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CHARACTERIZATION, PURIFICATION AND APPLICATION OF NARINGINASE FROM *Candida tropicalis*

by

D.SARANYA

Reg No. : 0720203008

of

KUMARAGURU COLLEGE OF TECHNOLOGY,
COIMBATORE-641 006.
(An Autonomous Institution affiliated to Anna University Coimbatore)

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NAME OF THE STUDENT	TITLE OF THE PROJECT	NAME OF THE SUPERVISOR WITH DESIGNATION
D.SARANYA (0720203008)	**CHARACTERIZATION, PURIFICATION AND APPLICATION OF NARINGINASE FROM *Candida tropicalis***	**Dr. S. SHANMUGAM Assistant Professor**

The report of the project work submitted by the above student in partial fulfilment for the award of Master of Technology in Biotechnology of Anna University was evaluated and confirmed to be the report of the work done by the above students and then evaluated.

(INTERNAL EXAMINER) **(EXTERNAL EXAMINER)**

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LIST OF ABBREVATIONS

MGYP	Malt extract, Glucose, Yeast extract, Peptone
DNS	DinitroSalicylic
BSA	Bovine Serum Albumin
SDS-PAGE	Sodium Dodecyl sulfate- PolyAcrylamide Gel Electrophoresis
kDa	KiloDaltons
BLAST	Basic Local Alignment Search Tool
DEAE	Diethyl Amino Ethyl
rpm	Rotation Per Minute

ABSTRACT

ABSTRACT

The presence of the bitter compound naringin in citrus fruits is a major problem in the processing of citrus fruits in juice industry. The flavonone, naringin can be hydrolysed by an extracellular enzyme naringinase(E.C.3.2.1.40). In the fruit juice industry the application of enzymes like naringinase, pectinase and cellulase are important to remove naringin, pectin and cellulose respectively. The microrganisms like yeast and molds were screened for the production of naringinase, pectinase and cellulase activities. It was found that the *Candida tropicalis,* possess better activity in all the three enzymes. The enzyme was purified by Ultrafiltration, Ammonium sulfate precipitation, Ion exchange chromatography, Gel filtration chromatography and, SDS-PAGE. About 9.3 fold purification and 22.5 fold total purification were obtained by these steps. The purified enzyme was characterized and its optimum pH was 3.0, optimum temperature was 25°C and its km value is obtained as 0.19mM. The crude enzyme was immobilized using two different supports hen egg white and sodium alginate. It was characterized to study the application in fruit juices. The application of the enzyme was studied in citrus fruits like *Citrus lemon* (lemon), *Citrus limetta* (musambi), *Citrus sinensis* (sweet oranges), *Citrus reticulata* (orange) and, *Vitis vinifera* (grapes). The soluble as well as immobilized naringinase was used to study its application. After 6 hrs the reduction in naringin concentration was recorded grapes- 56%, for soluble enzyme, similarly reduction of 62.9% was observed for sweet orange by using sodium alginate beads and reduction of 92.8% for grapes was observed by using hen egg white beads. The sequences coding for the two enzyme subunits were found and its structure was predicted by *In-silico.*

INTRODUCTION

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).

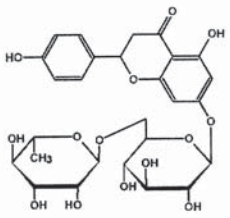


Figure: 1.1 Structure of Naringin

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*).

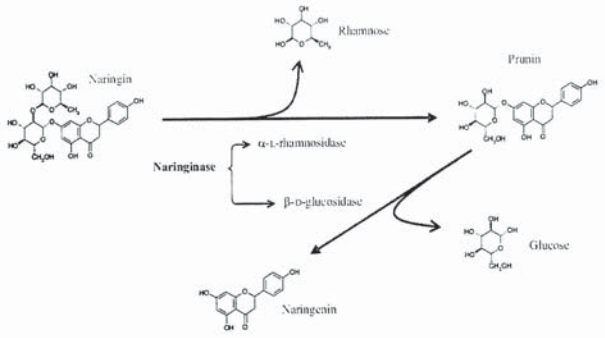


Figure: 1.2 Hydrolysis of naringin into prunin, rhamnose, glucose and naringenin by naringinase containing α-L rhamnosidase activities and β-D –glucosidase activities

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).

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. Many studies were carried out in the immobilization process.

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.

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

OBJECTIVES

To screen for naringinase producing fungi (*Aspergillus sp., Candida tropicalis, Kluyveromyces sp.,)*

- To purify the enzyme using different chromatographic techniques
- To characterize the crude and purified enzyme
- To study the application of naringinase on de-bittering of fruit juice with crude enzyme and immobilize enzyme
- To find the sequences coding for the enzyme Rhamnosidase and Glucosidase in *Candida sp.,*
- To model the structure of the enzyme using the sequences

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

Microorganism	References
Aspergillus niger, Aspergillus oryzae, A.usamii	Bram and Solomns, 1965; Kishi,1955
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 *Penicillicum 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.*, 2004) 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 tempearature is at 50°C. (Saha and Bothast, 1996)

2.6.3 Substrate Concentration

The Km Value reported as 1.7mM in *Penicillium decumbens* (Nourouzian *et al.*, 2004) and in *Aspergillus niger* 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 of 0.27 mmol l-1 and 64.6 U mg-1, 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 Purification of enzymes

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

2.8.1 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 monitored using SDS-PAGE and the single band were obtained at 102 kDa (Manzanres *et al.*, 2000)

The enzyme alpha-L-rhamnosidase activity was purified by anion exchange chromatography from an *Aspergillus niger* for commercial preparation. The alpha-L-rhamnosidase was shown to be N-glycosylated, and had a molecular mass of 85 kDa on sodium dodecylsulfate-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)

2.8.2 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 beta-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)

2.9 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.*, 2004).

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 (Sangeeta talang., e-thesis).

2.10 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.

2.10.1 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).

2.10.2 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)

2.10.3 Naringinase

The molecular weight 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)

2.11 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)

2.12 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 optimum 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 catalytic 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 cross-linking 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).

2.13 Application in fruit juice industry

At different time interval 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).

2.14 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.

2.14.1 α-L rhamnosidase

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

2.14.2 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)

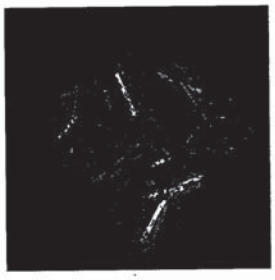


Figure 2.2: Crystal structure of rhamnosidase of *Bacillus sp.*, (Cui *et al.*, 2007)

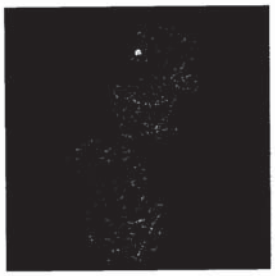


Figure 2.3: X-Ray structure of human acid-covalent beta-glucosidase (Prem kumar *et al.*, 2004)

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

MATERIALS AND METHODS

3. MATERIALS AND METHODS

Commercial naringin was obtained from sigma, USA, the other culture media (Potato dextrose broth, MGYP media and Agar) used was obtained from Hi-Media laboratories, Mumbai, India and, all other chemicals were of analytical grade. Citrus fruits were purchased from the local supermarket. *Candida tropicalis*-3522 was obtained from IMTECH Chandigarh, India.

3.1 Microorganisms and its cultivation conditions

The species of fungi were isolated from the Western ghats includes *Aspergillus sp., Candida tropicalis, Kleuyveromyces sp.,* The isolated species were grown on appropriate culture medium and culturing conditions. *Aspergillus sp.,* was grown on Potato Dextrose Broth and it was incubated at 28°C for 5 days. *Candida tropicalis, Kleuyveromyces sp.,* were grown in the MGYP medium.

3.2 Isolation of microorganisms and enzyme extraction

The isolated organism was screened for the production of naringinase, pectinase and cellulase activities. The isolated culture of different microorganism was subcultured and it was used to grow in the media. The isolated cultures were subjected to microscopic examination and it was used. The five-day-old culture media were centrifuged and the supernatant were collected and used for further activities. The organism having better activities in all the three enzymes was used for characterization and application. The subculture was maintained as stock in agar slants (MGYP agar) and it was used for the whole processes.

3.3 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.

3.4 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).

3.5 Assay for pectinase activity

The assay mixture comprises 0.2ml of 0.15m NaCl, 0.2ml of tris acetate buffer, pH 4.5, 0.1ml of 0.01m $CaCl_2$ and 0.5 ml of 1% solution of polygalatouronic acid and incubated the mixture for 1 hr at 37°C. The reducing sugar method by Nelson- somogyi was used (Sadasivam and Manickam., 2003).

3.6 Assay for cellulase activity

Pipette out 0.45ml of 1 % carboxymethyl cellulose solution at a temperature of 55°C for 15 minutes and 0.5ml of DNS reagent were added and heated the mixture in a boiling water bath for 5 minutes to the tubes, 1.0ml of potassium sodium tartarate solution were added and water is added to make up the volume of 5ml. The absorbance was measured at 540 nm (Sadasivam and Manickam, 2003).

3.7 Characterization of crude enzyme

3.7.1 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 6.5.

3.7.2 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.

3.7.3 Substrate Concentration

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

3.8 Substrate Specificity

Rutin (0.1%) and naringin (0.1%) the two flavonones were used as substrates to find the crude naringinase substrate specificity.

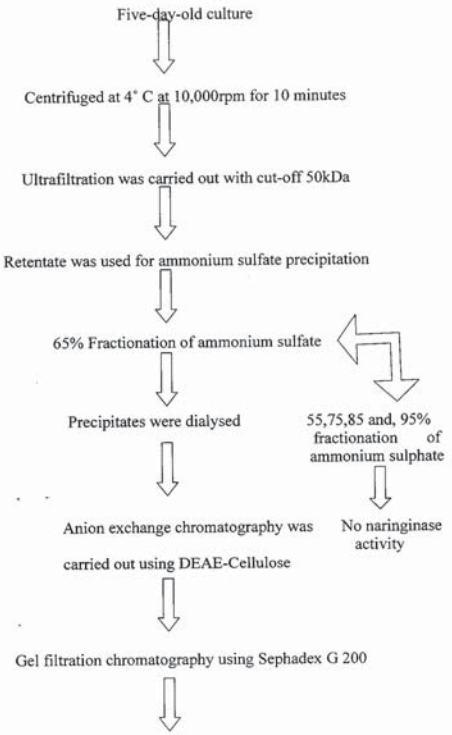
3.9 Effect of nitrogen sources and naringin

To the MGYP medium, the three studies were conducted to increase the activity of naringinase.

1. Effect of yeast extract & Ammonium sulfate with $CaCO_3$ and without $CaCO_3$
2. Effect of Peptone
3. Effect of naringin

3.10 Purification of enzyme

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



Purity was checked by SDS-PAGE

3.11 Steps in Brief

Under aseptic conditions the MGYP media was inoculated with the *Candida tropicalis*. 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 ultrafiltartion

Ultrafiltration

The molecular weight cut-off of 50kDa was used for the filtration purpose. Carefully the retentate was collected and checked for the naringinase activity and protein concentration. The filtrate was also assayed for naringinase activity.

Ammonium sulfate precipitation

The retentate was saturated with ammonium sulfate fractionation of different concentration of ammonium sulfate like 55, 65, 75,85 and 95% 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 phosphate buffer and stored at 4° C

Dialysis

The proteins were dialysed against phosphate buffer at pH 7.0 at 4 °C

Anion exchange chromatography

The dialysed proteins were used as analyte for the chromatography. The column was packed with DEAE-Cellulose of about 10 cm in height. The column was equilibrated with phosphate buffer of pH 7.0.The sample was

added to the column. The sample fraction was eluted with (0.1-1.5 M NaCl) in phosphate buffer of pH 7.0.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 is used as an analyte for the next step of purification.

Gel filtration chromatography

The Sephadex G 200 column was packed and equilibrated with phosphate buffer and the sample was eluted using different concentrations of NaCl. The eluted sample was assayed for naringinase activity. The sample with higher activity was pooled together and it was dialysed to remove the salts.

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

3.12 Characterizing the purified enzyme

The purified enzyme was characterized to know its optimum temperature, optimum pH, its Km value with the same specification used for the crude enzyme.

3.13 Immobilization of crude naringinase enzyme

The crude enzyme was used to study its application on different fruit juices like two varieties of oranges, lemon, musambi and grapes. Two different immobilization supports were used.

- Sodium alginate beads
- Hen egg white beads.

3.13.1 Preparation of sodium alginate beads

Different concentration of sodium alginate beads like 1, 2, 3 and 4% was used. Equal amount of enzyme and water was used to prepare the sodium alginate beads. Drop-by-drop sodium alginate was placed into cold 0.2 M $CaCl_2$ for the formation of uniform sized beads. The peristaltic pump was used at the low flow rate to form the beads in correct size. Then the beads were kept at 4°C for half an hour then it was used for protein quantification and naringinase activity. The beads were filtered and washed with water for further use. It was kept at 4°C for 15 days to study its stability. The protein concentration and naringinase activity was measured after 15 days.

3.13.2 Preparation of hen egg white beads

To the 5ml of egg white, 0.5 ml of enzyme was used. The hen egg white and enzyme were mixed well using the magnetic stirrer. The peristaltic pump was used at the low flow rate to form the beads in correct size. The cold mixture of 2% glutaraldehyde was prepared in Toluene and chloroform (3:1) mixture. The uniform sized beads were formed after two hours and it was washed with water. Then the water is analysed for protein quantification and naringinase activity. It has been kept at 4 °C for 15 days to study its stability. The protein concentration and naringinase activity was measured after 15 days.

3.14 Characterization of immobilized enzymes

3.14.1 Effect of pH

The optimal pH of the immobilized naringinase in Sodium alginate and Hen egg white beads were found by using the naringin of 0.1% in sodium acetate buffer (0.1M) of pH varies from 2.5 to 5.5.

3.14.2 Effect of Temperature

With the optimum pH of the immobilized naringinase in sodium alginate and hen egg white beads, the optimum temperature was found by incubating the beads with the substrate concentration of 0.1% at varying temperature ranges from 20 to 55°C.

3.15 Application of crude soluble and immobilized enzyme on citrus fruit juices

The citrus fruits like *Citrus lemon* (lemon), *Citrus limetta* (musambi), *Citrus sinensis* (sweet oranges), *Citrus reticulata* (orange) and, *Vitis vinifera* (Grapes) were used. All these fruits having naringin were reported (Hawolkog-dong *et al.*, 1999; Kim, 1993; Manalo *et al.*, 1980).

3.15.1 Extraction of juice from fruits

The fruits were peeled to remove the skin. The pomace with the juice was smashed well and it is centrifuged at 4°C for 15 minutes at 10,000 rpm. The supernatant obtained from the samples were estimated for the naringin content by Davis method. The juices were stored at -20 °C and hence it can be used for further analysis (Laboratory manual for fruit processing).

3.15.2 Assay to find the naringin content in citrus fruit juices

The initial naringin content was analysed by taking 100 µl of juice in 5ml of 90% diethylene glycol and it is allowed at room temperature for 10 minutes. The resultant yellow colour was appeared because of the naringin in fruits was measured at 420nm.

The soluble crude enzyme of 200 µl was used for 1ml of juice. The fruit juices were incubated at various temperatures and the results were analyzed. The samples were tested at various temperature and time intervals for the effective results (Olsen and Hill, 1964).

In case of immobilized enzymes, 0.25 g of wet weight of sodium alginate beads and 0.45 g of wet weight of hen egg white beads were used for 1ml of juice respectively. Then the juices were incubated at optimum temperature of the beads. Every one hour the juices were analysed for the naringin content. The reduction in naringin content was observed for every 1 hour up to 6 hours.

3.16 Predicting the structure of naringinase (*In-Silico*)

The alpha-L-rhamnosidase and Beta-D-glucosidase coding sequences were identified in *Aspergillus sp..,* With the help of similarity search tool BLAST, the protein sequences coding by *Candida sp.,* for those enzymes were identified. The sequences were used to predict the model of the protein by using Homology modeling method. The software PROTOPARAM was used to know its Primary structure details.

Many tools like GENO3D, 3digjsaw and Easypred was used to predict the structure. The Bacterial alpha-rhamnosidase was used as template to find the structure using Homology modeling method.

RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION

The culture supernatant from the three organisms was estimated for the protein concentration by Lowry method (Lowry *et al.*, 1951). The maximum protein concentration was observed in 5 day old culture. Hence the five-day-old culture supernatant was used for all the activities.

4.1 Activities of enzymes

The culture supernatant from the three organisms were taken and assayed for the production of the three enzymes. In that *Candida tropicalis* has shown a better activity in all the three enzymes (Table: 4.1). It is known that the cellulase and pectinase were produced from the *Candida tropicalis.* Hence *Candida tropicalis* supernatant was used for the characterization and application(Amba - Awua, etal., 1997)

4.2 Characterization of crude naringinase enzyme

4.2.1 Effect of pH

The optimum pH of crude soluble naringinase was found to be pH 3.0 (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 low pH as reported in *Penicillium decumbens* pH 4.5 (Nourouzian *et al.*, 2004) and in *Aspergillus niger* pH 4.0 (Munish Puri *et al.*, 2005).

4.2.2 Effect of temperature

The optimum temperature was found as 25°C (Figure: 4.2). As the temperature is near to room temperature this enzyme can be easily used. The temperature obtained was comparatively lower than other organisms. As reported in *Penicillium decumbens* 55°C (Nourouzian *et al.*, 2004) and in *Aspergillus niger* 45°C (Munish Puri *et al.*, 2005).

Table 4.1: Screening the microorganism for the activities of naringinase, pectinase and, cellulase

Organisms	Naringinase (U/ml)	Pectinase (U/ml)	Cellulase (U/ml)
Aspergillus sp.,	0.119	0.05	0.035
Candida tropicalis	0.107	0.09	0.08
Kleuyveromyces sp.,	0.08	0.07	0.085

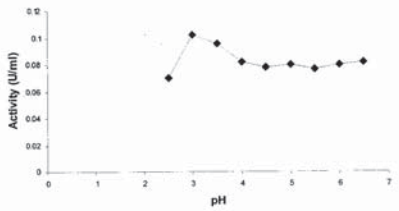


Figure 4.1: Optimum pH for the crude naringinase enzyme

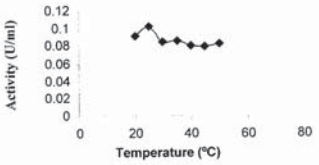


Figure 4.2: Optimum temperature for the crude naringinase enzyme

4.2.3 Substrate concentration

The Km Value obtained as 0.19mM and the Vmax is 0.1 U/ml. This is the very low Km obtained for this enzyme. As reported in *Penicillium decumbens* as 1.7mM (Nourouzian *et al*., 2004) and in *Aspergillus niger* 1.9mM (Munish Puri *et al*., 2005).

4.3 Substrate specificity

The crude naringinase enzyme hydrolysis both the substrates rutin (0.1%) and naringin (0.1%). The extent of hydrolysis of rutin was more than the naringin. Hence the rutin has more specificity than naringin. The enzyme obtained from the *Aspergillus niger* were more specific towards the naringin than rutin (Munish Puri *et al*., 2005).

4.4 Effect of nitrogen sources and naringin

- In the first case, the cells were grown but there is no production of naringinase in both the media with $CaCO_3$ and without $CaCO_3$
- In the second case, there is an increase in the enzyme activity when the peptone of 0.1% was added to the medium.
- In the third case when naringin used at higher concentration (0.5-2%), the cells were not grown. It shows the inhibition action. So slightly decreased the naringin content as (0.1% -0.5 %) were used. Only at the low concentration (0.3%), the activity was comparatively better (Figure: 4.3)

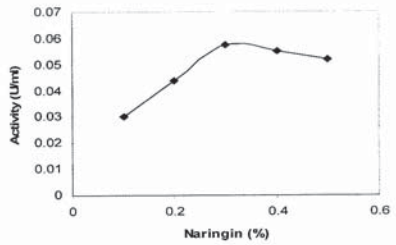


Figure 4.3: Effect of increase in naringin content in the culture media

4.5 Enzyme Purification

The retentate obtained after the ultrafiltration was subjected to ammonium sulfate concentration of various saturation concentration. The 65% 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 0.175M and 0.625M NaCl. The eluted protein in both molar concentration of NaCl has high naringinase activity. Both the eluted proteins were loaded into gel filtration DEAE-Sephadex column. The protein was eluted at two different molar concentration of Phosphate buffer. The protein eluted from the 0.05M of phosphate buffer 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 73 and 78 kDa. The value obtained was closer to the molecular weights reported as 80 and 85 kDa (Munish Puri *et al*., 2005). The purified a-L-rhamnosidase showed 102 kDa (Manzanares *et al*., 2000). The purified ß-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 4.4.

4.6 Characterization of Purified naringinase

4.6.1 Effect of pH

The optimum pH of purified naringinase was found to be pH 3.0 (Figure 4.5). The pH obtained for crude was also the same. This pH obtained is

comparatively low pH as reported in *Penicillium decumbens* pH 4.5 (Nourouzian *et al.*, 2004) and in *Aspergillus niger* pH 4.0 (Munish Puri *et al.*, 2005).

4.6.2 Effect of temperature

The optimum temperature was found as 25°C (Figure 4.6). As it is near to room temperature this enzyme can be easily used. The temperature obtained was comparatively lower than other organisms. As reported in *Penicillium decumbens* 55°C (Nourouzian *et al.*, 2004) and in *Aspergillus niger* 45°C (Munish Puri *et al.*, 2005).

4.6.3 Substrate Concentration

The purified and crude naringinase showed the same Km values. The Km Value obtained as 0.19mM and the Vmax is 0.1 U/ml. This is the very low Km obtained for this enzyme. As reported in *Penicillium decumbens* as 1.7mM (Nourouzian *et al.*, 2004) and in *Aspergillus niger* 1.9mM (Munish Puri *et al.*, 2005).

4.7 Substrate specificity

The enzyme naringinase hydrolysis both the substrates rutin (0.1%) and naringin (0.1%). The extent of hydrolysis of naringin was more than the rutin. The enzyme obtained from the *Aspergillus niger* was more specific towards the naringin than rutin (Munish Puri *et al.*, 2005).

Figure 4.4: Protein shown in SDS-PAGE

Lane: 1Casein
Lane: 2 BSA
Lane: 3 Lysozyme
Lane: 4 Protein eluted by 0.05 M of phosphate buffer
Lane: 5 Protein eluted by 0.1M phosphate buffer

Table 4.2: Purification table

Purification steps	Protein (mg/ml)	Total protein (mg)	Activity (U/ml)	Total activity (U)	Specific Activity (U/mg)	Total activity recovery (%)	Purification fold	Total purification fold
Crude extract	0.908	454	0.053	26.5	0.058	100	1	1
Ultra filtration	0.847	169.4	0.04	8	0.059	30.1	1.01	1.01
Ammonium sulfate Precipitation	0.324	3.24	0.02	0.2	0.061	0.75	1.03	1.05
Anion exchange chromatography	0.138	1.104	0.02	0.16	0.14	0.603	2.29	2.41
Gel filtration chromatography	1.0153	0.107	0.01	0.14	1.307	0.528	9.3	22.5

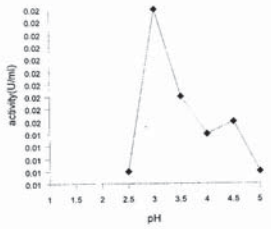


Figure 4.5: Optimum pH for the purified naringinase

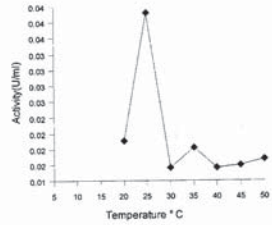


Figure 4.6: Optimum temperature for purified naringinase

4.8 Immobilization of crude naringinase enzyme

4.8.1 Sodium alginate and stability studies

The protein entrapped by the beads was quantified by using Brad ford's assay. About 75% protein was trapped by sodium alginate beads. The sodium alginate of different concentration varied from 1 to 4% was used. The protein trapped and the activity of naringinase was high in only 2% sodium alginate beads. Once the beads were formed in $CaCl_2$, then it is washed with water and it was stored at 4°C. The activity was lost only after 15 days. Even at very high temperature there were not any physical changes in the beads.

4.8.2 Hen egg white and stability studies

There was about 71% of protein was trapped by hen egg white. The beads formed was washed with water and stored at 4°C. It was stable for up to 5 days. When compare with sodium alginate the stability was poor. The removal of naringin content on fruit juice was relatively higher than sodium alginate.

4.9 Characterization of naringinase immobilized in sodium alginate and hen egg white

4.9.1 Effect of pH

The optimum pH was found as pH 5.0 (Figure 4.7) and pH 3.0 (Figure 4.8) for Sodium alginate and Hen egg white respectively. The activity remains good from the pH 3.0 for sodium alginate. As most of the citrus juices pH are from 3.0 to 5.0, these two supports can widely used for its application in fruit juices. The optimum pH of calcium alginate beads was pH 4.0 (Norouzian *et al.*, 1999).The optimum pH for sodium alginate beads was found as pH 4.0 (Munish Puri *et al.*, 1996).

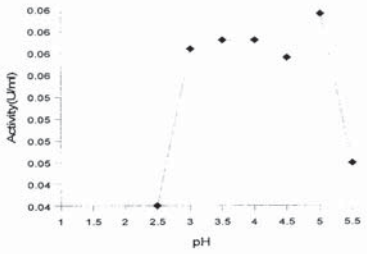


Figure 4.7: Optimum pH for the immobilized naringinase in sodium alginate

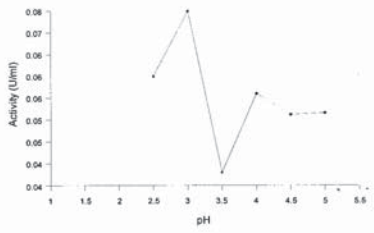


Figure 4.8: Optimum pH for the immobilized naringinase in hen egg white beads

4.9.2 Effect of Temperature

The optimum temperature was found as 50°C for both (Figure 4.9 & 4.10) sodium alginate and hen egg white. These two supports can be used for its application in fruit juices. The optimum temperature of calcium alginate beads and Hen egg white was found as 65°C (Norouzian *et al.*, 1999). The optimum temperature for sodium alginate beads was found as 55-60°C (Munish Puri *et al.*, 1996). These results showed that the temperature used for this is very less comparatively hence for the hydrolysis of naringin can be done extensively without the visible disintegration of beads upto the 55°C.

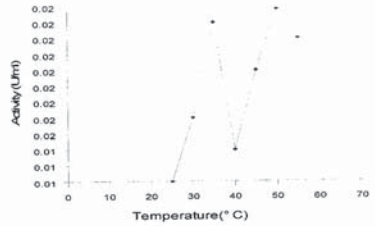


Figure 4.9: Optimum temperature for immobilized naringinase in sodium alginate

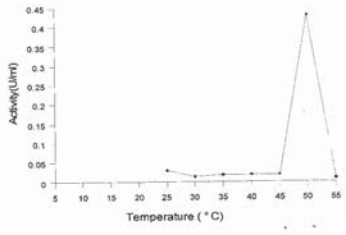


Figure 4.10: Optimum temperature for immobilized naringinase in hen egg white beads

4.10 Application of naringinase in citrus fruit juices

The five citrus fruits were used to reduce its naringin content by adding crude soluble naringinase and immobilized naringinase in two supports like sodium alginate and Hen egg white. The fruit juices pH were measured and it was given in the table. The pH showed that this enzyme can be used for the naringin hydrolysis. Initially the fruit juices were incubated with Enzyme to know its optimum temperature for the reduction on naringin. For the crude and immobilized enzyme showed optimum as 50°C for the reduction of naringin content.

The soluble enzyme showed that 56% reduction of naringin in grapes. The other fruits have also shown that there was considerable reduction of naringin in citrus fruits. Lemon-43%, Sweet Orange-41%, Musambi-36%, Grapes-56%, Orange-42%.The results were shown in figure 4.11.

The immobilized enzyme support was used to treat all the citrus fruit juice at its optimum temperature. The sodium alginate beads showed that there was reduction of 63% of naringin in sweet oranges. Till six hours of incubation of sodium alginate beads, there was not any visible disintegration of beads. Hence it can be used to reduce naringin content. The reduction of naringin in decreasing order is Sweet oranges>Orange>Musambi>Lemon>Grapes. The result obtained was shown in figure: 4.12.

The immobilized naringinase in Hen egg white beads showed more active reduction of naringin in citrus fruit juices. On all those citrus fruits grapes showed considerable reduction in naringin in six hours of about 92%.The colour was reduced for the grapes and hence it is suspected that there might be some kind of changes happened to the juice. The reduction of naringin in fruit juice was shown in decreasing order as Grapes> Orange> Lemon> Musambi> Sweet orange. The results were shown in figure: 4.13.

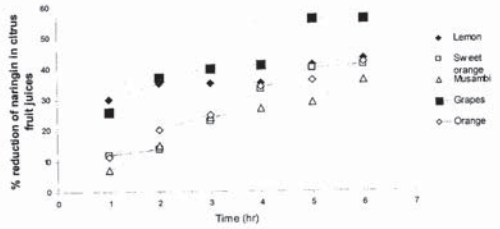


Figure 4.11: Percent reduction of naringin in citrus fruit juices by crude soluble naringinase

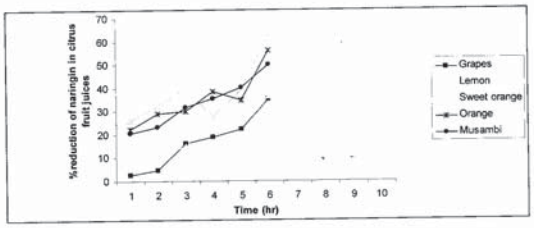


Figure 4.12: Percent reduction of naringin in citrus fruit juices by immobilized naringinase in sodium alginate beads

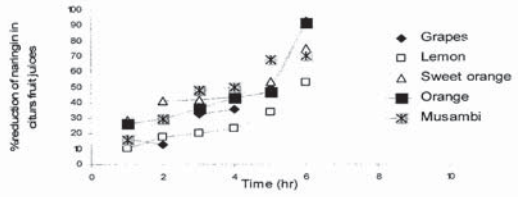


Figure 4.13: Percent reduction of naringin in citrus fruit juices by naringinase immobilized in hen egg white beads

4.11 Structure prediction by *In-Silico*

The protein model was predicted by Geno3D software which can be acceptable. The template used was Bacterial alpha rhamnosidase. Its energy is -8256.69 kcal/mol. The protein allowed region for the ramachandran plot was high and disallowed region is less. The predicted structure was shown in the figure. The structure has

- Number of helices- 2
- Number of H-bonds-93
- Number of strands-12
- Number of turns-29

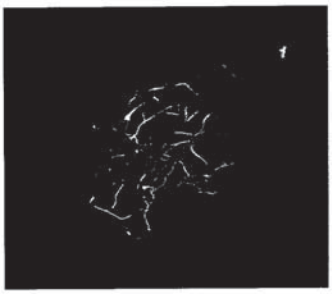


Figure 4.14: Predicted naringinase structure

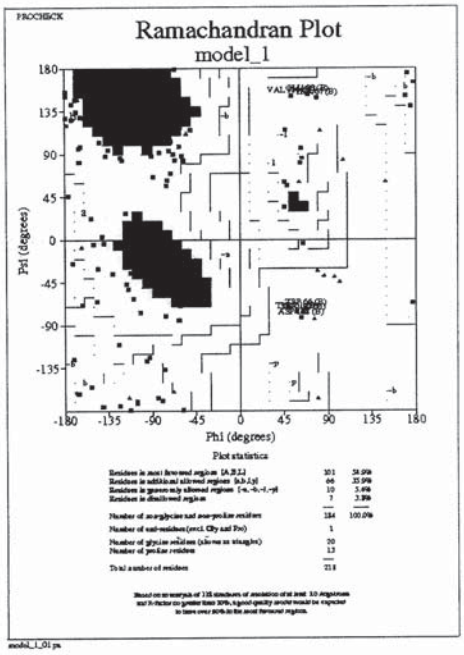


Figure 4.15: Ramachandran plot for modeled protein

CONCLUSION

5.0 Conclusion

- The yeast, *Candida troipcalis* was found to produce the enzymes cellulase, pectinase, naringinase
- It has been cultured to purify the enzyme
- The purification steps includes ultrafiltration, Ammonium sulfate precipitation, Anion exchange chromatography, Gel filtration chromatography, SDS-PAGE
- About 9.3 fold purification was obtained
- The Protein was found as dimmer and its molecular weight is 73 and 78 kDa
- The purified naringinase was characterized, its optimum pH was found as 3.0, optimum temperature was 25°C and its km value is obtained as 0.19 mM
- The immobilized supports sodium alginate and hen egg white bead was used
- The application was studied with citrus fruits like C*itrus lemon* (lemon), *Citrus limetta* (musambi), *Citrus sinensis* (sweet oranges), *Citrus reticulata* (orange) and, *Vitis vinifera* (Grapes)
- Both the supports and crude soluble naringinase were used to remove the naringin content in citrus fruit juices
- For soluble enzyme the reduction was observed as Grapes> Lemon> Orange> Sweet Orange>Musambi
- For the sodium alginate support the reduction of naringin was found as Sweet oranges>orange>Musambi>lemon>grapes

- For the hen egg white support the reduction of naringin was found as Grapes> Orange> Lemon> Musambi> Sweet Orange.
- Hen egg white was found to be efficient in removal of naringin in citrus fruit juices.
- The results obtained were clearly shown that this is highly economical if it is used in fruit juice industry
- The large scale production of this enzyme can be done

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7. APPENDICES

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	6	3.8
41	9	4.0
36.8	13.2	4.2
30.5	19.2	4.4
22.5	24.5	4.6
20	30	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

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

MGYP media composition (g/l):

malt extract – 3.0

yeast extract – 3.0

glucose – 10

peptone – 5.0

pH - 6.4-6.8

The culture was incubated at 28°C for 5 days

Appendix IV

Sequence of naringinase protein

MKVENIRLDLTLPGPLQGTLNTTRPSVSWKTVGDEKNWFQHGYQIR
VRYNNENWTEYEEQMSERSTFVSWPGRDLRSRESFEVSVRVKGPAG
FSDWSAPVLGQVGFLAGQEKWPAAFIAAKNQPRAEGPTAPETLFRK
TFSVSKKVVSATVWSTALGIYELEINGKKVGSDYLSPGWTTYEKRLL
HQTYDVTALIDENVQENVIGARVGAGWYSGKFGFDGGLTNIYGEKR
AISAVLRLVFEDNSSVEIVSDNSWQSSPGPIVAAGLYDGESYDANREI
PGWSLAAENGSGSWAGVDVVPFETSRIEPQTFPHVTVQRKISPKQIFT
TPKGKTVVDFGENIVGFVEFQNATAPKGYQIQFKHAEVMEHGELGT
RPLREAKATDTYTFKGEKSGESYAPRFTFHGFRYCQVEDVSKALSLE
DLRAVVISTNMAQTGEFSCDNNLLNQLHDNVIRSTRGNFITLPTDCP
QRDERMGWTGDIALFGQTAAFLFDCSSMLSSWLKDLWCEQELKAD
SKFPYAPPVTVPNVIKYMKHFWDDQISAIWQDCAVFLPKKLYDSIGA
TFVLADQYESMEKWIECIPKIPGKVRWNKDKVPHLGDWLDPEAPPE
NPFQALTSAYLVADAFLYQVLCYMTEISAQVAPQNKEKYAAMAAQ
CKSDFHDAYIESSGQLSSDSQTAYALVICFGLYKTSEQVEYGGKRLA
AIVEKNGFKIGTGFAGTPFVAKALATTGHLASAYSMLLQKECPSWL
YPVSMGATTIWERWDSMKPDGSINPGEMTSFNHYALGAVASTMHE
VIGGLELVSPGYKEFKVKPQVGGDLKRCRVSHECPYGTIVSSWKIED
GKFSLDVTVPLNTRATIELPDGTQSETGSGVYSFECKAMKFTRATVL
AFAALSMAAPAFDEKLQKKRDGENCDETRVHSHHKHKRAVVYDY
AYVTVTVDAKGNPVTTESAVTSVASTAETDETSSTSTDVSSTTTIVL
DESLTSNEPKTLSLGTGTVTRSTSEETSAETSSSSGSSSGSDNGIYGDL
SAFSDPTEEFEDGVLSCDEFPVGQGVIALDHLGFGGWSGIYNSDTST
GGSCKEGSYCSYACQSGMSKTQWPEDQPSNGVSVGGLLCKNGKLY
KTNSRSNYLCEWGVNKNVVSKLSKSVAICRTDYPGTENMVIPTVVD
GGSSSVITVVDQDTYYTWRGGATSAQYYVNNVAWKDGCLWGTAG
SGVGNWAPLNFGAGYADGVAYLSLIPNPNNYDSLNFNVKIVAQDG
ASVSGSCVYKDGKYNGNGSDGCTVGVTSGAASFVLYE