of them reported as monomer. The result was shown in the figure 4.17.

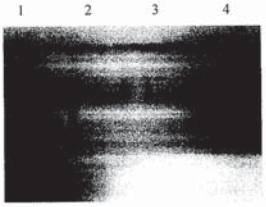


Figure : 4.15 Native PAGE of Ammonium Sulphate Fractionation

Lane 1 – Crude, Lane 2- 20% Ammonium Sulphate Fractionation

Lane 3 - 40% Ammonium Sulphate Fractionation

Lane 4 – 60 % Ammonium Sulphate Fractionation

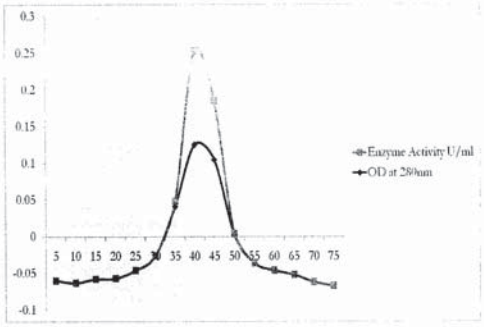


Figure : 4.16 Elution profile of anion exchange chromatography

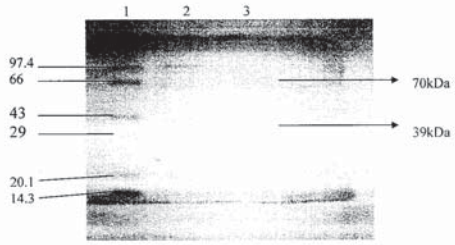


Figure : 4.17 Protein shown in SDS – PAGE

Lane 1 – Marker (14.3 kDa – 97.4 kDa)

Lane 2 – crude sample

Lane 3 – Protein eluted using 25mM acetate buffer of pH 4.5

Table : 4.11 Purification Table of naringinase from *A.flavus*

Purification steps	Activity (U/mL)	Total Activity (U)	Protein (mg/mL)	Total Protein (mg)	Specific Activity (U/mg)	Fold purification	Yield (%)
Crude	3.6	1800	1.9	950	1.894	1	100
Ammonium sulphate	3.8	152	0.5	20	7.6	4.01	8.44
Anion exchange chromatography	3.95	15.8	0.24	0.96	16.458	8.689	0.877

Conclusion & Future enhancement

5.1 Conclusion

- The organism, *Aspergillus flavus* was found to produce the enzymes naringinase
- It has been characterized, its optimum pH was found as 4.5 and optimum temperature was 50°C
- Naringenin was determined as best inducer of naringinase increasing 0.2% of activity
- Mg2+ and Fe2+ increases the naringinase activity at 10mM concentration
- Optimization was done by Plackett-Burman and Response Surface method to find the best suited culture medium for higher activity.
- Confidence level of above 90% was obtained in medium optimization.
- Naringinase has been purified.
- The purification steps includes ammonium sulfate precipitation, anion exchange chromatography, SDS-PAGE
- About 8.6 fold purification was obtained
- The Protein was found as dimmer and its molecular weight is 39 and 70 kDa

5.2 Future Enhancement

- Optimization of other environmental conditions like Temperature, Shaking rate etc can be determined by using Plackett-Burman and Response Surface method
- Purified enzyme can be characterized
- Immobilization of naringinase using calcium alginate beads can be done
- Appilcation of naringinase in citrus fruit juices and the naringin reduction can be idenfied
- Structure prediction of naringinase from *A.flavus* can be done
- A large scale production of naringinase can be done

Appendix I

Sodium acetate buffer composition

Stock Solution

Solution A: 0.2 M of acetic acid (11.55ml in 1000ml)

Solution B: 0.2M of Sodium acetate (16.4g in 1000ml)

"x" ml of solution A and "y" ml of solution B is diluted to 100ml with distilled water

x	Y	pH
48.2	1.8	3.2
46.3	3.7	3.6
44.0	6.0	3.8
41.0	9.0	4.0
36.8	13.2	4.2
30.5	19.2	4.4
22.5	24.5	4.6
20.0	30.0	4.8
14.8	35.2	5.0
10.5	39.5	5.2
8.8	41.2	5.4
4.8	45.2	5.6
3.6	45.4	5.8
2.4	47.6	6.0

Appendices

Appendix II

Estimation of protein by Lowry's method

Reagents

Stock standard: Bovine serum albumin

Dissolve 100mg of BSA and make up to 100ml with distilled water

Working standard solution:

Take 10ml of stock standard and dilute to 100ml with distilled water

2% sodium carbonate in 0.1 N Sodium hydroxide (Reagent A)

0.5% Copper sulfate in 1% potassium sodium tartarate (Reagent B)

Alkaline copper solution

Mix 50ml of A and 1ml of b prior to use

Folin coicalteau reagent(1:1) should be freshly prepared.

Estimation of protein by Bradford method

Dye concentrate

-Dissolve 100 mg of coomassie brilliant blue G250 in 50 ml of 95 % ethanol. Add 100 ml of concentrated (ortho) phosphoric acid. Add distilled water to a final volume of 200 ml. Store refrigerated in amber bottles; the solution is stable at least 6 months.

-Mix 1 volume of concentrated dye solution with 4 volumes of distilled water for use.

Appendix III

Activity (U/ml) = (Substrate-Test)* 1.2 / 60 *0.1

Rf = Distance migrated by the protein / Distance migrated by the dye front

References

6. REFERENCES

1. Adinarayana, K. and Ellaiah, P. (2002) 'Response surface optimization of the critical medium components for this production of alkaline protease by a newly isolated *Bacillus* sp.', J. Pharm. Pharmaceut. Sci, Vol.5 No.3, pp.272-278.

2. Akira Konno, Masaru Misaki, Jun Toda, Takeo Wada and Katsuharu Yasumatsu. (1982) 'Bitterness reduction of naringin and limonin by β-cyclodextrin', Agricultural biology and chemistry, Vol.46, No.9, pp.2203-2208.

3. Amoa-Awua, W.K. Frisvad, J.C. Sefa-Dedeh, S. and Jakobsen, M. (1997) 'The contribution of moulds and yeasts to the fermentation of 'agbelima' cassava dough', Journal of applied microbiology, Vol. 83, pp.288–296.

4. Andrea Belanic, Ziya Guanta, Marie Jose Vallier and Eduardo Agosin. (2003) 'Beta-Glucosidase from the grape native yeast *Debaryomyces varijie*: purification and characterization and its effect on monoterpene content of a Muscat grape juice', Journal of agricultural food chemistry, Vol.51, No.5, pp.1453-59.

5. Bailey, D.G. Dresser, G. K. Kreeft, J. H. Munoz, C. Freeman, D. J. and Bend, J .R. (2000) 'Grapefruit-felodipine interaction: effect of unprocessed fruit and probable active ingredients', Clinical pharmacology and therapectiucs, Vol.68, No.5, pp.468-77.

6. Becker, M. Mueller, J.J. Heinemann, U. and Seckler, R. (2007) 'Low temperature structure of P22 tailspike protein fragment (109-666) mutant V450a', Nucleic acid research, Vol.35, pp.421-27

7. Bram, B. and Solomons, G. L. (1965) 'Production of the enzyme naringinase by *Aspergillus niger*', Applied microbiology, Vol.13, No.6, pp.842-845.

8. Busto, M.D. Meza, V. Ortega, N. and Perez-Mateos, M. (2007) 'Immobilization of naringinase from *Aspergillus niger* CECT 2088 in poly(vinyl alcohol) cryogels for the debittering of juices', Food chemistry, Vol.104, pp.1177–1182.

9. Cui, Z. Maruyama, Y. Mikami, B. Hashimoto, W. and Murata, K. (2007) 'Crystal structure of glycoside hydrolase family 78 alpha-L-rhamnosidase from *Bacillus sp.*, GL1', Journal of molecular biology, Vol.374, pp.384-398.

10. Davis, D.W. (1947) 'Determination of flavonones in citrus juice', Analytical chemistry, Vol.19, pp.46–47.

11. Ferreira-Dias, S. Correia, A. C. and da Fonseca, M. M. R. (2003) 'Response surface modelling of glycerolysis catalyzed by Candida rugosa lipase immobilized in different polyurethane foams for the production of partial glycerides'. Journal of Molecular Catalysis B-Enzymatic. Vol.21. pp.71–80.

12. Fukumoto, J and Okado, S (1973) 'Naringinase production by fermentation', Japanese Patent 7306554.

13. Giovanni, M. (1983) 'Response surface methodology and product optimization', Food Technology, Vol.37, pp.41–45.

14. Habelt K, Pittner F. (1983) 'A rapid method for the determination of naringin, prunin and naringenin applied to the assay of naringinase', Anal Biochem Vol.134, pp.393–7.

15. Hau-yang tsen and Gee-kaite yu. (1994) 'Limonin and naringin removal from grapefruit juice with naringinase entrapped in cellulose triacetate fibers', Journal of food science, Vol.56, No.1, pp.31-34.

16. Hawolkog-dong and Seongbuk-gu Seoul. (1999) 'Naringin and naringenin as inhibitor of acyl coa-cholesterol-o-acyltransferase, inhibitor of macrophage-lipid complex accumulation on the arterial wall and preventive or treating agent for hepatic diseases', pp.136-130.

17. Helder, A. L. Pedro, António J. Alfaia, João Marques. Helder J. Vila-Real, António Calado and Maria H.L. Ribeiro. (2007) Design of an immobilized enzyme system for naringin hydrolysis at high-pressure', Enzyme and microbial technology, Vol.40, No.3, pp.442-446.

18. Isabel A. Ribeiro. and Maria H.L. Ribeiro. (2008) 'Naringin and naringenin determination and control in grapefruit juice by a validated HPLC method', Food control, Vol.19, pp.432–438.

19. Ito, T. and Takiguchi, Y. (1970) 'Naringinase production by *Cochiobolus miyabeanus*', Japanese Patent 7014875.

20. Kapat, A. Rakshit, S. K. and Panda, T. (1996) 'Optimization of carbon and nitrogen sources in the medium and environmental factors for enhanced production of chitinase by Trichoderma harzianum', Bioprocess Engineering, Vol.15, pp.13–20.

21. Kim, S. (2003) 'Naringin suppresses the mitogenic effect of lysophosphatidylcholine on vascular smooth muscle cells', Nutrition research, Vol. 23, No.12, pp.1671-1683.

22. King, V. A. (1993) 'Studies on the control of the growth of Saccharomyces cerevisiae by using response surface methodology to achieve effective preservation at high water activities', International Journal of Food Science and Technology. Vol.28. pp.519–529.

23. 'Laboratory manual for processing of citrus fruit', Manual No. 054R10020.000-fourth edition, by Citrus Systems, FMC Food Tech.

24. Lhomme, B. and Roux, J.C. (1991) 'Utilization of experimental designs for optimization of *Rhizopus arrhizus* culture', Bioresour. Technol, Vol.35, No.3, pp.301-312.

25. Lowry, O.H. Rosebrough, N.J. Farr, A.L and Randall, R.J. (1951) 'Protein measure-ment with the folin phenol reagent', Journal of biological chemistry, Vol.193, pp.265–75.

26. Luís Ferreira, Cristina Afonso, Helder Vila-Real, António Alfaia and Maria H.L Ribeiro, (2008) 'Evaluation of the effect of high pressure on naringin hydrolysis in grapefruit Juice with naringinase immobilised in calcium alginate beads', Food technology and biotechnology, Vol.46, No.2, pp.146-150.

27. Manalo, G.D. Garcia, E.N. and Chua, M.T. (1980) 'Isolation of naringin from *Citrus maxima L.* and dihydrochalcone sweeteners', Publication AGRIS Subj. Cat. Plant physiology and biochemistry, Record number- XB8120732.

28. Manjon, A. Bastida, J. Romero, C. Jimeno, A. and Iborra, J.L. (2005) 'Immobilization of naringinase on glyophase coated porous glass', Biotechnology letters, Vol.7, No.7, pp.477-82.

29. Manzanares, P. orejas, M. Ibanez, E. Valless. and Ramon D. (2000) 'Purification and characterization of an α-L rhamnosidase from *Aspergillus nidulans*', Letters of applied microbiology, Vol.31, No.3, pp.198-202.

30. Manzanres, P. Degraff, L.H. and Vise, J. (1997) 'Purification and characterization of an from *Aspergillus niger*', FEMS Microbiology letters, Vol.152, No.2, pp.279-83.

31. Montserrat, A. Inaki, R. Franc_ois, O. Francesco, G. and Carles, C. (1993) 'Application of factorial design to the optimization of medium composition in the batch cultures of Streptomyces lividans TK21 producing a hybrid antibiotic', Biotechnology Letters, Vol.15, pp.559–564.

32. Munish Puri, Marwaha, S.S. and Kothari, R.M. (1996) 'Studies on the applicability of alginate -entrapped naringinase for the de-bittering of kinnow juice', Enzyme and microbial biotechnology, Vol.18, pp.281-285.

33. Munish Puri, Anirban Banerjee, U.C. and Banerjee. (2005) 'Optimization of process parameters for the production of naringinase by *Aspergillus niger* MTCC 1344', Process biochemistry, Vol.40, pp.195-201.

34. Munish Puri and Sukirti Kalra. (2000) 'Purification and characterization of naringinase from a newly isolated strain of *Aspergillus niger* 1344 for the transformation of flavonoids', World journal of microbiology and biotechnology, Vol.21, pp.753-758.

35. Munish Puri and Uttam Chand Banerjee. (2000) 'Production, purification, and characterization of the debittering enzyme naringinase', Biotechnology advances, Vol.18, pp.207-217.

36. Nomura, D. (1965) 'Studies on the naringinase produced by *Coniothyrium diplodiella* I. The properties of naringinase and the removal of co-existing pectinase from the enzyme preparation', Enzymologia,Vol.29, pp.272–82.

37. Noguchi, J. Hayashi, Y. Baba, Y. Okino, N. Kimura, M. Ito, M., and Kakuta Y., (2008) 'Crystal structure of the covalent intermediate of human cytosolic beta-glucosidase', Biochemical and biophysical research communications, Vol.374, pp.549-552.

38. Norouzian, D. Osseinzadeh A. Inanlou D.N. Moazami N. (1999) 'Various techniques used to immobilize naringinase produced by *Penicillium decumbens* PTCC 5248', World journal of microbiology and biotechnology, Vol.15, pp.501-502.

39. Norouzian, D. Osseinzadeh, A. NouriInanlou, D. and Mozami, N. (2000) 'Production and partial purification of naringinase from *Penicillium decumbens* PTCC 5248', World journal of microbiology and biotechnology, Vol.16, No.5, pp.471-473.

40. Olsen, R.W. and Hill, E.C. (1964) 'Debittering of concentrated Grapefruit juice with Naringinase', Process flow state Hort, Vol. 77, pp.321-325.

41. Ono, M. Tosa, T. and Chibata, I. (1977) 'Preparation and properties of naringinase immobilized by ionic binding on DEAE-Sephadex', Journal of Fermentation Technology, Vol.55, pp.493-500.

63. Vuataz, L. (1986) In J. R. Piggott editor. 'Statistical procedures in food research', London, Elsevier. pp.100–123.

64. Weisberg, S (1985) 'Applied linear regression', New York, Wiley. pp.217.

65. Zhang, J. Marcin, C. Shifflet, M.A. Salmon, P. Brix, T. Greasham, R. Buokland, B. and Chartrain, M. (1996) 'Development of a defined medium fermentation process for physotigmine production by *Streptomyces griseofuscus*', Appl. Microbiol. Biotechnol, Vol.44. No.5, pp.568-575.