# STUDIES ON NARINGINASE, A DEBITTERING ENZYME, FROM *PENICILLIUM* SP.

By

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# CERTIFICATE

Certified that the work incorporated in the thesis '**Studies on naringinase, a debittering enzyme, from *Penicillium* sp.**' submitted by Mrs. Sangeeta Telang was carried out by the candidate under my supervision / guidance. Such material as has been obtained from other sources, has been duly acknowledged in the thesis.

Gadre 5/6/98

Dr. R. V. Gadre

Research Guide

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Sangeeta Telang

# ABSTRACT

The flavonoid glycoside, naringin (4', 5, 7-trihydroxyflavanone-7-rhamnoglucoside), chiefly contributes to the bitterness in citrus fruits thereby affecting its keeping quality. Enzymatically naringin is cleaved by naringinase, an enzyme complex with an α-L-rhamnosidase (E.C. 3.2.1.40) and a flavonoid dependent β-D-glucosidase (E.C. 3.2.1.21) activity. The two components act sequentially cleaving the parent naringin to give naringenin, a tasteless compound.

A fungal isolate identified as *Penicillium citrinum* was selected for the present study. Naringinase from this isolate was not inhibited by the various components of the juice, i.e. sugars and citric acid, a problem faced by enzymes reported so far.

The routine assay performed for enzyme activity was a spectrophotometric method using DNSA to estimate reducing sugars. However, the enzyme was also assayed by HPLC to account for the activity in terms of total naringinase as well as the individual enzyme components.

Shake flask level experiments for media optimisation resulted in a yield enhancement from a basal level of 20 U/L to 600 U/L. Naringinase was found to be an extracellular enzyme produced in the late log phase. Media optimisation with respect to components of media, their concentration and physiological conditions, like pH and temperature was carried out. Naringinase production was higher in organic media and addition of glucose further enhanced enzyme production. Growth patterns were similar in the pH range from 5.5 to 7. However, maximum enzyme production occurred at pH 6.5. A temperature of 30 °C was found most suitable for growth and enzyme production. Naringin acted as an inducer for enzyme production and its addition after growth phase proved most beneficial. In the fermenter a similar pattern of enzyme production was obtained.

The enzyme was purified to homogeneity by a series of chromatographic steps involving IEC on DEAE-methacrylate, HIC on methylmethacrylate and GPC through Sephacryl S-200. A fold purification of 1397 with a specific activity of 47.5 U/mg was

obtained. The enzyme had a molecular weight of 105 kD as deduced by SDS-PAGE. Naringinase had a pH optimum between pH 3.5 and 4 and a temperature optimum between 40 and 45 °C. Moreover, in the pH range between 4 to 12 the enzyme retained more than 90% activity at 4 °C and more than 65% activity at 40°C for a period of 24 h. The $K_m$ and $V_{max}$ values for naringinase as determined by DNSA assay were 2.45 mM and 145.5 U/mg protein and that as determined by HPLC were 2.9 mM and 138 U/mg protein respectively. The enzyme was not inhibited by glucose, fructose, sucrose or citric acid and retained greater than 80% activity in the presence of 2% w/v rhamnose.

The enzyme was immobilized on Matrex PAE silica 1000L by adsorption and stabilized by 1.5% glutaraldehyde treated for 1 h. The immobilized enzyme had a pH optimum between 3 and 3.5 and temperature optimum between 50 and 55 °C. In the pH range between 4 and 8.5 the enzyme retained greater than 70% activity at 4 °C for a period of 24 h. The kinetic constants $K_m$ and $V_{max}$ as determined by DNSA assay were 1.12 mM and 0.82 U/g of matrix and by HPLC were 1.42 mM and 0.34 U/g of matrix, respectively. The immobilized enzyme was also not inhibited by glucose, fructose, sucrose and furthermore even by rhamnose at low and high concentrations. This property is a report, first of its kind.

The debittering studies on naringin and simulated juice were carried out using a fluidized bed reactor. For 30 mL of naringin solution or juice processed, 90% naringin was removed in 2 h at 40 °C at a flow rate of 2 mL/min. The enzyme retained 100% activity for 7 runs in 7 days.

# 1. INTRODUCTION

Chemically naringin is a 4', 5, 7 trihydroxy flavanone-7- rhamnoglucoside with an α 1→ 2 linkage between rhamnose and glucose and a β 1 → 7 linkage between glucose and the flavanone moiety. Naringinase carries out stepwise cleavage of naringin. In the first step α-L rhamnosidase [E.C. 3.2.1.40] cleaves the rhamnose from naringin to yield prunin. In the next step β glucosidase [E.C. 3.2.1.21] cleaves the glucose to yield the tasteless compound, naringenin (Figure 1.1).

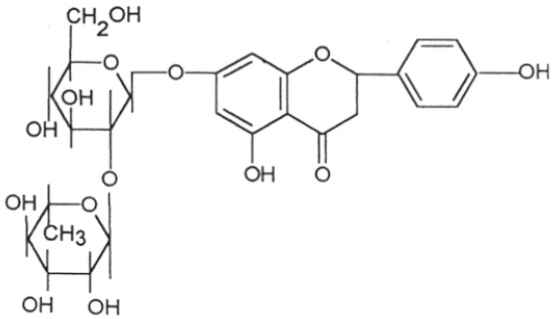
The enzyme plays a significant role in debittering of citrus juice thereby increasing the keeping quality of juice. The enzyme also finds other applications like the synthesis of sapogenins, quercitrin, selinone and derivatives of chloropolysporin.

## 1.1 Objectives

The naringin content in citrus juice varies from 0.1 mg/ml of rough lime to 0.3 mg/ml of grapefruit juice. A few commercial preparations are available for debittering purposes which include those from *Penicillium decumbens* (marketed by Sigma Chemical Company, USA), Naringinase Amano from Amano Pharmaceutical Co., Sanzyme N from Sankyo Co. and from *Aspergillus niger*. These enzymes face an inherent problem of inhibition by the components of the juice itself. Even immobilization of these preparations did not overcome the problem of inhibition by sugars. Furthermore, work on production, purification and characterization of naringinase is rather scanty.

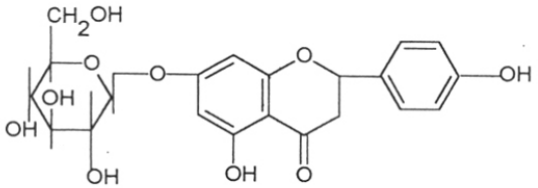
Thus, this study was undertaken to

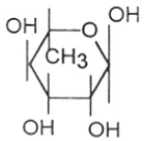
* Isolate a naringinase producing microbial strain and study the various physiological conditions and components of media affecting the enzyme production.
* Purify the enzyme and characterize its different properties.
* Immobilize the enzyme and compare the properties of the immobilized preparation with that of the free enzyme.
* Carry out studies on debittering of juice with respect to efficiency of method and stability of the immobilized enzyme.



NARINGIN

↓ α - L rhamnosidase

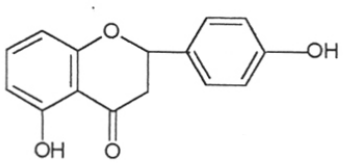


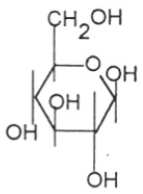


PRUNIN

RHAMNOSE

↓ β - D glucosidase





NARINGENIN

GLUCOSE

**Figure 1.1 :** Stepwise action of naringinase on naringin

## 1.2 Thesis presentation

The thesis has been divided into three parts :-

### *Review of literature*

This part includes a report on citrus fruits, their availability, market and processing as well as a survey of the studies carried out so far on naringinase as regards its production, purification, characterization and debittering of juice by various immobilized preparations.

### *Materials and Methods*

In this section, the protocols for the experiments carried out and the materials used for the study are described.

### *Results and Discussion*

This chapter includes the outcome of the various studies carried out and a comparison between the properties of the enzyme preparations available commercially as described in literature with those of the naringinase prepared from the strain isolated during the present study.

# 2. LITERATURE REVIEW

## 2.1 Citrus

Citrus juice is the most popular processed fruit juice owing to its natural flavour, colour and aroma. Commercially important citrus fruits can be classified into the following main groups: oranges, tangerines, grapefruits, lemons and limes.

Citrus fruits are grown in the tropical and sub-tropical regions of the world. Some of the principal countries where these fruits are grown on a large scale are USA, Brazil, India, China, Pakistan, Egypt, Israel, Morocco, Algeria, Italy, Spain, Mexico, Australia and Japan. The total production of citrus fruits in the world during 1993 was 79,518 thousand tons, of which sweet oranges were 57,520, mandarins 9,010, lemons and limes 7,708, and grapefruit and pummels were 5,280 thousand tons (Figure 2.1).

India is the third largest producer of fruits, next only to Brazil and USA, accounting for 8% of the global fruit production. The world grapefruit market is dominated by the US accounting for 42% and most of it is produced in Florida and California in an almost 2:1 ratio (FAO Yearbook, 1990). In the year 1991, Florida processed 90% of its produce while California processed around 15% and marketed it as juice (Kimball, 1991). Brazil has emerged as a major contender to the US, closely followed by The Peoples' Republic of China and Israel.

In India the citrus industry is one of the major fruit industries after mango and banana. During the year 1993 India produced 2,000 thousand tons oranges, 600 thousand tons lemons and limes, and 50 thousand tons grapefruit and pummels (Mankad, 1994). The figure for oranges includes both sweet oranges and mandarins. The data on export of different citrus fruits during 1992-93 are given in Table 2.1.

## 2.2 Nutritive value of citrus

Citrus fruits are rich in vitamin C and mineral salts. They contain citric acid, malic acid, sugars, fatty acids, essential oils, glycosides, pectins, β-carotene and pigments.

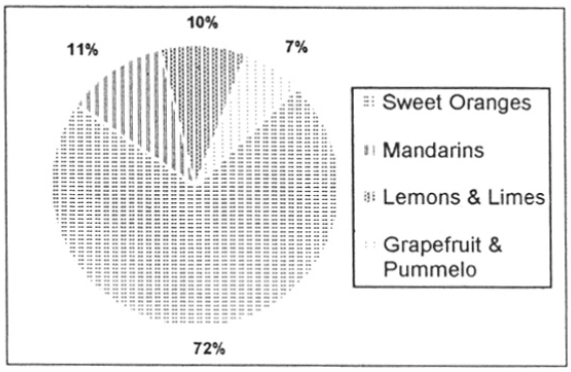



**Figure 2.1** : Production of different citrus fruits in the world in 1993

| Fruit | Quantity (Tons) | Value (Rs. Lakhs) |
|---|---|---|
| Sweet Orange | 8185 | 412 |
| Lemon and Lime | 325 | 16 |
| Grapefruit | 20 | 5 |
| Other citrus fruits | 340 | 30 |

**Table 2.1** : Export of Different Citrus Fruits from India in 1992-93

| Fruit | Protein | Fat | Carbohydrate | Fibre |
|---|---|---|---|---|
| | g / 100 g of edible portion (wet mass) | | | |
| Acid lime | 1.5 | 1.3 | 11.0 | 1.9 |
| Lemon | 1.0 | 0.9 | 11.1 | 2.2 |
| Pummelo | 0.5 | 0.05 | 10.2 | 1.1 |
| Grapefruit | 0.9 | 0.05 | 10.0 | - |
| Mandarin orange | 0.9 | - | 10.3 | 0.45 |
| Sweet lime | 0.7 | 0.6 | 9.0 | 0.45 |
| Malta | 0.7 | 0.8 | 8.0 | 0.3 |

(Mankad, 1994)

**Table 2.2** : Chemical composition of some citrus fruits

The sweetness of the juice is attributed to the sugars namely glucose, fructose and sucrose that vary from as less as 1% in certain limes to as high as 15% in some oranges. The fruits also contain coumarins, psoralins, flavonoids and limonoids. The chemical composition of citrus fruits is summarized in Table 2.2.

## 2.3 Processing of fruits

Different citrus fruits, especially oranges, are mainly processed for their juice from which many products like single strength canned juice, beverages, concentrates, etc. are obtained. In addition, by-products like volatile oil, pectin and cattle feed are also obtained from these fruits. The second and third grade, ripe, sound oranges are used for processing while the fresh fruits of first grade are for table consumption.

In India, various products are manufactured mainly from the mandarin varieties such as 'Nagpur' santra, 'Coorg', 'Assam' and 'Khasi' oranges and 'Kinnow'. The sweet orange varieties 'Malta', 'Malta Blood Red', 'Mossambi' and 'Sathgudi' are used to a limited extent in the preparation of squashes in some areas, mainly for blending, when mandarins are in short supply. Of the various fruits, Kinnow Mandarin, a hybrid of *Citrus nobilis* and *Citrus deliciosa* has emerged as one of the main fruit crops of Punjab and accounts for 45% of Punjab's total fruit production.

For the extraction of juice the unwholesome fruits and attached stems are removed manually. The fruits are washed in running chlorinated water to remove dirt and microorganisms. After this, the fruits are sent to the extractor. Juice from oranges is extracted either by the application of pressure to the whole fruit or by pressing the halves of crosswise cut fruits on a suitable rosette.

Rotary juice extractors, Brown models, Taglith, Citromart and Polycitrus juice extractors are the different types of extractors used. In all these, the fruits are cut into halves. In Brown model the juice is extracted by reaming. In the Taglith, the halves carried in the depression of drums are pressed by perforated heads while in Citromat the fruit halves are drawn by the drum, containing small spikes, towards the screen and the juice is squeezed out as the distance between the drum and the screen become narrower. In the

FMC extractor, the fruit is initially placed between finger-like cups when a perforated steel tube cuts a hole in the bottom of the fruit and the juice flows through the cut into the perforated tubes as the fruit is pressed by the upper cup. In CFTRI citrus juice extractor, the cutting of mandarin fruits into halves and extraction are accomplished in the same machine thereby saving labour. This machine can handle up to 10,000 fruits per hour. The juice is collected in two separate streams, one free from peel oil and the other mixed with traces of peel oil.

The next step in processing involves screening of the juice. The juice is screened to remove pieces of peel, membrane, coarse pulp and seeds, retaining only the natural fruit cells. The presence of fruit cells gives an attractive appearance to the juice and exerts a marked effect on the mouth feel. In screening, the coarse particles are removed by passing the juice through a 3 mm perforated screen. The juice so obtained is called raw juice and can be used in the preparation of squash and in some cases, pasteurized juice. For the production of concentrates, further screening through finer screens is necessary. Incorporation of excess air in the juice is avoided. One of the best methods of screening consists of passing the juice through a long, slightly inclined, slowly revolving, stainless steel screen in the form a drum. The screened juice flows into the blending tanks where it is tested for soluble solids, colour, etc. and is sweetened or acidified as permitted.

## 2.4 Products from citrus

The following products are manufactured from Mandarin oranges: squash, syrup, crush, beverages, sweetened juice, concentrates, segments, jam, marmalade, pectin and peel oil, the last two being the by-products.

### *2.4.1 Squash*

Preparations from cloudy natural juice are called squashes while cordial are made from clarified juice. According to the Indian Standard Specifications (ISI-5800, 1970) such beverages should contain 25% juice, 40-45% soluble solids, acidity 1.2-1.5% and

preserved with sulfur dioxide (350 ppm) or benzoic acid (600 ppm) or a combination of both. Another variation of squashes is 'Barley Water' that contains 0.25% barley starch.

### *2.4.2 Sweetened juice*

The sweetened juice contains 85% juice having soluble solid content not less than 10%. Generally, the soluble solids are maintained at 16% and acidity at 0.5%. Canned juice does not contain any preservative.

### *2.4.3 Comminuted beverage*

In a process developed by CFTRI Mysore, the fully ripe, sound Mandarin oranges are selected, washed, steamed and cut into pieces which are then mixed with a known quantity of dilute sugar solution and blended. The blended material is then passed through a pulper to get a homogenous mass and the extract thus obtained is formulated and bottled.

### *2.4.4 Ready to serve beverage*

It is prepared from fresh or pasteurized, concentrated orange juice. It must have a minimum of 10% juice, not less than 10% soluble solids, and 0.5% acidity as anhydrous citric acid (w/w) and should not contain more than 70 ppm of $SO_2$ or 120 ppm of benzoic acid. Permitted food colours and flavouring are also added.

### *2.4.5 Concentrates*

They are very popular in several parts of the world, especially in USA and Europe. Three types of concentrates are manufactured commercially: frozen, canned and chemically preserved, of which the canned concentrates are used in the soft beverage, pharmaceutical and infant food industries.

### *2.4.6 Citrus juice powders and canned segments*

Fruit juice powders are commercially manufactured by puff drying and foam mat drying techniques. The powders have a porous structure free from lumps and dissolve readily in water to produce a juice that is reasonably similar to fresh juice in colour and flavour. In the dehydrated orange juice powder, the Brix : Acid ratio lies between 12:1 and 18:1; recoverable oil between 0.006 and 0.012 mL/100 mL of the reconstituted juice.

Although orange segments are canned to a limited extent in India, there is a good scope for export market for the product. The tight skinned oranges 'Malta' and 'Sathgudi' and loose skinned 'Nagpur' and 'Coorg' oranges yield good product.

### *2.4.7 Jams, jellies and marmalades*

Jam manufacture involves dissolution of pectin, addition of fruit pulp, juice or extract and sugar followed by cooking to a reasonably thick consistency (usually 68-70 $^{\circ}$ Brix), firm enough to hold the fruit tissue in position. The ratio of fruit pulp to sugar is 45:55 (w/w).

Jelly is prepared by boiling the fruit with or without water, straining the extract and mixing the clear extract with sugar and boiling the mixture to a stage at which it will set to a clear gel.

Marmalade is a fruit jelly in which slices of the fruit or of the peel are suspended. Marmalades may be either clear (jelly marmalade) or opaque (jam marmalade).

### *2.4.8 By-products*

#### *2.4.8.1 Essential oils*

The citrus oils comprise a number of important essential oils, extensively employed in the flavouring and perfumery trade which impart a refreshing flavour and are indispensable ingredients in the manufacture of alcoholic and non-alcoholic beverages. The oils also

serve as flavouring agents for baked goods, candies, chewing gums as well as perfumed and aerated waters. The commercially important oils are bergamot, citron, grapefruit, lemon, lime and mandarin oils. The yield of cold pressed oil expressed as Kg/ton of fruit, is 3.5 to 6.1 in orange, 2.5 to 3.0 in grapefruit, 3.7 in lime and 6.9 in lemon.

#### *2.4.8.2 Pectin*

Citrus peel from which essential oil has been removed and the pomace from which the juice is extracted, form the raw material for commercial extraction of pectin. Citrus peels and residues contain 2.5-5.5 % pectin.

Pectin is chiefly used as a gelling agent in the manufacture of jams, jellies and marmalades. It is used as a clarifying, thickening, stabilizing and foam forming agent. In the pharmaceutical field pectin is used as a haemostatic agent for treating wounds as a blood plasma substitutes well as in the treatment of gastrointestinal disorders. It is also used in pastes, cosmetics, soaps. Pectin finds further applications in the manufacture of explosives, lacquers and in textile sizing.

## 2.5 Factors determining quality of juice

The fresh fruits for table consumption should have an attractive colour, fresh and uniform appearance without blemishes, be easy to peel, and have high juice content. These characters are conditioned by the climate of the growing area and vary from place to place.

The citrus products used as supplementary foods and beverages are appreciated more for their sensory qualities and to some extent for nutrition. Sensory qualities like taste, texture, aroma, mouth-feel and freedom from defects are important in processed products.

The sugar : acid ratio of the juice and beverages is an important factor in processing. The total soluble solids (TSS) impart sweetness to the fruit and the total titrable acidity (anhydrous citric acid) gives them sourness. The ratio of these two important constituents is usually taken as a reliable index of the quality of the fruit. The soluble solids in citrus

juices primarily consist of 80% sugars, 10% citric acid and its salts. The remainder is made of nitrogenous compounds and other minor soluble substances.

Brix expresses the percent by weight of sucrose in a solution. However, in citrus ° Brix reflects all the soluble solids and not only the sugars. It is determined using a hydrometer or refractometer. Other quality control parameters include pulp content, pectin esterase activity, gelation and recoverable oil.

Microbial analysis is generally not done because of low pH of juices which prevents the growth of pathogens. However, development of off-flavour, off-colour, and off-odour are important for which rapid detection methods are available.

A very important factor affecting the shelf life of citrus juice as well as its consumer acceptability is the 'bitterness' and 'delayed bitterness' of citrus contributed by naringin and limonin, respectively.

## 2.6 Limonin

The delayed bitterness, an outcome of juice processing, is contributed by limonin (Kefford, 1959). Chemically limonin is a highly oxygenated triterpene derivative consisting of a furan ring, two lactone rings, a five membered ether ring, and an epoxide as seen in Figure 2.2. The opening and closing of the lactone rings determine the bitterness of the compound. In intact fruits limonin exists as its tasteless precursor limonate, a ring lactone in which the D ring is open. However, in citrus juice which has an acidic pH, the D ring closes to form the bitter substance limonin (Kimball, 1991; Maier and Margileth, 1968). The limonin content of commercial orange juice ranges from 1.9 to 7.2 mg/L and that for grapefruit juice from 2.1 to 11.4 mg/L.

## 2.7 Naringin

Bitterness in citrus juices is primarily imparted by the substituted saccharide, naringin. The proportion and contribution of naringin towards the bitterness of citrus juice is more significant. Naringin is located chiefly in the albedo (inner white portion) of fruit. A

schematic representation of a section of grapefruit is given in Figure 2.3. Its concentration varies in different sections of the fruit as well as in different varieties of fruit.

Furthermore, mature and ripe fruits show lesser amounts of the flavonoid. Naringin content in a few citrus varieties is summarized in Table 2.3

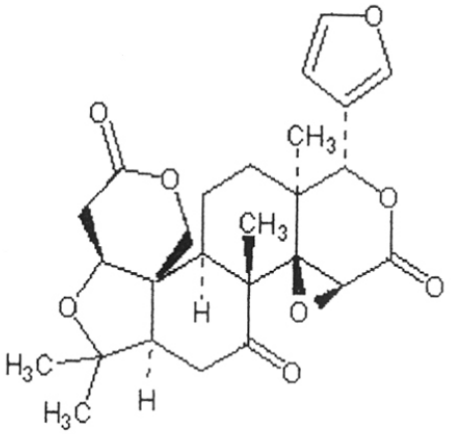
### *2.7.1 Structure and Chemistry*

Naringin belongs to the family of compounds termed as flavonoids. Flavonoids are $C_{15}$ compounds arranged as $C_6$-$C_3$-$C_6$ and the central group is usually linked with oxygen. Depending on the oxidation state of the central $C_3$ unit the flavonoids are classified as catechins, flavanones, flavones and flavonols, in the order of increasing oxidation (Figure 2.4). In citrus the flavonoids usually exist as glycosides. Chemically, naringin is 4',5,7 trihydroxyflavanone-7-rhamno glucoside (Figure 2.2). The flavanone portion of the molecule is tasteless or slightly sweet (Horowitz, 1964). Bitterness is the outcome of a specific linkage between the sugars and the flavanone. When the $C_1$ in the rhamnose is linked to the $C_2$ in glucose, the resulting disaccharide is termed as neohesperidinose and this, in turn, when linked to the flavanone via the 7-hydroxy position yields the intensely bitter compound naringin. However, if the same sugars are linked via $C_1$ to $C_6$ to form rutinose 6-O-rhamnopyanosyl glucopyranose and linked to the flavanone in the same fashion, the resulting molecule "narirutin" is tasteless [Figure 2.5 (Koeppen, 1968)]. Moreover, reduction of the flavanone to its corresponding flavone also destroys bitterness. Removal of rhamnose from naringin results in formation of prunin, which retains only 33% bitterness of the parent naringin molecule (Guadagni *et al.*, 1976).

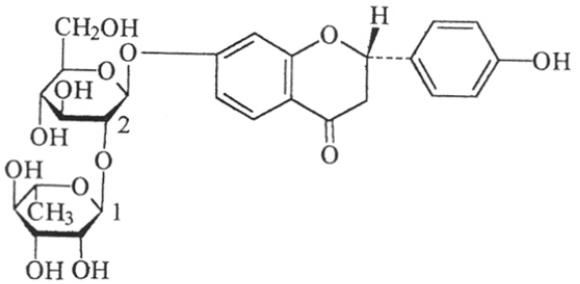
## 2.8 Methods for assay of naringin, its breakdown products and the hydrolytic enzyme, naringinase.

### *2.8.1 Davis method: Use of DEG and NaOH*

One of the earliest reports of detection of flavanones is based on the spectrophotometeric determination of flavanones according to the alkaline diethylene glycol method of Davis (1947).



LIMONIN



NARINGIN

**Figure 2.2** : Bitter components in citrus

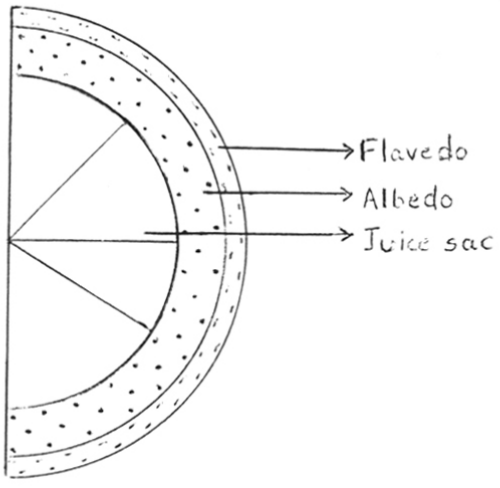
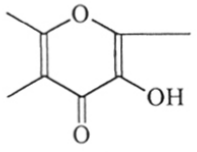


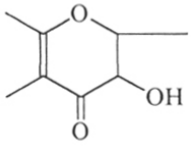

**Figure 2.3** : Schematic representation of section of a grapefruit

| Fruit | Component | Naringin Content (mg/g) or (mg/mL) | Reference |
|---|---|---|---|
| Pummelo | Skin | 3.90 | Salmah *et al.*, 1990 |
| | Juice | 0.22 | ,, |
| Rough Lime | Skin | 0.52 | ,, |
| | Juice | 0.10 | ,, |
| | Seed | 0.03 | ,, |
| Grapefruit | Juice | 0.31 | Hagen *et al.*, 1965 |

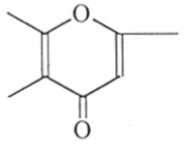
**Table 2.3** : Naringin content in a few citrus varieties




FLAVONOLS




FLAVANONOLS

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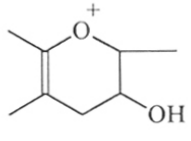


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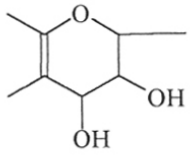


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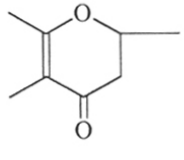


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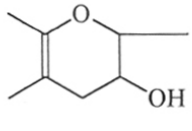
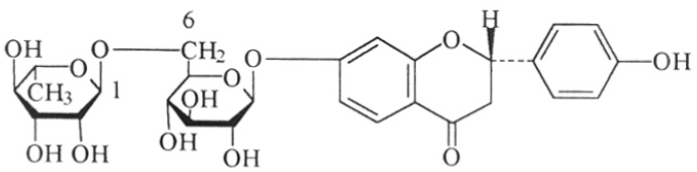
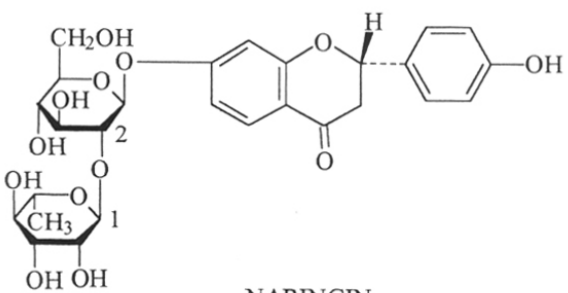



CATECHINS

**Figure 2.4** : Flavonoid structures as determined by the central $C_3$ unit



NARIRUTIN



NARINGIN

**Figure 2.5** : Differentiation in flavanone structure based on sugar linkage

The solutions of naringin, prunin and naringenin give stable, yellow colours in the presence of strong alkali due to the formation of phenolate ions. This method takes advantage of the increase in absorbance of the flavanones in the presence of alkali in the 420 nm region.

In the assay procedure 10 mL of a 90% diethylene glycol solution is mixed with 0.2 mL of the test solution containing the flavanone. Absorbance at 420 nm is measured with a diethyl glycol blank. After this 0.2 mL of 4 N NaOH solution is added to the mixture and the tubes are read once again after 5 minutes.

The major disadvantage of this method is that since all flavanones have a common absorbance maximum of 420 to 430 nm, (though naringenin has less absorbance as compared to naringin and prunin) it fails to distinguish between the individual flavanone components in a reaction mixture.

### *2.8.2 Habelt and Pittner's modification of Davis' method*

In order to overcome the inability of the Davis method to distinguish between naringin, prunin and naringenin, Habelt and Pittner (1983) suggested a method by combining two spectrophotometric procedures. This method has two steps.

1. Treatment with strong alkali to determine the amount of naringenin as well as sum of naringin and prunin.

2. Assay of the liberated aldohexoses with o-aminodiphenyl.

From the data thus obtained, the amount of remaining substrate (naringin), amount of the intermediate (prunin) as well as the product (naringenin) at a given time can be calculated.

In the first step, the mixture containing the three flavonoids is incubated with NaOH and read after 20 min at 375 nm (to get sum of naringin and prunin) and at 310 nm to get amount of naringenin in the mixture. In the second step, i.e. during the enzyme assay the aliquots of assay mixture are assayed for their reducing sugar content. Thus, with the help of the data obtained, prunin content can be deduced. The drawback of the method is that in the first part of the assay, other phenolates may interfere with the estimation.

### *2.8.3 Detection of reducing sugars*

Okada and Inoue (1969) proposed a method whereby the reducing sugars liberated upon degradation of naringin and prunin could be spectrophotometrically detected by the Somogyi Nelson method. Thus, the enzyme could be assayed in terms of the μmoles of reducing sugar, *i.e.* in terms of the product formed.

### *2.8.4 HPLC*

This is the most reliable, accurate and sensitive method available for determination of the specific flavanones. The naringin analysis by HPLC is carried out by the method devised by Fisher and Wheaton (1976). The column used was a Waters Associates 30 cm x 4 mm i.d. Bondapack reversed phase, C-18 column (octadecyl trichlorosilane chemically bonded to < 10 μm porasil packing).Acetonitrile-water in a proportion of 20:80 v/v was used as the mobile phase. Naringin was detected by absorbance at 280 nm and its area was correlated to naringin content by means of a previously calculated calibration curve.

This method is used by several workers for both naringin analysis and enzyme assay by HPLC (Gray *et al.*, 1979; Gray and Olsen, 1981; Tsen, 1988; Tsen *et al.*, 1988; Tsen and Yu, 1991).

### *2.8.5 Use of p-nitrophenyl-α-L-rhamnoside (pNPR) for assaying naringinase.*

In this method (Romero *et al.*, 1985) a specific synthetic substrate p-nitrophenyl-α-L-rhamnopyranoside was prepared to specifically assay the α-rhamnosidase activity of the enzyme. The enzyme activity is expressed in terms of μmol of pNP liberated. [$\varepsilon^{400}$ *p*NP : 21.44 $mM^{-1}cm^{-1}$]

The advantages of this method are

- It did not change the pH, temperature or ionic strength optima of the enzyme.

- There was an increase in the sensitivity thus allowing the detection of low levels of enzyme even in the presence of inhibitors.
- It is a quick, cheap and reproducible method for evaluating the α-rhamnosidase activity.

### *2.8.6 Other methods*

Verstig *et al.* (1977) tried to separate the reaction mixture products by TLC and the quantification of spots was performed either by direct spectrofluorodensitometry or by elution followed by spectrophotometric measurement of the scraped amounts. However, the process is both tedious as well as prone to errors.

Another method is based on determination of rhamnose by using rhamnose dehydrogenase (Bruel *et al.* 1979). However, this method requires an additional step, an additional enzyme and $NAD^+$ thereby making it time consuming and expensive.

## 2.9 Debittering of citrus juices

In order to reduce the bitterness in citrus juices below the threshold level for consumer acceptability, a number of physicochemical and enzymatic treatments have been devised, primarily for debittering grapefruit juice.

### *2.9.1. Physicochemical approaches*

#### *2.9.1.1. Adsorptive debittering*

Applicability of variety of adsorbents such as cellulose acetate, nylon based matrices, porous polymers and ion exchangers has been explored to reduce bitterness and acidity in grapefruit juice. Johnson and Chandler (1982) have comprehensively discussed the results of an Australian programme devised in 1968 on the adsorptive removal of naringin from citrus juices. They reported removal of about 63% of naringin by Amberlite XAD-7 and 20% by Deacidite- FFIP.

Treatment of grapefruit juice with activated magnesium silicate (Florisil) in a batch mode significantly reduced limonin, naringin, narirutin and total acids without adversely affecting its nutritional quality (Barmore *et al.*, 1986).

### *2.9.1.2. Debittering by passage through polystyrene DVB resins*

Puri (1996) has reported around 80% removal of naringin and 90% removal of limonin in batch operations with the use of cross-linked divinyl-benzene styrene resin. Processing navel orange juice with these resins had no effect on the minerals, acid and amino acid content of the juice (Kimball, 1990). Several commercial debittering processes in the citrus juice industry are comprised of centrifugation of orange juice to separate suspended pectinacious solids and passage of clarified juice through polystyrene divinylbenzene resins followed by reconstitution.

### *2.9.1.3. Debittering by β-cyclodextrin*

Beta-cyclodextrin at 0.5% concentration has been used to remove 58% of the initial bitter taste of juice from grapefruit, iyo orange and *Citrus natsudaidai* (Konno *et al.*, 1982). The decrease in bitterness was due to formation of an inclusion complex of β-CD with naringin or limonin. This information led to utilization of a β-CD polymer in a batch/continuous mode to remove limonin and naringin from citrus juices. The important feature observed was that while components such as naringin, naringenin, 7-β rutinoside, coumarins and flavonoids were removed, the brix, acidity and ascorbic acid content remained unaltered. The regeneration of the β-CD polymer by extraction with an organic solvent encouraged its use for scale-up trials. Using a continuous fluid flow column, significant quantities of limonin, nomilin and naringin from grapefruit juice as well as limonin and nomilin from navel orange juice were removed. These results led to a scaled up application of β-CD-polymer on a pilot scale fluidized bed column, enhancing the possibilities of using such a system for commercial operations (Wagner *et al.*, 1988).

#### *2.9.1.4 Limitations of physicochemical approaches*

Although physicochemical techniques are used for debittering of citrus juice they faced several shortcomings like,

1. The methods altered the chemical composition of the juice either by some chemical reaction or removal of flavour, nutrients, colour, etc.
2. The methods being non-specific in nature resulted in batch to batch variation in processed juice quality due to variations in raw juice.
3. The chemicals used in certain processes could not be recycled and hence debittering and disposal of pollutants was cost prohibitive.
4. The methods were cumbersome, lacked reproducibility and gave lower yields with partial loss of desired nutrients in the process of debittering.
5. The quality and type of polymers have to pass requirements of Food and Drugs Administrations in terms of leaching of the polymer or stabilizers.

Owing to the problems encountered in the non-specific physicochemical approach the need for a specific biochemical one arose such as use of enzymes acting on naringin and limonin. Thus, limonin dehydrogenase and naringinase in the form of whole cell preparations, soluble enzymes and immobilized preparations have been studied (Puri *et al.*, 1996).

### *2.9.2 Enzymatic approach of debittering*

Naringin can be hydrolyzed enzymatically and thereby debittered by naringinase. Naringinase is an enzyme complex consisting of an α-L-rhamnosidase [EC 3.2.1.40] component and a flavonoid dependent β-glucosidase [EC 3.2.1.21] component. The substrate naringin is first acted upon by the α-L-rhamnosidase component to yield prunin [4',5,7 trihydroxyflavanone 7 glucoside] and rhamnose. Prunin is only 33% bitter as compared to naringin. Prunin is further acted upon by the flavonoid dependent

β-glucosidase to yield naringenin (4',5,7 trihydroxyflavanone) and glucose. Naringenin is a tasteless compound.

## 2.10 Substrate specificity of naringinase and mode of action

### *2.10.1 Specificity of naringinase from Aspergillus niger*

It was seen that the enzyme hydrolyzed only those substrates containing an α-L-rhamnopyranosidic linkage while those having β-L-rhamnopyranosidic or α-L-mannopyranosidic linkage remained unhydrolyzed. Thus, the enzyme specifically recognized or required an α-L-rhamnosidic linkage for action. In order to study the substrate specificity of α-L-rhamnosidase several disaccharides were synthesized. The action of the enzyme on 2-O α-L rhamnopyranosyl-α-D glucopyranose, methyl 4-O-α-L rhamnopyranosyl-β-L-arabinopyranoside, methyl 2-O-α-L-rhamnopyranosyl α-L-rhamnopyranoside and 6-O-α-L mannopyranosyl β-D-glycopyranose and 15 other disaccharides containing α or β-L-rhamnopyranosidic bond or an α-L-mannopyranosidic bond was examined (Kamiya *et al.*, 1985a; Kamiya *et al.*, 1985b).

### *2.10.2 Specificity of naringinase from Penicillium decumbens*

Several synthetic substrates were prepared and analysed for their ability to serve as substrates for naringinase from *Penicillium* (Michon *et al.*, 1989). Of all the possible methyl rhamnopyranosides, only methyl α-L-rhamnopyranoside was a substrate for naringinase indicating that the enzyme is specific for α-L-rhamnopyranosyl linkage. However, the enzyme failed to act on methyl-1-thio-α-L-rhamnopyranoside, a compound showing the required conformation. The reason may be attributed to the fact that 1-thioglycosides are inhibitors of glycosidases. Further, the enzyme could also cleave α-L-mannopyranoside a property not exhibited by the corresponding enzyme from *Aspergillus niger.* The enzyme site was independent of the linkage sites in rhamnobioses and cleaved α 1-2, α 1-3 and α 1-4 linked rhamnose disaccharides with the same affinity. This

property was further confirmed by treating the enzyme with rhamnose trisaccharides. The enzyme cleaved all the substrates with varying α-L linkages namely sequential α 1-2 or α 1-4 linkages and a combination of α 1-2, followed by α 1-4 linkage. In addition, di and trisaccharides with terminal α-L- mannopyranosyl residues were as effectively hydrolyzed as their corresponding analogs with terminal non-reducing α-L-rhamnopyranosyl residues. All these observations led to the conclusion that the α-L-rhamnosidase from *Penicillium decumbens* works as an exoglyconase rather than a glycosidase.

### *2.10.3 Mode of action*

In order to explain the action of naringinase on the substrate naringin two theories have been proposed.

- According to the first theory proposed by Kishi (1959) the substrate naringin is first acted upon by the glucosidase to yield naringenin and rutinose. In the next step the rhamnosidase decomposes the disaccharide to give glucose and rhamnose.

- Smythe and Thomas (1960) proposed the other theory that naringin is first acted upon by α-L-rhamnosidase to give prunin and rhamnose. Prunin is subsequently acted upon by β-D-glucosidase to give naringenin and glucose.

Okada *et al.* (1964) found that naringin is first split into prunin and rhamnose and the former is degraded to naringenin and glucose. Roitner *et al.* (1984) carried out experiments to determine the exact mode of action. Both spectrophotometric and chromatographic analysis of the hydrolysis products of naringin were carried out. These assays revealed presence of prunin, glucose and rhamnose as intermediates. Rutinose could never be found. Hence their findings support the second theory and this confirmed the mode of action of naringinase.

A schematic representation of the mode of action of naringinase on naringin is given in Figure 2.6.

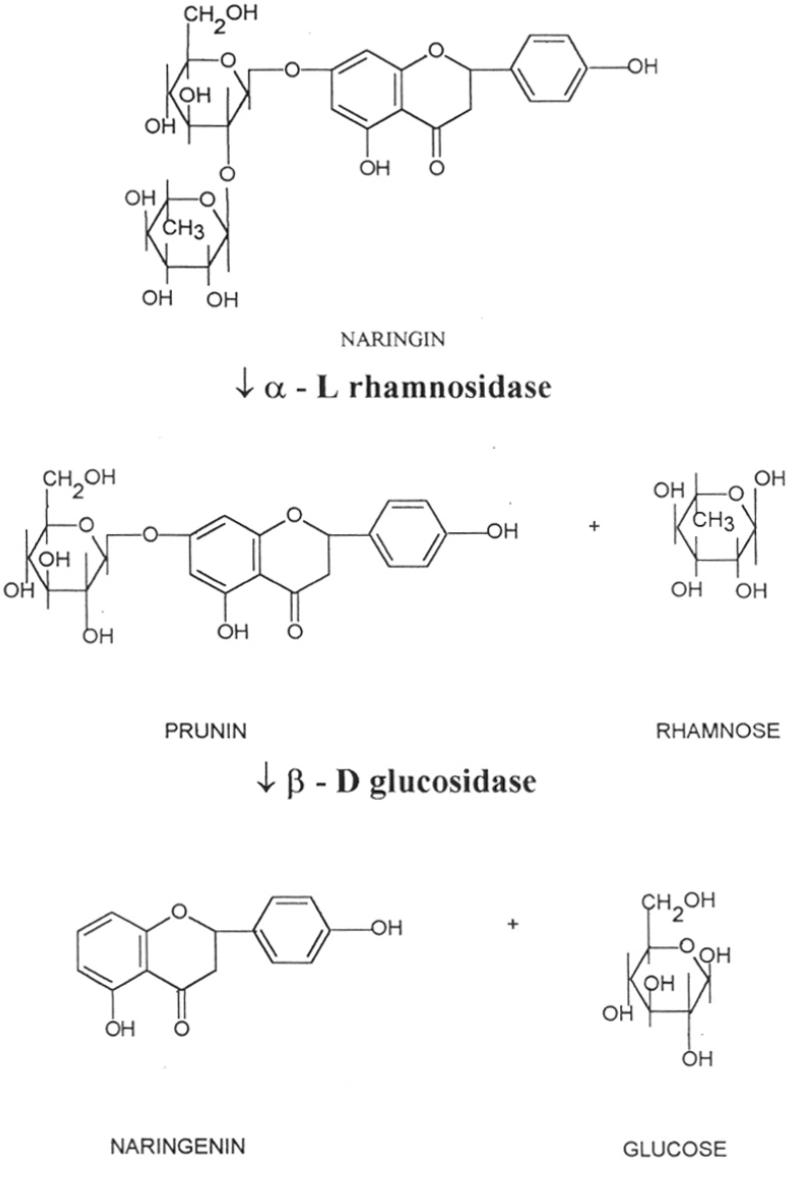



**Figure 2.6** : Schematic representation of stepwise hydrolysis of naringin by naringinase

## 2.11 Production of naringinase

### *2.11.1 Microorganisms producing naringinase*

Fungi belonging to several genera have been reported to produce naringinase, of which enzyme preparations from *Penicillium sp.* and *Aspergillus sp.* are of more common occurrence. Naringinase from *Penicillium sp.* H-3 Ferm P6807 (Amano Pharmaceutical Co. Ltd., 1984), *Penicillium capsulatum* (Fitho, 1991.), *Penicillium DSM 6825 and Penicillium DSM 6826* (Johannes *et al.*, 1994), *Aspergillus niger* NRRL 72-4 (Bram and Solomons, 1965), *A. niger* (Juichiro and Shigetaka, 1972), *A. niger GrM3*, *A. oryzae* and *A. glaucus* (Juichiro and Shigetaka, 1973) have been reported. Besides these, enzyme production by brewers yeast (Tadasa and Hiroshi, 1988), *Rhizopus nigricans* (Shanmugan and Yadav, 1995), *Talaromyces emersonii* (Fitho, 1991), *Cochliobolus miyabeanus*, *Rhizoctonia solani* and *Phomopsis citri* (Tadashi and Yoo, 1975) have also been reported.

### *2.11.2 Media*

Naringinase production in shake flasks as well as fermenters has been studied. In all the reports it has been seen that naringinase is an extracellular enzyme produced in the late log phase. In most cases complex media with low carbohydrate content were employed. Fukumoto and Okada (1972) reported enzyme production from *Aspergillus niger* GrM3 in a medium containing rhamnose and lactose. More recently Shanmugan and Yadav (1995) also reported naringinase production from *Rhizopus nigricans* in a medium containing sucrose and ammonium acetate with an enzyme titer of 1500 U/L.

Generally it has been seen that enzyme production has a requirement for complex and not easily utilizable organic carbon and nitrogen sources. Moreover, glucose, sucrose and starch suppress enzyme synthesis although they support excellent growth (Bram and Solomons 1965). Thus, media with varying concentration and combinations of organic components like soybean meal, corn steep liquor, wheat bran, yeast extract, rhamnolipid,

casamino acids and citrus peel have been formulated for naringinase production. Naringin or hesperidin in pure or crude preparations are used to induce higher yields of enzyme.

In shake flasks titers as high as 10,000 to 15,000 U/L have been obtained from *Aspergillus niger* GrM3 and other *Aspergillus* species in a medium containing soybean residue powder, corn steep liquor and raw hesperidin as an inducer in a period of five days at 30°C (Fukumoto and Okada, 1973).

Bram and Solomons were one of the first to report production of naringinase in fermenters. From their preliminary shake flask experiments they concluded that a medium with CSL-Yeast extract (low carbohydrate content), and naringin added as small aliquots at regular intervals yielded highest enzyme titers. *Aspergillus niger* NRRL 72-4 when grown in a 10 L fermenter with agitation set to 500 rpm, temperature 30 °C and pH 5.5 yielded enzyme of about 460 U at the end of 87 hours as against 410 and 360 U at agitation of 700 and 900 rpm, respectively. The decrease in yield of enzyme at higher agitation could be due to shearing of cells at high speeds. The production was little affected by pH in the range from 4.5 to 7.5, but the yields fell markedly at pH values below 4.

More recently Johannes *et al.* (1994) have claimed production of naringinase by *Penicillium* DSM 6825 and *Penicillium* DSM 6826 in a medium containing rhamnolipid, alkyl esters of rhamnolipid or rhamnolipid mixtures (all obtained from *Pseudomonas aeruginosa*), ammonium sulfate and casamino acids. Fermentation was carried out in a 10 L fermenter with the agitation set at 300 rpm, an aeration of 0.6 vvm, pH maintained at 5.5 and temperature at 27°C for a period of 5 to 10 days yielding an enzyme titer of 5000 U/L.

Naringinase production by stationary culture method on homogenized citrus peel by brewers yeast (Tadasa and Hiroshi, 1988), wheat bran by *Penicillium* H-3 (Amano Pharmaceutical Co. Ltd., 1984) and steamed soybean by *Cochliobolus miyabeanus Rhizoctonia solani* and *Phomopsis citri* (Tadashi and Yoo, 1970) have also been carried out. Activity was seen after 5 days of incubation with a specific activity of about 1100 U/g.

## 2.12 Purification of naringinase

Naringinase from several sources has been isolated and purified by conventional chromatography. The first step involved the concentration of broth by either fractional ammonium sulfate precipitation or ultrafiltration. This was followed by ion exchange chromatography and gel permeation chromatography.

### *2.12.1 Naringinase from Penicillium sp.*

#### *2.12.1.1 Naringinase from Penicillium decumbens*

Hsieh and Tsen (1991) subjected the commercial enzyme preparation to ion exchange chromatography through DEAE-cellulose and later gel filtration on a Sephadex G-200 column with a 2.16 fold purification. The enzyme was found to be a glycoprotein with a molecular weight of 105 kD with no requirement for metal ions. It was inhibited by rhamnose, glucose and fructose in a competitive manner.

Further work was carried out by Gunata *et al.* (1991) who purified the α-rhamnosidase from the commercial preparation by chromatofocussing on PBE 9 Polybuffer 74 ion exchanger. The dialyzed enzyme was loaded onto the column at pH 7.4. The bound proteins were eluted by a pH gradient from 7.4 to 3.7 created during the migration of ampholines at a flow rate of 44 mL/h. The α-rhamnosidase activities were eluted in the pH gradient and 3 isoenzymes with a pI of 6.2, 5.7 and less than 3.7 were obtained. The overall recovery of the α-rhamnosidase activity was 62%.

A detailed analysis of the α-L-rhamnosidase from *Penicillium decumbens* was carried out by Young *et al.* (1989). The enzyme preparation from Sigma was first subjected to ion exchange chromatography with a Pharmacia FPLC equipment and a Mono Q anion exchange column, eluted at 1 mL/min with 0.02 M histidine buffer pH 6.0 and a 30 min gradient from 0 to 0.35 M NaCl in the same buffer. The glycopeptides were prepared by pronase digestion of the α-rhamnosidase. The products were separated on a column of Sephadex G-100 using 0.02 M ammonium acetate buffer at pH 5.0 to give glycopeptide I

which was redigested and purified on Sephadex G-100 and glycopeptide II which was separated from other peptides by chromatography on a column of Bio-Gel P-4. The composition of the enzyme and glycopeptides I and II were determined by amino acid analysis on a Durrum D-500 Analyzer (Table 2.4). The glycopeptides were characterized by $^{1}H$ and $^{13}C$ NMR spectroscopy.

The native enzyme gave a single, somewhat diffused band on SDS-PAGE with an apparent molecular weight of 97 kD. Gel filtration on Sephadex G-100 indicated a molecular weight of 88 kD while size exclusion HPLC on Zorbax GF-250 gave a value of 52 kD.

The larger glycopeptide contained galacto-furanose and mannose residues together with lesser proportions of ethanolamine and glucose. The smaller glycopeptide was of the N-linked type with 10-12 mannose residues per asparagine (Table 2.5).

| Amino acid | Residue (%) | Amino acid | Residue (%) |
|---|---|---|---|
| Asp | 8.9 | Ile | 4.7 |
| Thr | 10.2 | Leu | 8.7 |
| Ser | 14.2 | Tyr | 4.5 |
| Glu | 6.8 | Phe | 3.4 |
| Pro | 5.0 | His | 1.8 |
| Gly | 10.7 | Lys | 3.3 |
| Ala | 10.8 | Arg | 2.3 |
| Val | 4.7 | Ethanolamine | 11.8[a] |

[a] Mol / 100 mol of amino acids

**Table 2.4**: Amino acid analysis of α-L rhamnosidase purified by Mono Q chromatography

| | Mannose | Glucose | Galactose | GlcNAc | Ethanolamine |
|---|---|---|---|---|---|
| Enzyme | 1.89 | 0.35 | 1 | 0.03 | 0.2 |
| Glycopep I | 1.30 | 0.2 | 1 | 0 | 0.17 |
| Glycopep II | 8.4 | trace | 1 | 0.6 | 2.03 |

Values expressed relative to galactose

**Table 2.5** : Carbohydrate analysis of α-L rhamnosidase and derived glycopeptides

| Peptide | N-terminal sequence |
|---|---|
| naringinase (Sigma) 96,000 | ASVPXGEXILAPSSIELIPT |
| α-L rhamnosidase Peptide I 96,000 | DTNDQTSAKVDRGTFDDPAARL |
| α-L rhamnosidase Peptide II 83,500 | FFGSCys θ SLYLKLVLKFGTLFDXA |

X→ amino acid not certain

**Table 2.6** : N terminal sequencing of naringinase and α-L rhamnosidase

#### *2.12.1.2 Naringinase from Penicillium sp. DSM 6826*

The enzyme was purified by a series of steps consisting of concentration using 10 kD molecular weight cut-off membrane, chromatography on Sepharose Q Mono and finally on Superose 12. Ultrafiltration of the enzyme yielded a preparation with a naringinase activity of 50 U/mL. This was loaded on a Sepharose Q chromatography at pH 7.6 and eluted with a linear gradient of NaCl from 0 to 0.5 M. The active fraction with a specific activity of 62 U /mg was loaded on Mono P column and the activity was obtained in fractions from pH 5.6 to 5.8. This fraction was then passed through a Superose 12 column. The enzyme was thus purified to homogeneity and found to have a molecular weight ranging from 60-100 kD as determined by SDS-PAGE, a pH optimum between 5 and 5.5, temperature optimum between 50 and 55 °C and pI 5.6-5.8 ( Johannes *et al.*, 1994). Both the native enzyme and the α-L rhamnosidase peptides were subjected to N-terminal sequencing (Table 2.6).

### *2.12.2 Naringinase from Aspergillus sp.*

Naringinase from *Aspergillus* has been purified using similar procedures like column chromatography using DEAE cellulose, and Sephadex G-200 matrices.

The enzyme from *A. aculeatus* was characterized as a protein of 87 kD (by SDS-PAGE) with a pH optimum between 5.5 to 6.0 and temperature optimum of 60 °C (Mutter, 1994). The naringinase from *A. niger* is a 90 kD protein with a pH optimum of 4.5 (Hsieh and Tsen, 1991). The same enzyme was also studied by Roitner *et al.* (1984) who characterized the enzyme as oligomeric with the smallest active subunit having a molecular weight of 95 kD. The rhamnosidase activity was found to be independent of the pH in the range from 3 to 7 whereas the glucosidase showed a distinct optimum varying between pH 4 and 6. The oligomers showed either both the enzymatic activities or mere rhamnosidase action.

## 2.13 Immobilization of naringinase

Although enzymes find a high potential for use in several industries their actual use has been restricted owing to their inherent instability, high cost of isolation as well as purification and the inability to recover the enzyme from the reaction mixture. In order to overcome these shortcomings several processes whereby the enzyme is immobilized by attachment or entrapment to a water insoluble solid support have been developed.

Some of the advantages of immobilized enzymes over their soluble counterparts are

- The enzymes can be reused
- The process could be run continuously with fine control over several parameters
- The products are easily separated
- Effluent problems and materials handling are minimised
- In certain cases the enzyme properties are altered favourably by immobilization.
- Process cost is considerably reduced.

Immobilization of naringinase on several matrices by adsorption, entrapment or covalent linkage is reported. An account of reactor performance, efficiency of debittering and properties of immobilized naringinases is summarized in Tables 2.7, 2.8 and 2.9.

### *2.13.1 Adsorption*

One of the earliest reports of adsorption of naringinase was the binding of *Aspergillus* naringinase to DEAE- Cellulose (Nobuaki *et al.*, 1975). Later on the binding of the same enzyme onto DEAE- Sepharose was studied by Ono (1977). The conversion of naringin was studied both as a batch as well as a continuous process. The immobilized preparation retained good activity when tested for naringin solution. In the continuous mode it retained 50 % activity after 60 days at 20 °C and for 44 days at 37 °C for a 0.2% naringin solution. For a 0.5% naringin solution, 50% activity was seen after 9 days at 37 °C.

Moreover, when used for natsudaidai juice the enzyme rapidly lost its activity within a day due to inactivation of the enzyme by the sugars in the juice.

## *2.13.2 Covalent binding*

### *2.13.2.1 Chitin*

Both *Penicillium* and *Aspergillus* naringinases have been immobilized on chitin using sodium borohydride and glutaraldehyde (Tsen, 1984; Tsen and Tsai, 1988). In case of the *Aspergillus* enzyme, the column got rapidly inactivated by citrus juice. The *Penicillium* enzyme was stable for at least 6 days with no loss of activity but the enzyme effected the removal of only 30% naringin in a period of 2 h. Citrus juice caused blocking of the column due to the presence of suspended particles. Both the enzymes faced problem of inhibition by the sugars naturally present in the juice. Glucose and fructose were non-competitive inhibitors for *Aspergillus* naringinase while behaved as competitive inhibitors in case of the *Penicillium* enzyme. This reduced the efficiency of the immobilized column with time.

### *2.13.2.2 Porous glass*

Alfonso *et al.* (1987) immobilized naringinase on controlled pore glass (Glycophase G$^{TM}$ CPG from Pierce, USA, 46 nm nominal pore size). For the enzyme immobilization the controlled pore glass was activated by glycol by a periodic acid oxidation to give an aldehyde derivatized support. This was further derivatized by coupling *p*-phenylenediamine to give an arylamine glass support to which the enzyme was covalently bound after a nitrous acid oxidation step. The debittering of simulated juice was carried out in a packed bed reactor. The column showed an operational stability of greater than 99% after 145 hours of continuous operation with a naringin conversion of about 95 to 98%.

### *2.13.3 Entrapment*

#### *2.13.3.1 Hollow fiber*

Olson *et al.*(1979) studied the immobilization of *Aspergillus* naringinase in hollow fiber. The enzyme was loaded in the shell of a Romicon HF PM10 Cartridge having anisotropic polysulfone hollow fibres. The enzyme was stable with no deterioration for a period of 3 days with a use of 8 h per day. However, it caused only 40% removal of naringin.

The enzyme was similarly packed in a Romicon HFXS unit incorporating an identical PM-10HF cartridge (Gray and Olson, 1981). In this case the naringin content in juice with 885 μg/mL naringin could be reduced marginally to 785 μg/mL at a flow rate of 14 mL/min at 45 °C.

#### *2.13.3.2 Cellulose triacetate fiber*

Naringinase was immobilized in cellulose triacetate (CTA) by fiber entrapment method. CTA dissolved in methylene chloride was mixed with the enzyme and the emulsion was extruded through spinnerets to get fine fibres with a diameter of 0.75 μm. In the studies carried out by Tsen *et al.* (1989) the naringinase entrapped in the CTA fibres was packed in a column in the form of parallel ropes and the reactor was operated as a packed bed reactor. The column effected about 31-35% removal of naringin from grapefruit juice and both the rate of removal of naringin as well as the extent of removal remained the same after 20 runs in 20 days (Tsen and Yu, 1991).

| Matrix | Method of immobilization | Column configuration | Enzyme loaded | Juice volume | Flow rate | Temp °C | Substrate conversion (%) | References |
|---|---|---|---|---|---|---|---|---|
| 1) DEAE-Sephadex | Adsorption | column packed with 0.1 g enzyme + 4.9 g plain matrix | 133 U | 5 bed volumes | 0.43-0.68 mL/min | 37 | 100% | Ono et al., 1977 |
| 2) Chitin | Covalent | 8 mm i.d., 5.47 g of enzyme | 12.1 U | 30 mL | 0.8 mL/min | 30 | pNPR 60% in 2 h<br>naringin 40% in 2 h | Tsen *et al.*, 1988 |
| 3) Chitin | Covalent | 8 mm i.d. | 0.8 U | 50 mL | 6.5 mL/min | 40 | Run 1 - 42%<br>Run 2 - 30% (after 1 h) | Tsen, 1984 |
| 4) CTA fiber | Entrapment | 1 g fibre, cut into 1-2 cm, column (i.d. 0.8 cm) x 10 cm | not available | 30 mL | a) 1 mL/min<br>b) 0.5 mL/min | 30 | a) 40-50% in 2 h for pNPR<br>b) 30% in 2 h for pNPR<br>80% in 2 h for naringin | Tsen *et al.*, 1989 |
| 5) Hollow fibre | Entrapment | Romicon HF 1.1 with 45 anisotropic polysulfone hollow fibres, i.d. 1.1 mm, shell 63.5 x 2.5 cm | 4 U<br>112 mg of commercial preparation | 1000 mL | 300 mL/min | 25 | 40% after 3 h | Olson *et al.*, 1979 |
| 6) Hollow fibre | Entrapment | same as above | 145 U | 2 L | 140 mL/min | 45 | From 885 µg naringin to 700 µg in 3 h. | Gray *et al.*, 1981 |
| 7) Calcium alginate | Entrapment | batch reaction | 30 U | 50 mL | | 40 | For naringin 72% in 3 h<br>82% in 7 h<br>Kinnow juice<br>56% in 24 h<br>56% in 48 h | Puri *et al.*, 1996 |

Table 2.7 : **Debittering studies on immobilized naringinase preparations**

| Source | Material | Stability and performance | Reference |
|---|---|---|---|
| *Aspergillus niger* | DEAE Sephadex | 50 % activity after 60 days at 20°C<br><br>and 43.8 days at 37°C for a 0.02% naringin solution | Ono *et al.*, 1977 |
| *Penicillium decumbens* | S-MDA resin (derivatized starch) | 4 months at 4°C for grapefruit juice | Victor *et al.*, 1973 |
| *A. niger* | Chitin | Rapidly inactivated by citrus juice | Tsen, 1984 |
| *Penicillium* sp. | Chitin | 4 months at 4°C. No loss of activity after 5 runs in 5 days | Mei *et al.*, 1988 |
| *A. niger*<br>*P. decumbens* | Chitin | Inactivated by juice,<br>stable for 6 runs in 6 days with no loss of activity | Tsen *et al.*, 1988 |
| *A. niger* | Hollow fiber | No loss of activity after 3 days with 8 h/day use | Olson *et al.*, 1979 |
| *Penicillium* sp. | CTA fiber | 35 runs in 35 days | Tsen and Yu, 1991<br><br>Tsen *et al.*, 1989 |
| *Penicillium* sp. | Calcium alginate gel | 3 months at 6°C.<br><br>50% loss of activity after 12 recycles | Puri *et al.*, 1996 |

**Table 2.8** : Stability and performance of immobilized naringinase preparations

| Matrix | Source | Properties | | | | Reference |
|---|---|---|---|---|---|---|
| | | Opt pH, Temp °C | pH, Temp. stability | $K_m$, $V_{max}$ | Inhibitors | |
| DEAE Sephadex | *A. niger* | 4.0, 60 | retains 80% activity at 55°C after 150 min | 0.48 mM | | Ono *et al.*, 1977 |
| Chitin | *A. niger* | | | | glucose, fructose, rhamnose | Tsen, 1984<br>Tsen *et al.*, 1988 |
| | *P. decumbens* | 3.7, 55 | pH 2 to 12 for 8 h | $K_m$ for<br>naringin 7.8 mM<br>pNPR 1.56 mM<br>$k_i$ for<br>glucose-178 mM<br>fructose-68mM<br>rhamnose-12mM | glucose, fructose, rhamnose | Mei *et al.*, 1987<br>Tsen *et al.*, 1988 |
| CTA fiber | *Penicillium* sp. | 3.7, 55 | | naringin -7.8 mM<br>pNPR- 5.8 mM | | Tsen *et al.*, 1989;<br>Tsen *et al.*, 1991 |
| Porous glass beads | *A. niger* | 5, 55 | pH 5 - 9 for 2 h<br>Activation energy 8.54 kcal/M | naringin-0.86 mM | | Park and Chang, 1979 |
| Calcium alginate | *Penicillium* sp. | 4.5, 55-60 | | 10mM, 8μMol | | Puri *et al.*, 1996 |

**Table 2.9** : Properties of Immobilized Naringinase

## 2.14 Uses of naringinase other than debittering of citrus juice

Naringinase finds several other applications besides debittering of citrus juice. It has been used for the production of a few specialty chemicals and for flavour enhancement in the food industry. A few applications are enlisted below

### *2.14.1 Production of saponins and sapogenins*

Incubation of whole seeds or ground seeds of fenugreek (*Trigonella foenumgraecum*) with naringinase gives higher yields of sapogenins (Elujoba and Hardman, 1985a; Elujoba and Hardman, 1985b). The sapogenin obtained was characterized as diosgenin (Elujoba and Hardman, 1987) which serves as an important steroid. Similarly a new bisnarhopa type genuine sapogenal was obtained by hydrolysis of spergulagenin A. The parent saponin mixture was extracted from the root of *Mollugo spergula* (Isao *et al.*, 1977). Furthermore naringinase from *Aspergillus niger* could also split protodiocin the main steroidal saponin from *Tribucus terrestris* (Perepelitsa *et al.*, 1975). Yet another family of saponins hydrolyzed by naringinase are the ginseng saponins and their related glycosides to yield the corresponding sapogenins. The parent compound was obtained either from the leaves of ginseng or roots of *Panax japonicus*. (Hiroshi and Osamu, 1975)

Ginsenosides are useful as analgesics, sedatives, cardiovascular or metabolism impairing agents.

### *2.14.2 Synthesis of selinone*

Selinone is chemically 4'-γ-γ-dimethylallyl naringenin. Thus, this compound was prepared by the condensation of 4,2,6-$RO(HO)_2C_6H_2AC$ (R = neohesperidosyl) with 4-$OHCC_6H_4OCH_2CH{:}CMe_2$ and hydrolysis of the condensation product with naringinase. (Chari *et al.*, 1970; Sheshadri and Vishapaul, 1970; Aurnhammer *et al.*, 1972).

### *2.14.3 Manufacture of monoglycosyl rutin from rutin*

Rutin when treated with naringinase or hesperidinase yields monoglucosylrutin and isoquercitrin. The former compound was separated from the latter by crystallization. α-glucosylated rutins have good water solubility and are useful as anti-inflammatory agents, antioxidant, UV-absorbant and are used in manufacture of cosmetics (Ida *et al.*, 1993).

### *2.14.4 Novel flavonoids as plant growth accelerators*

Hesperidin was treated stepwise with hesperidinase or naringinase and then with glucosyltransferase in the presence of dextrin to manufacture hesperidin-7-maltotrioside and other oligo-modified hesperidins. These products were soluble in water and stimulated growth of lettuce at ≥ 100 ppm. ( Koichiro, 1993)

### *2.14.5 Manufacture of partially deglycosylated derivatives of chloropolysporin*

Chloropolysporins A, B and C are novel glycopeptide antibiotics obtained from *Faenia interjicta*. These compounds are converted to their partially deglycosylated derivatives by hydrolysis with naringinase (Takatsu *et al.*, 1987; Haishi *et al.*, 1988).

### *2.14.6 Aroma production or enhancement*

Fungal enzymes including naringinase were added during the vinification of red and white wines to enhance the aroma by hydrolysis of terpene glycosides (Bayonove *et al.*, 1993). In the preparation of wine from whole citrus juice (*Citrus natsudaidai*) the juice is first treated with naringinase and then subjected to conventional wine fermentation. Thus the resultant product is citrus wine free from bitterness and with a unique pleasant flavour and aroma (Kumato Prefecture, 1985).

Grape monoterpenyl disaccharides are cleaved sequentially by naringinase to finally liberate monoterpenal thus enhancing the aroma of grape juice and its derived beverages (Gunata *et al.*, 1988; Gunata *et al.*, 1991). The precursor solutions of *Jasminum polyanthum* F, *Jasminum sambac* and *Cardenia jasminoides* E were treated with naringinase to obtain the aroma producing compounds. Thus, it was suggested that the intrinsic glycosidase levels of flowers are enhanced during flower opening that results in aroma formation (Watanabe *et al.*, 1993).

### *2.14.7 Use in food*

Fermented milk preparations with rich and long lasting flavour are manufactured by mixing milk containing materials with flavonoid glycosides like rutin, quercitrin, isoquercitrin, peltatoside or hyperoside (Tsunoda, 1992). These chemicals in turn are obtained from enzyme treatment of their precursors.

Treatment of meat (beef, pork, chicken, fish) with flavonoid glycosides (derived from rutin, quercitrin, isoquercitrin, peltatoside, hyperoside) helps in both softening as well as retaining the freshness and colour of meat (Tanaka, 1992).

Ascorbate browning (by formation of its derivatives) is prevented by addition of flavonoid glycosides. This is useful in pharmaceuticals, cosmetics and food (Inoue, 1992). Similarly in the preparation of citrus puree, citrus fruit candy, health beverages and juices the fruit, peel and/or juice are pretreated with naringinase to remove the bitterness and enhance the flavour and aroma of the product (Cruse and Lime,1973; Onozuka, 1980; Kumai, 1995).

# 3. MATERIALS AND METHODS

## 3.1 Chemicals and growth media

Chemicals and components of the culture media of extrapure or analytical grade were purchased from local suppliers. The salts used in media as well as for preparation of buffers were obtained from S.D. Fine Chemicals, Mumbai. The media components and the dehydrated media were purchased from HiMedia Pvt. Ltd., Mumbai. Naringin, naringinase and molecular weight standards required for gel electrophoresis were purchased from Sigma Chemical Company (St. Louis, USA) whereas Coomasie Brilliant Blue G250 and Imidazole were from Aldrich (USA). Dinitro salicylic acid was obtained from Fluka (Switzerland). Solvents for HPLC were procured from Merck India. Cellulose triacetate was obtained from Eastman Chemical Company (Tennessee, USA). The Glycidyl methacrylate based matrices were a kind gift from Dr. S.Ponnarathnam (Chemial Engineering Division, National Chemical Laboratory, Pune, India).

## 3.2 Screening for naringinase producing microorganisms

Soil samples from different locations were collected in sterile containers and screened for naringinase producing microorganisms. The isolation of cultures and screening was done as follows.

One hundred milligrams of soil sample was inoculated in 50 mL enrichment medium contained in 250 mL Erlenmeyer flasks with the following composition (in g/L) : soymeal, 10; yeast extract, 4; $K_2HPO_4$, 2; dried citrus peel, 2. The flasks were incubated on a rotary shaker at 240 rpm, at 30 °C for 72 h. Samples were withdrawn aseptically at every 24 h and plated on Potato Dextrose Agar (HiMedia) and Nutrient Agar (HiMedia), for isolation of yeasts, fungi and bacteria.

Cultures of *Penicillium* sp. were obtained from National Collection of Industrial Microorganisms (National Chemical Laboratory, Pune), because naringinase with favourable properties for debittering was obtained from a *Penicillium* isolate (Tsen and Tsai, 1988).

To check for naringinase production all the isolates were grown in the same medium mentioned above with 1 g/L naringin instead of the citrus peel. After 72 h of incubation

the solids were separated by centrifugation and the culture filtrate was assayed for naringinase activity.

## 3.3 Assay for naringinase activity

### *3.3.1 Naringinase assay by reducing sugar method*

Naringinase acts on the naringin molecule to liberate the reducing sugars rhamnose and glucose. The reducing sugars were estimated spectrophotometrically by the method prescribed by Miller (1959) with the use of the dinitro salicylic acid reagent.

***Reagents***

1. Substrate : 10 g/L naringin solution in 100 mM citrate buffer, pH 4.0
2. Dinitro salicylic acid reagent (DNSA) reagent : (DNSA 10 g, NaOH 16 g, sodium potassium tartarate 300 g, distilled water to make up the volume to 1L).
3. Glucose standard solution : 2 mg/mL

***Procedure***

One hundred microlitres of the enzyme preparation was mixed with 900 µL of substrate naringin solution and incubated at 40 °C for 30 min in a shaking water bath. After incubation, 1 mL of DNSA reagent was added to the reaction mixture, mixed well and placed in a boiling water bath for exactly 5 min. Contents of the tube were cooled, diluted with 10 mL distilled water, mixed well and absorbance of the solution was measured at 540 nm using a Shimadzu UV-Visible spectrophotometer - Graphicord 254 in 10 mm path length cuvette against a reagent blank.

The amount of reducing sugar produced was calculated from a plot of standard glucose solutions between 0 and 2000 µg/mL at 200 µg/mL difference. One unit of the enzyme was defined as the amount of enzyme that produced 1 µmol reducing sugar, per minute.

### *3.3.2 Naringinase assay by residual substrate estimation using HPLC*

The spectrophotometric method measures the activity on the basis of the product formed. In order to confirm the activity on the basis of residual substrate estimation, an HPLC assay was carried out which being highly sensitive even very low levels of activity could be detected. Naringinase activity was estimated on the basis of naringin hydrolyzed. The procedure of HPLC was according to that given by Fischer and Wheaton (1976) with further modifications.

***Reagents***

1. Citrate buffer 100 mM, pH 4.0
2. Substrate : 10 g/L naringin solution in 100 mM citrate buffer, pH 4.0

***Procedure***

One hundred µL of enzyme preparation was mixed thoroughly with 900 µL of naringin substrate and incubated at 40 °C for 30 min. After incubation 500 µL of of HPLC-grade acetonitrile was added to 500 µL of the reaction mixture in a centrifuge tube to stop the enzymatic reaction and precipitate the non-compatible substances in the sample. The contents of the tube were thoroughly mixed and centrifuged at 5000 g for 10 min. The supernatant was assayed for its naringin content. Twenty µL samples were injected into the column using Rheodyne 7225 injector fitted with a 20 µL fixed volume loop. Different dilutions of naringin were injected to calculate response factor, linearity and to validate the method of quantitation. The naringin was quantitated by external standard method. Units of the enzyme are defined in terms of µmoles of naringin hydrolyzed per minute.

**HPLC instrument**

- **Pump** : Perkin Elmer binary gradient pump LC 250 or Thermo Separation Products (TSP) P4000 quaternary gradient pump with vacuum degasser, SCM 1000.

- **Column** : Scientific Glass Engineering Company, Australia, Reversed Phase, C 18 ODS 2, Glass lined cartridge column, 100 mm x 4 mm, 5 μm particle size, 50 °A pore size, with a similar guard column of 10 mm x 4 mm.

- **Column Temperature** : 30 °C

- **Mobile Phase** : Acetonitrile : Water 20 : 80, filtered through Millipore PVDF membrane and degassed by helium sparging or by SCM 1000 vacuum degasser, flow rate of 0.8 mL/min.

- **Detector** : Perkin Elmer 135 C Photodiode Array Detector or UV 3000 Scanning Detector from Thermo Separation Products, at 285 nm.

- **Chromatography Software** : PC 1000 of TSP.

### *3.3.3 Naringinase assay by estimation of reducing sugars produced using HPLC*

Naringin, upon total hydrolysis, liberates rhamnose as well as glucose which can be quantified separately to give the α- L rhamnosidase and β- D glucosidase activities. HPLC offers a sensitive and dependable analytical technique for detection of these carbohydrates. The assay and sample preparation were carried out as given in the previous method. Different dilutions of glucose and rhamnose were injected to calculate the response factor and to validate the method of quantitation. The naringin was quantitated by external standard method. Units of α-L-rhamnosidase and β-D-glucosidase are defined in terms of μmoles of rhamnose and glucose liberated per minute respectively.

**HPLC instrument**

- **Pump** : P4000 Thermo Separation Products, quaternary gradient pump with vacuum degasser.

- **Column** : Biorad Aminex HPX-87 H, 300 mm x 7.8 mm, ion exchange column.

- **Column Temperature** : 40 °C maintained by Shimadzu Column Oven.

- **Mobile Phase** : 0.05 N $H_2SO_4$ in demineralised water, degassed by SCM 1000 online vacuum degasser, flow rate of 0.5 mL/min.

- **Detector** : Waters 410 Refractive Index detector.

- **Chromatographic Software** : Oracle 2 chromatography software from Indtech, Mumbai, for quantitation of the different compounds.

### *3.3.4 Estimation of naringin content in citrus fruits*

Several varieties of citrus fruits are available in India and a few of them are reported to contain naringin. The citrus varieties locally available were analyzed for their naringin content using HPLC. It was also attempted to determine the relative concentration of naringin in different parts of the fruits. Different portions of the fruit, i.e., flavedo (outer peel), albedo (inner white portion), juice sac and juice were analyzed. One gram (wet weight) of each component was suspended in 5 mL distilled water and placed in a water bath at 75 °C for five minutes. Contents of the tube were cooled and 5 mL HPLC grade methanol was added to each tube. The mixture was centrifuged and the supernatant was subjected to HPLC for naringin estimation following the procedure mentioned in 3.3.2.

## 3.4 Identification of the Isolate

The naringinase producing fungal isolate was identified on the basis of its morphological characters (Raper and Thom, 1949). The culture was identified to species level studying its growth on several recommended media and microscopic examinations.

In the preliminary step recommended by Raper and Thom (1949) the isolate was spot inoculated on three sets of media - Czapek Dox Yeast Extract Agar (CYA), Malt Extract Agar (MEA), and Glycerol Nitrate Agar (G25N).

Composition of CYA (in g/L) : $NaNO_3$, 3; $K_2HPO_4$, 1; $MgSO_4$ 7H2O, 0.5; KCl, 0.5; $FeSO_4$ 7H2O, 0.01; sucrose, 30; yeast extract, 10.

Composition of MEA (in g/L) : malt extract, 20; dextrose, 20; peptone 1.

Composition of G25N (in g/L) : same as CYA with 25 g glycerol instead of sucrose and yeast extract.

The culture was spot inoculated in order to obtain three colonies per plate and the plates were incubated at 25 °C for 7 days. At the end of 7 days all the plates were examined for growth and their colony characteristics were studied and noted.

Slide cultures were used for microscopic observations in order to study the branching and conidiation pattern of the isolate. The slide culture assembly consisted of two glass slides mounted on a triangular glass rod placed in a petri dish with moist cotton at its base to retain moisture. A thin piece of agar block was placed on each slide. The medium was spot inoculated with a spore suspension of the isolate and a cover slip was placed on each agar block. After 48 h incubation at 25 °C, the cover slips were lifted gently and placed on clean slides with a drop of glycerol and then sealed to get permanent preparations. These slides were then observed under microscope to note the morphological characteristics.

## 3.5. Shake flask studies on naringinase production

### *3.5.1 Naringinase production*

Ten flasks containing 50 mL medium with a composition (in g/L) : hydrolyzed soybean meal, 10; yeast extract, 5; $KH_2PO_4$, 2 and naringin, 4 contained in 250 mL flask were inoculated with $2 \times 10^7$ spores and incubated on rotary shaker at 30 °C. Two flasks were withdrawn every 24 h. One of the flasks was used for cell mass estimation and the other was processed for enzyme activity in the following way.

Filtered broth was centrifuged at 5000 g for 15 min and the supernatant was used for determination of extracellular naringinase. The pellet was resuspended in citrate buffer of pH 6.0 after suspension and centrifugation in the same buffer two times. This was then subjected to cell disruption by sonication for 15 min in a Branson Sonifier at a 50% duty cycle (Power requirement: 220 V/50-60 Hz., 3 Amp; Output Power: 400 W; Frequency: 20 kHz). The homogenate was centrifuged at 5000 g for 15 min and the supernatant was used to determine intracellular naringinase liberated in buffer upon cell disruption. The pellet was resuspended in citrate buffer of pH 6.0 and used to determine cell-bound enzyme activity.

### *3.5.2 Media optimization*

The naringinase production studies were carried out in two stages. The first stage covered media optimization which was carried out in shake flasks.

#### *3.5.2.1 Effect of inorganic nitrogen sources and sugar / starch as carbon source on naringinase production*

At the beginning, suitability of inorganic nitrogen and sugars for naringinase production was studied. The nitrogen sources studied were $(NH_4)_2SO_4$, $NH_4NO_3$, $NaNO_3$ and $(NH_4)H_2PO_4$ at concentrations such that they were equal in terms of total nitrogen.

The carbon sources chosen were starch, sucrose and glucose at a concentration of 5 g/L. Twelve combinations of media were prepared and 500 mL flasks with 100 mL medium were inoculated with $3.2 \times 10^7$ spores and incubated on a rotary shaker at 30 °C. Cell mass, enzyme activity and pH were determined for all flasks.

#### *3.5.2.2 Effect of organic nitrogen and carbon on naringinase production*

Media optimization studies primarily revolve around obtaining an optimum carbon to nitrogen ratio which are the energy source and building blocks for cell growth. The choice of the components is governed by several factors like ease of availability, consistency of nutrient content, cost effectiveness and in certain cases requirement for certain specialty chemicals as precursors or inducers. Most commercial media contain agricultural byproducts or wastes as the chief carbon and nitrogen sources. Thus, soybean meal, cotton seed meal, corn steep liquor, as well as wheat bran are commonly used in fermentation media.

A basal medium containing the following components (in g/L) : soybean meal hydrolysate (SBH), 10; $KH_2PO_4$, 2; and naringin, 4 was used. To this, the corresponding organic nitrogen was added on an equal nitrogen content basis in a range from 0.7 to 1.4 g/L. The nitrogen sources tested were yeast extract, peptone, corn steep liquor, casein digest and Pharmamedia (cotton seed meal from Traders Proteins, USA). A flask with only basal medium was also run as control.

Flasks containing 100 mL medium in 500 mL flasks were inoculated with $1 \times 10^7$ spores and incubated on a rotary shaker at 220 rpm at 30 °C. Samples were withdrawn and analyzed for cell mass, enzyme activity and pH. The experiment was repeated with two fold quantities of the organic components to see the effect of double strength medium.

#### *3.5.2.3 Comparison between casein digest and corn steep liquor with and without glucose in enhancing naringinase production*

Of the several organic components tested, corn steep liquor and casein digest gave higher yields of enzyme. Hence a comparative study with these components was made to

check which was more effective. Furthermore a rationale was to be obtained if the hydrolyzed protein (casein digest) was better than complex protein (CSL) as regards yielding greater enzyme levels.

Basal medium (in g/L) : SBH, 10; $K_2HPO_4$, 2 and naringin, 4 with 2.66 g/L corn steep liquor, 1.48 g/L casein digest and two sets of same media with 5 g/L glucose were prepared. Five hundred mL flasks containing 100 mL medium were inoculated with $1 \times 10^7$ spores and incubated on rotary shaker at 30 °C. Samples were withdrawn and analyzed for cell mass and enzyme activity.

#### *3.5.2.4 Comparison between soybean meal (SBM) and soybean hydrolysate (SBH) in naringinase production*

In order to compare the usefulness of soybean meal and hydrolysate, the media were formulated with varying concentrations of SBM and SBH to study their effect on growth profile and enzyme production. The basal medium contained (in g/L) : CSL, 3; glucose, 7; $KH_2PO_4$, 2 and naringin, 4 to which SBM or SBH was added at 20 or 25 g/L concentration. Samples were withdrawn and analyzed for cell mass and naringinase activity.

#### *3.5.2.5 Effect of varying concentrations of soybean meal and glucose on naringinase production*

##### *i) Experiments in presence of corn steep liquor (CSL)*

Basal medium containing (in g/L) : CSL, 3; $KH_2PO_4$, 2 and naringin 4 was prepared. Media were divided into two sets. Two sets with glucose concentration of 7 and 8 g/L were prepared. To both the sets SBM at concentrations of 5, 10, 15, 20, 25 and 30 g/L was added. Five hundred mL flasks containing 100 mL medium were inoculated with $2 \times 10^7$ spores and incubated on rotary shaker at 30 °C. Samples were withdrawn and analyzed for cell mass and activity.

*ii) Experiments in absence of CSL*

Experiments were performed to study the effect of varying concentrations of SBM and glucose on enzyme production in a medium devoid of CSL. The SBM concentrations tested were 15 and 20 g/L and that of glucose were 7 and 8 g/L. One hundred mL medium in 500 mL flasks was inoculated with $2 \times 10^7$ spores and incubated on rotary shaker. Cell mass and enzyme activity were determined.

*3.5.2.6 Effect of surfactant on naringinase secretion*

*(i) Effect of Tween 80 and Triton X-100*

Media containing (in g/L) SBM, 20; glucose, 7; $KH_2PO_4$, 2 and naringin 4 were prepared and divided into two sets. To the first set, Tween 80 or Triton X-100 at a concentration of 2 g/L was added at the beginning in the medium and to the other set the surfactants at the same concentrations were added after 48 h incubation. A control flask with no surfactant was also run. Inoculations were carried out in the manner described above. Cell mass and enzyme activity were determined.

*(ii) Effect of varying concentrations of Tween 80 on naringinase secretion*

Media were prepared as described in (i). Flasks were inoculated and after 48 h of incubation Tween 80 at increasing concentration of 0, 1, 2, 3, 4 and 5 g/L were added to the flasks.

*3.5.2.7 Effect of incubation temperature on naringinase production*

Effect of temperature was studied in a medium containing (in g/L) SBM, 20; glucose, 7; $KH_2PO_4$, 2 and naringin 4. One hundred mL medium in 500 mL flasks was inoculated with $1 \times 10^7$ spores and incubated in a controlled environment incubator shaker at 25, 30 and 35 °C.

### *3.5.2.8 Effect of medium pH on naringinase activity*

Media were prepared as in previous experiment, except that they were in $NaH_2PO_4/Na_2HPO_4$ buffers with pH ranging from 6 to 8 at 0.5 unit intervals. A control flask was prepared in which media components were dissolved in distilled water and contained no buffering salts. Composition of 100 mL buffer solutions are given in the following table (Mc Kenzie, 1978).

| pH Value | Volume of 0.1 M $Na_2HPO_4$ (mL) | Volume of 0.1 M $NaH_2PO_4$ (mL) |
|---|---|---|
| 6.0 | 12.3 | 87.6 |
| 6.5 | 32 | 68 |
| 7.0 | 61 | 39 |
| 7.5 | 84 | 16 |
| 8.0 | 94.7 | 5.3 |

### *3.5.2.9 Effect of different patterns of naringin addition on naringinase production*

To determine the effect of naringin on naringinase production, studies were conducted by periodic addition of naringin to shake flasks. The medium composition was maintained as in section 3.5.2.7 except for naringin. The addition pattern of naringin is summarized in the following table.

| Flask | Time of naringin addition (h) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0 | 12 | 24 | 36 | 48 | 60 | 72 | 84 | 96 |
| | Naringin added (g/L) | | | | | | | | |
| A | 5 | | | | | | | | |
| B | 1 | 1 | 1 | 1 | 1 | | | | |
| C | | | | | 1 | 1 | 1 | 1 | 1 |
| D | 1 | | 1 | | 1 | | 1 | | 1 |

Samples were withdrawn and assayed for enzyme activity. Residual naringin was estimated by HPLC.

## 3.6 Fermenter studies

Growth and enzyme production of *Penicillium citrinum* was studied in a 1 L Gallenkamp fermenter having working volume 700 mL and 14 L New Brunswick Scientific Company's 'Laboferm' automated fermenters having working volume 10 L.

The Gallenkamp fermenter had the following features.

- Volume : 1000 mL total ; 700 mL working
- Vessel Height (H) : 16 cm
- Vessel Diameter (D): 8 cm
- H/D : 2:1
- Impeller Type : Bottom driven, magnetically coupled

The New Brunswick Scientific Co. Fermenter of following specifications was used in the studies.

•Volume : 14 L total ; 10 L working capacity

•Vessel Height : 44 cm

•Vessel Diameter : 21 cm

•H/D : 2.1

•Impeller Type : Three Rushton turbine disks with six blades each

•Impeller Diameter D1 : 7.5 cm

•D/D1 : 2.8

•pH control : Ingold pH combination probe (Switzerland)

•DO : Ingold Polarographic probe with amplifier

•Foam Control : Polypropylene glycol based antifoam UCN of HICO, Mumbai

The fermenters containing the medium (in g/L) : SBM, 20; glucose, 7; $KH_2PO_4$, 2 and naringin 4 were sterilized at 121 °C for 45 min. The temperature during fermentation was maintained at 30 °C with a Julabo circulating water bath. The pH was controlled by addition of 2 N NaOH and 2 N HCl using a pH-stat (NBS). A time delay of 30 seconds was used to avoid titration of the acid and alkali in the fermenter. Single diaphragm combination pH probe was used.

### *3.6.1 Inoculum preparation for fermenter*

A spore suspension with a count of approximately $1 \times 10^7$ spores/mL of *Penicillium citrinum* was prepared. Two hundred fifty mL flasks containing 50 mL medium were inoculated with a 1% (v/v) spore suspension and incubated on a shaker for 48 h at 30 °C and 220 rpm. The contents of the flasks were then transferred to 2 L flasks containing 450 mL medium and incubated on the shaker at 30 °C for 24 h. This served as the inoculum for the 14 L fermenter. The pre-inoculum and inoculum for the 1 L fermenter were prepared in a similar manner with a volume of 10 mL and 70 mL respectively. The

inoculum size was maintained at 10% v/v. The well grown inocula were checked microscopically for purity of the culture .

### *3.6.2 Sampling*

Fermentation broth samples were withdrawn aseptically from the fermenter using a Watson Marlow peristaltic pump. A sterile air purge sample system described by Jogdand and Karanth (1983) was employed. Samples were withdrawn every 12 h for a period of 84 h. Sample sizes for the Gallenkamp and NBS fermenters were 20 mL and 50 mL, respectively.

### *3.6.3 Batch culture studies*

Fermenter studies were carried out as batch experiments for a period of 84 h. The aeration was maintained at 0.5 vvm and the agitation speed was increased from 100 rpm to a maximum of 600 rpm to maintain suitable dissolved oxygen tension in the broth during growth. Enzyme activity was measured by the reducing sugar method using DNSA. The growth was expressed in terms of Packed Cell Volume (PCV). Ten mL of broth was centrifuged in a graduated centrifuge tube at 3000g for 10 minutes in a swing out centrifuge and the volume of the cell pellet was recorded.

### *3.6.4 Effect of pH on enzyme production*

*Penicillium citrinum* was cultivated at different pH values in 1 L Gallenkamp fermenter. The pH was controlled at 5, 5.5, 6, 6.5, 7, 7.5 and 8 using 2 N HCl and 2 N NaOH in different otherwise identical fermentation runs. A run without pH control was also carried out whose starting pH was 5.7. Samples were withdrawn at periodic intervals and analyzed with respect to PCV and enzyme activity.

### *3.6.5 Effect of temperature*

Batch fermentations at varying temperatures were carried out in 1 L fermenter. The temperature was maintained at 25 °C, 30 °C and 35 °C. In one fermentation run it was maintained at 30 °C for the initial 24 h and then decreased to 27 °C. Samples were withdrawn at 12 h interval and estimation of PCV and enzyme activity was carried out.

## 3.7 Purification of naringinase

### *3.7.1 Solids separation and enzyme concentration*

After fermentation in the NBS fermenter the culture broth was filtered through filter cloth and Whatman No. 1 filter paper, under vacuum. If required, Celite was used as a filter aid in the form of a coat on the filter cloth.

The filtrate obtained was subjected to microfiltration using a tubular ceramic membrane with the following features

- Module: Kerasep K00W5085 Techsep (Cranbury, NJ, USA )
- Area: 1200 $cm^2$
- Length : 943 mm
- Diameter : 20 mm
- Particle cutoff size: 20 μm
- Permeate flux : 70 mL/min
- Peristaltic Pump : Watson Marlow 701 U/R (Serial No. 123768)

The permeate (filtrate) obtained from microfiltration was concentrated by ultrafiltration in a polysulfone hollow fibre membrane cartridge with a 20 kD molecular weight cut off at 4 °C. The membrane had the following features

•Module: NTU 3250 C1RC, Nitto Denko Corporation, Japan

•Area: 0.1 $m^2$

•Peristaltic pump: Watson Marlow 503 U

•Retentate outlet pressure: 1 bar

The permeate flux obtained was 50 mL/min and the broth was concentrated to one-tenth of its original volume. Samples of retentate and permeate were withdrawn during the processing and assayed for naringinase activity. The retentate was used as the starting material for further steps of purification.

## *3.7.2 Ion exchange chromatography*

### *3.7.2.1 Use of DEAE-Sepharose*

DEAE-Sepharose, a weak anion exchanger was the first matrix tried. To determine the optimum pH for binding of the enzyme, 1.5 mL of the gel was taken in 25 mm x 150 mm clean Borosil glass tubes. The gel was washed and equilibrated with buffers of different pH values (from 5.0 to 9.0 at 0.5 unit interval). One mL of sample was loaded on each gel, mixed well for one minute and the gel was allowed to settle. The supernatant in each case was assayed for free, unbound naringinase activity. The pH most suitable for binding was found to be 6.5 in case of naringinase.

In the second step the chromatography was carried out using a column (15 x 2.5 cm) packed with DEAE-Sepharose having a bed volume of 30 mL. The sample (filtered broth) was loaded onto the column at a flow rate of 0.3 mL/min with a Pharmacia LKB Pump P1 (Code No. 19-4610-02). After loading, washing with 1 bed volume of buffer (imidazole HCl, pH 6.5) was performed. Elution of the protein was done using five bed volumes of the same buffer employing a linear gradient of 0 to 0.5 M NaCl in the buffer at a flow rate of 0.3 mL/min and fractions of 2 mL each were collected using a Pharmacia fraction collector (UV 100). Protein content in the washings as well as in the fractions collected was estimated by reading at 280 nm in a Shimadzu UV-Visible Spectrophotometer.

### *3.7.2.2 Use of Macro Prep DEAE-Methacrylate matrix*

Macro Prep DEAE-Methacrylate (Biorad Catalogue No: 156-0020) matrix is a weak anion exchanger with a nominal particle size of 50 μm, and a pore diameter of 1000 °A.

The matrix was packed in a column (12.5 x 1.5 cm) with a bed volume of 15 mL. It was washed and equilibrated with the starting buffer, 0.5 M imidazole-HCl at pH 6.5. Fifty mL of membrane concentrated broth was loaded onto the column with a Pharmacia pump at 0.3 mL/min. The column was then washed with one bed volume of 0.5 M imidazole-HCl buffer at the same flow rate. The protein was eluted with a linear gradient of 0 to 0.5 M NaCl in 0.5 M imidazole-HCl buffer of pH 6.5 using a Pharmacia GM gradient mixer. The total volume used for elution was 75 mL (5 bed volumes) and the fraction size was 2 mL each. Protein content in the washings as well as fractions collected was estimated by measuring absorbance at 280 nm in a Shimadzu UV-Visible Spectrophotometer.

The peak fractions as well as samples from the wash fractions were assayed for enzyme activity. The active fractions were pooled, subjected to polyacrylamide gel electrophoresis (PAGE) and then to the next step of purification.

## *3.7.3 Hydrophobic interaction chromatography*

The matrix used was Macro Prep HIC support with a methyl functional group, a nominal particle size of 50 μm (Biorad Catalogue No.: 156-0080). It was packed in a column (9.5 x 1.5 cm) with a bed volume of 9 mL. The column was washed with the initial buffer, i.e. 0.5 M imidazole-HCl buffer of pH 6.5 containing 20% w/v $(NH_4)_2SO_4$. The pooled active fractions of ion exchange chromatography, saturated overnight with 20% $(NH_4)_2SO_4$ were loaded onto the column at a flow rate of 0.3 mL/min. It was then washed with five bed volumes of initial buffer. The elution was carried out using a descending linear gradient 20 to 0% $(NH_4)_2SO_4$ in 0.5 M imidazole-HCl buffer of pH 6.5 using a Pharmacia GM gradient mixer at 0.3 mL/min and fraction size of 1.0 mL.

Protein estimation of fractions and washings was carried out using UV-absorbance as described earlier. Activity in peak and wash fractions was determined. The active fractions were pooled, desalted and subjected to PAGE.

### *3.7.4 Gel permeation chromatography*

The molecular weight of the naringinase from different sources reported so far are in the range of 70-120 kD. Sephacryl S-200 with a fractionation range between 5 and 250 kD was therefore selected for gel permeation chromatography. The matrix was packed in a column (100 x 1.6 cm) with a bed volume of 180 mL. The active fractions of HIC were desalted and concentrated using an Amicon UF cell with a YM 30 membrane having a cut-off of 30 kD and were loaded on the column. Thus, 2 mL of ultrafiltered sample was loaded on the column and isocratic elution at 0.3 mL/min with 180 mL buffer (1 bed volume) was carried out. Three mL fractions were collected. Protein estimation of fractions using UV-absorbance was carried out and enzyme activity of the peak fractions were determined. The active fractions were pooled and subjected to PAGE.

### *3.7.5 Protein estimation*

Total proteins in the fractions were estimated by Lowry's method (Lowry *et al.*, 1951) or by absorbance at 280 nm using bovine serum albumin (fraction V, Sigma Chemical Company) as standard.

### *3.7.6 Electrophoresis*

Polyacrylamide gel electrophoresis (PAGE) of protein samples was performed according to the method of Ornstein (1964) and Davis (1964). The gels were stained as recommended by Neuhoff *et al.* (1988).

***Reagents***

a) Acrylamide : 30 g; N,N-methylene bis acrylamide 0.8 g; distilled water 100 mL

b) N,N,N',N'-tetra methylene diamine: (TEMED)

c) Ammonium per sulphate: 1.5 g; distilled water to 5.0 mL, prepared fresh.

d) Stacking gel buffer: Tris-HCl buffer 0.0625 M, pH 6.8

e) Running gel buffer: Tris-HCl buffer, 0.375 M, pH 8.9

f) Electrode buffer: Tris-glycine buffer, 0.05 M, pH 8.3

g) Tracking dye: Bromophenol blue 5 mg; Ethanol 10 mL.

h) Fixing solution: Methanol 40% v/v in distilled water.

i) Staining solution: Coomassie Brilliant Blue G-250 100 mg; $H_3PO_4$ 2 g; $(NH_4)_2SO_4$ 10 g; Methanol 20 mL; distilled water to 100 mL

j) Destaining solution: Methanol 25% v/v in distilled water

k) Storing solution: $(NH_4)_2SO_4$ 25 g; distilled water 100 mL

The reagents were mixed in the following proportion to cast the gel.

| **Nature of gel** | **Reagent** | | | | |
|---|---|---|---|---|---|
| | a | b | c | d | e |
| Stacking | 1.0 mL | 10 µL | 10 µL | 9 mL | - |
| Running | 2.65 mL | 10 µL | 10 µL | - | 7.35 mL |

***Procedure***

A 1 mm thick gel was prepared and loaded with protein samples (5-20 µL) containing 10% glycerol and 5% tracking dye. The separation was carried out at 100 volts using an electrophoresis power supply. When the tracking dye had reached 1 cm from the bottom of gel, the gel was removed and placed into fixing solution, overnight. The gel was

washed twice with distilled water and stained in reagent (i) for 8 h. It was then briefly washed in solution (j) and stored in solution (k).

## 3.8 Enzyme characterization

### *3.8.1 Molecular weight determination*

The molecular weight of naringinase was estimated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) method (Laemmeli, 1970).

***Reagents***

All reagents were prepared as described for PAGE except that solutions (a), (d), (e) and (f) contained 1% w/v SDS as well.

***Sample preparation***

The samples were pretreated to denature all proteins.

Sample buffer was composed of 1.51 g Tris and 20 mL glycerol dissolved in 35 mL distilled water and pH adjusted to 6.75 with conc. HCl. To this buffer, 4 g SDS, 10 mL 2-mercaptoethanol and 0.002 g bromophenol blue were added. The volume was made to 100 mL with distilled water.

Equal volumes of sample and buffer were mixed together and the mixture was incubated in a boiling water bath for 60 s. This sample was loaded onto the gel and processed as described before. The molecular weight was then calculated by plotting the relative mobility of proteins of known molecular weight against logarithm of molecular weight, and interpolating values for the unknown protein.

The standard proteins used in SDS-PAGE were from Sigma Chemical Co. and consisted of a mixture of carbonic anhydrase (29 kD), egg albumin (45 kD), bovine albumin (66 kD), phosphorylase B (97 kD), β-galactosidase (116 kD) and myosin (205 kD).

### *3.8.2 pH optimum of the enzyme*

The purified enzyme was dialyzed against distilled water (Sigma dialysis bag catalogue number : D- 0655; Lot : 73H0905 ). A cocktail buffer in the pH range of 2 to 12 with 0.5 unit interval was prepared (citric acid 1.92 g; $NaH_2PO_4$ 1.77 g; Tris 1.21g; glycine 0.75 g; distilled water 500 mL. Titrate with 0.2 M NaOH to the required pH).

Fifty microlitres of enzyme preparation was mixed with 950 μL of naringin solutions (10 mg/mL) in buffers at the respective pH. The mixture was incubated at 40 °C in a Julabo shaking water bath at 200 rpm for 30 min. After incubation, the samples were assayed for reducing sugar as described earlier. Activity of enzyme in the different tubes was estimated and a graph of percent activity vs. pH was plotted.

### *3.8.3 pH stability of naringinase*

The stability of naringinase is affected considerably by pH of the enviornment. Activity of the present naringinase was studied after exposure to different pH values for a fixed time as follows.

One hundred microlitres of the dialyzed, purified enzyme preparation was mixed with 100 μL of cocktail buffer in the range of pH 2 to 12 . One set of tubes was incubated at 40 °C and the other at 4 °C for a period of 24 hours. After this, 50 μL of the enzyme sample was mixed with 950 μL of substrate solution at pH 4.0 and the enzyme activity was estimated by reducing sugar method.

### *3.8.4 Temperature optimum of the enzyme*

Fifty microlitres of purified enzyme was mixed with 950 μL of naringin solution (10 mg/mL) in citrate buffer of pH 4.0 and the mixture was incubated at varying temperatures between 20 and 80 °C at 5 °C interval for a period of 30 minutes each in a Julabo shaking water bath. After incubation the activity was estimated as described earlier.

### *3.8.5 Temperature stability of the enzyme*

One mL of the enzyme at pH 4.0 was incubated at different temperatures (10, 30, 40, 50, 60, 70, 80 °C) for 2h. At intervals of thirty minutes aliquots were withdrawn and assayed for naringinase activity.

### *3.8.6 Determination of $k_m$ and $V_{max}$ of enzyme*

Varying concentrations of substrate, i.e. naringin, in the range of 0.3 to 18 mM in the final assay mixture of 1 mL were prepared. Naringin was dissolved in citrate buffer, pH 4.0.

Fifty microlitres of the purified enzyme was mixed with 950 µL of substrate in buffer and incubated for 30 min at 40 °C. After incubation, activity of enzyme was determined by the DNSA method and HPLC.

### *3.8.7 Effect of sugars (glucose, fructose, sucrose and rhamnose) on naringinase activity*

The sugars were dissolved in 0.5 M citrate buffer, pH 3.4, containing naringin (1 mg/mL). The concentrations of the sugars tested were as follows

| Sugar | Concentration (g/100mL) |
|---|---|
| Glucose | 1, 2, 4, 6 |
| Fructose | 1, 2, 4, 6 |
| Rhamnose | 0.5, 0.75, 1, 1.5, 2 |
| Sucrose | 2, 4, 6, 8 |

Two hundred and fifty microlitres of the enzyme preparation were mixed with 750 µL of the sugar-substrate mixtures and incubated at 40 °C. At 0, 10 and 20 minutes, aliquots of the assay mixture were withdrawn and added to equal volume of acetonitrile to stop the reaction. The mixture was centrifuged and the supernatant was assayed for residual naringin content by HPLC. Control for the assay was prepared which did not contain any sugar in the assay mixture.

Activity of the enzyme was calculated as µmole of naringin hydrolyzed/min/mL of enzyme.

## 3.9 Immobilization of naringinase

### *3.9.1 Covalent binding*

Glycidyl methacrylate beads (GMA) crosslinked to either divinyl benzene (DVB), trimethylol propane trimethacrylate (TRIM) or penta erythritol triacrylate (PETA) with varying degrees of hydrophobicity were used for immobilization of naringinase. These matrices were in the form of beads with a particle size of 200-500 µm, 40-100 mesh and pore size of 50-1000 °A. The matrices had oxirane functional groups by which the enzyme could be covalently coupled by its amino or carboxyl functional group. Five hundred milligrams of matrix was washed ten times with 10 mL of 0.5 M phosphate buffer, pH 7.0. Two mL of naringinase in 0.5 M phosphate buffer pH 7.0 was mixed with the matrix and incubated on the shaker at 100 rpm at 30 °C for 72 h. The supernatant was assayed for enzyme activity and matrices with greater than 70% binding was assayed for enzyme activity.

Another modification tested was binding of enzyme substrate complex to the matrix. Five mL of broth was mixed with 50 mg of naringin and incubated in the cold room at 4 ° C for 1 h. Two and a half mL of the mixture (enzyme-substrate complex) was mixed with 500 mg of matrix GMA-TRIM (Glycidyl methacrylate-trimethylol propane trimethacrylate) and incubated at 4 °C for 72 h after which both supernatant and matrix were assayed for enzyme activity.

### *3.9.2 Entrapment*

Another method tried for immobilization of naringinase was entrapment of enzyme in cellulose triacetate. The concentration of cellulose triacetate was adjusted for each experiment in order to suit the requirement.

#### *3.9.2.1 Entrapment in cellulose triacetate ( CTA) fibre*

Cellulose triacetate (0.27 g) was dissolved in methylene dichloride (2.73 mL) and to it 0.4 mL of enzyme solution was added. The mixture was stirred well and fibres extruded through a syringe and needle into toluene bath. The fibres were then air dried to remove solvent. The fibres were then assayed for entrapped naringinase activity.

#### *3.9.2.2 Entrapment in CTA membrane*

One gram of CTA was dissolved in 19 mL methylene dichloride. To this one mL of enzyme solution was added dropwise and the mixture was stirred for 1 h. The membranes were hand casted on glass plates and dipped in toluene broth. After drying, the membrane was peeled off, cut into pieces, washed well in buffer and assayed for naringinase activity. The procedure was repeated with a short mixing time before casting of membrane, after addition of the enzyme.

#### *3.9.2.3. Entrapment by surface coating of membrane*

CTA at a concentration of 0.4 g was dissolved in 19 mL methylene dichloride. To this, 1 mL of enzyme solution was added dropwise and stirred well to get a fine emulsion. Mylar (Polymethyl methacrylate) sheets, 500 μm thick, pinned on glass plates were dipped in the emulsion for 1 min, after which, the membranes were air-dried and then assayed for naringinase activity.

In another modification of this method the emulsion was coated on precast cellulose triacetate membranes.

### *3.9.3 Adsorption*

The hydrophobic matrices used for adsorption of enzyme were XAD2, XAD4, XAD7, XAD9 (Rohm and Hass, USA), NPA1( Ion Exchange India Ltd., Mumbai), silica gel mesh 80-120 (S.D. Fine chemicals, India) and PAE-Silica 1000L (Amicon, USA). The XAD matrices had an average particle size between 0.3 to 0.45 mm, mesh 20 to 50 and pore diameters of 85 to 90, 50, 80 and 90 °A for XAD 2,4,7,and 9 respectively. The PAE-Silica 1000L matrix had a particle size of 50 μm and pore diameter of 1000 °A. In case of XADs and NPA1, wetting of the matrix was carried out in absolute methanol. It was then washed free of the solvent using ten volumes of distilled water over a period of 2 days. One gram of the matrix was equilibrated with citrate buffer, pH 4.0. Then 10 mL of the enzyme solution (pH adjusted to 4.0) was mixed with the matrix and incubated at 30 °C for 2 h on the rotary shaker. The matrix was washed with buffer and assayed for enzyme activity. The binding of naringinase on PAE-Silica 1000 L was carried out at pH 7.0 since the matrix is an anion exchanger.

### *3.9.4 Stabilization of immobilized enzyme with glutaraldehyde*

Stabilization of the immobilization with glutaraldehyde was attempted. In the first set of experiments, after binding of enzyme to the matrix PAE-silica 1000L as described in 3.9.3, glutaraldehyde at a concentration of 2.5% v/v was added to the matrix - enzyme slurry and incubated on the shaker for 2 h. After incubation the matrix was washed with buffer and the matrix was tested for batch debittering of citrus juice. A control flask without glutaraldehyde was also prepared and tested simultaneously.

In the batch experiment, the matrix was washed with citrate buffer, pH 3.4. Then 10 mL of simulated juice (glucose 3 g; fructose 2 g; sucrose 5 g; naringin 0.02 g; citric acid 1.5 g; trisodium citrate 0.8 g; distilled water 100 mL) was mixed with the slurry and incubated on the rotary shaker for 30 min at 40 °C and 200 rpm. After incubation the supernatant was subjected to HPLC for estimation of residual naringin. In this way 12 runs for both control and glutaraldehyde-treated matrix were performed to check for

debittering performance and stability of the immobilized enzyme. Before each fresh batch, the matrix was washed with citrate buffer, pH 3.4.

In the next set of experiments, optimization of both concentration as well as time of treatment of glutaraldehyde was carried out. In this case the matrix was treated with varying concentrations of glutaraldehyde ranging from 0.5% v/v to 2.5% v/v for a fixed period of 2 h and in another set for varying time intervals ranging from 0.5 to 2.5 h with a fixed concentration of 2.5% v/v. The rest of the experiment was carried out in a similar fashion.

### *3.9.5 Characterization of the immobilized naringinase*

All characterization studies were carried out in shake flasks. The basic protocol followed was as follows.

Five mL of enzyme was immobilized on 500 mg of the PAE-Silica 1000L matrix and then treated with 1.5% glutaraldehyde for 1 h. The immobilized enzyme was washed first with phosphate buffer, pH 7.0 and then with the appropriate buffer in which the enzyme was to be assayed. For the assay, 6 mL of the substrate solution in buffer was added to the matrix, of which 1 mL was withdrawn immediately to get the blank. After incubation at 40 °C and 150 rpm for a specified time interval, 1 mL sample was withdrawn for test. Naringinase was assayed either by reducing sugar method or by HPLC.

The immobilized enzyme was characterized with respect to (a) optimum pH, (b) pH stability, (c) optimum temperature, (d) $K_m$, $V_{max}$, (e)Effect of glucose, fructose, sucrose and rhamnose on enzyme activity as studied for the free enzyme, (3.8.2 to 3.8.7) described earlier.

### *3.9.6 Debittering of citrus juice in a fluidized bed reactor with the immobilized enzyme*

After studies on batch debittering of juice in shake flasks, debittering of juice in a column containing immobilized enzyme was attempted. A glass column with dimensions of

(12.5 x 1.2 cm) was packed with 5 mL PAE-Silica immobilized enzyme. The column was washed with buffer at a flow rate of 2 mL/min in an upflow manner. This led to the expansion of the bed upto 10 mL. The temperature of the column was maintained at 40 °C using a circulating bath connected to the jacket of the column. After washing with buffer, the simulated juice was passed through the column at the same flow rate. An initial volume of 50 mL was allowed to pass through the column after which 50 mL of fresh juice was recirculated through the column for 3 h at 40 °C at a rate of 2 mL/min. Samples were withdrawn during operation every 30 min and analyzed for their naringin content by HPLC. After each cycle, the column was washed with phosphate buffer, pH 7.0 and stored at 4 °C. Seven runs over a period of 7 days for debittering of simulated juice were carried out to study the efficiency of debittering and stability of the column.

# 4. RESULTS AND DISCUSSION

## 4.1 Screening

The soil samples screened for naringinase producers yielded a few fungal isolates. On the basis of their morphology they were tentatively identified as *Penicillium* sp., *Aspergillus* sp., *and Rhizopus* sp. These isolates and a few *Penicillium* isolates from NCIM, when grown in broth, yielded varying amounts of enzyme as given in Table 4.1. A *Penicillium* isolate gave maximum naringinase activity under the given set of conditions and was selected for further work. Literature available on screening for naringinase producers is scanty. Johannes *et al.* (1994) used the chromogenic substrate para nitrophenyl α-L-rhamnoside (pNPR) for screening on agar plates, whereby a yellow zone around the colony indicated a naringinase producer. However, owing to the difference in specificity for pNPR and naringin, pNPR was not used in this study.

## 4.2 Identification

Table 4.2 describes the growth characteristics of the isolate on different culture media. The colonies on CYA were leathery with a size of about 30 mm. Sporulation was rather late (after 10 days) and the spores were olive green in colour. Conidiophores were smooth walled throughout and arose from the substratum with some branching. Divergent metulae bearing sterigma with conidia were seen. Conidia were smooth and globose.

Colonies on MEA were dense, velvety, heavy sporulating throughout, grayish green with conidiophores arising from substratum in a dense stand and without exudate.

Growth on G25N was dense, floccose, with poor conidiogenesis and no exudation.

On the basis of the morphological and microscopic observations (Pitt, 1979; Raper and Thom 1949; Rayner, 1970) the isolate was identified as *Penicillium citrinum* (Figures 4.1 and 4.2). *Penicillium citrinum* grows on a wide variety of substrata including cotton and other fabrics, dairy and food products, tobacco, leather goods and various vegetative materials undergoing slow decomposition. They occur regularly in soil. Many strains produce citrinin,

| Culture | Naringinase Activity (U/L) after 72 h |
|---|---|
| *Aspergillus niger* | 5.16 |
| *Rhizopus* sp. | 0.0 |
| *Penicillium* sp. | 20.0 |
| *Penicillium* sp. (NCIM) 765 | 4.36 |
| *Penicillium* sp. (NCIM) 766 | 1.74 |
| *Penicillium* sp. (NCIM) 1065 | 4.3 |
| *Penicillium* sp. (NCIM) 1066 | 2.8 |
| *Penicillium* sp. (NCIM) 1067 | 6.28 |

**Table 4.1** : Naringinase production by different isolates and NCIM strains

| Growth Characteristics at 25 °C | CYA | MEA | G25N |
|---|---|---|---|
| Size | 33 mm | < 30 mm | 18-20 mm |
| Depth | low | low | medium |
| Growth density | dense | dense | dense |
| Surface | sulcate | sulcate | sulcate |
| Mycelium | leathery | velutinous | floccose |
| Height | 1-2 mm | < 3 mm | 3-5 mm |
| Margin | entire | entire | sub surface and entire |
| Conidiogenesis | no | pale olivaceous green | poor conidiation |
| Exudate | nil | nil | nil |
| Soluble Pigment | - | - | - |
| Reverse | light yellow | colourless | white |

**Table 4.2** : Growth characteristics of the isolate on different media after 7 days

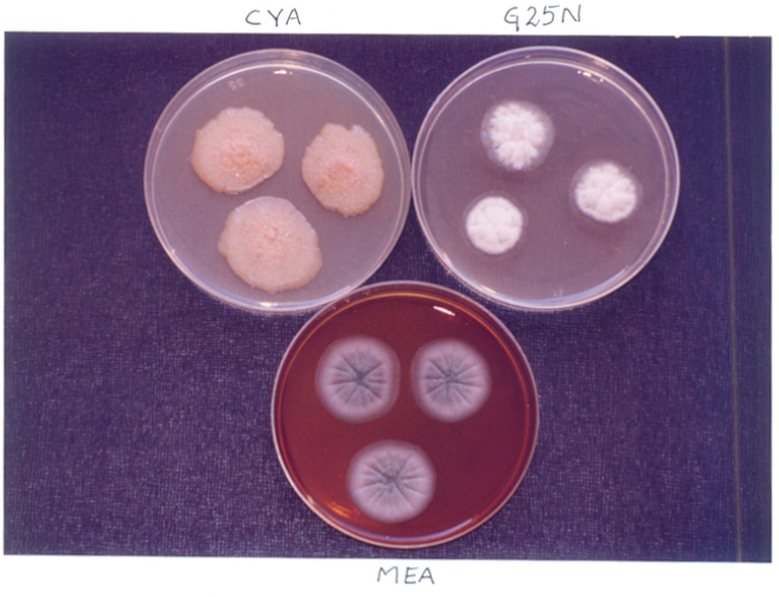



**Figure 4.1** : Photograph showing growth of the isolate after 7 days on CYA, MEA and G25N

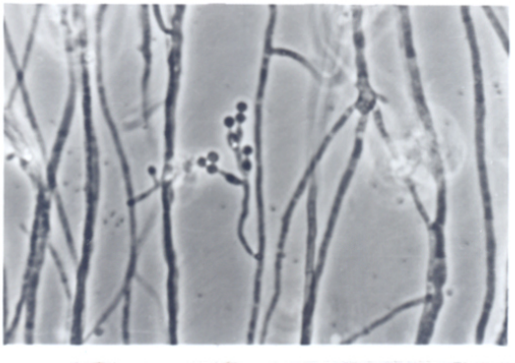


**Figure 4.2** : Photograph showing morphology of *Penicillium citrinum* on CYA

($C_{13}H_{14}O_5$ ), a laevorotatory monobasic acid. Citrinin has selective action against Gram positive bacteria. Some Gram negative bacteria are inhibited at low concentrations. Citrinin is also reported to act against a few pathogenic fungi (Melouk and Aken, 1987). Citrinin is toxic to rats, mice and guinea pigs but does not cause irritation in man. Enzymes such as lipase (Maliszewska and Zboinska, 1996), nuclease P1 (Suh *et al.*, 1987) and protease (Majima *et al.*, 1987) from *P. citrinum* are also reported.

## 4.3 Naringinase assay

The enzyme was routinely assayed with the use of the DNSA reagent as given by Miller (1959). The estimation of naringin as well as the enzyme assay was also carried out by HPLC. The assay by HPLC was used to estimate the activity in terms of the residual naringin (Figure 4.3) as well as to obtain the activity in terms of its α-L-rhamnosidase and β-D-glucosidase components (Figure 4.4). As seen in Table 4.3 there is a good correlation between the spectrophotometric and HPLC assay. In case of the naringinase from the *Penicillium citrinum* isolate, the enzyme is predominantly a rhamnosidase. For every unit of naringinase as assayed by residual substrate method, 0.96 unit is expressed as α- L rhamnosidase and 0.04 unit as β-D glucosidase as deduced by HPLC analysis of sugars.

Other methods for naringin analysis described in literature include the Davis method (Bram and Solomons, 1965; Park and Chang, 1979; Ono, 1980; Puri *et al.*, 1996); the use of the chromogenic substrate *p*NPR (Tsen *et al.*, 1988; Tsen and Tsai, 1988; Young *et al.*, 1989; Gunata *et al.*, 1991; Tsen and Yu, 1991; Johannes *et al.*, 1994) and HPLC analysis as given by Fisher and Wheaton (Olson *et al.*, 1979; Gray and Olson, 1981; Tsen *et al.*, 1988; Tsen and Tsai, 1988; Tsen and Yu, 1991). A severe drawback of the Davis method is its lack of specificity for naringin. Naringinase has a lower $k_m$ value for the chromogen pNPR and its use reflects only the rhamnosidase activity. Hence, our studies employed the DNSA method for total naringinase and HPLC studies for confirmation of results.

| Assay Method | Enzyme Activity (U/mL) |
|---|---|
| Spectrophotometric : DNSA | 0.103 |
| HPLC : Residual substrate | 0.101 |
| HPLC : Sugar analysis | 0.105[a] and 0.004[b] |

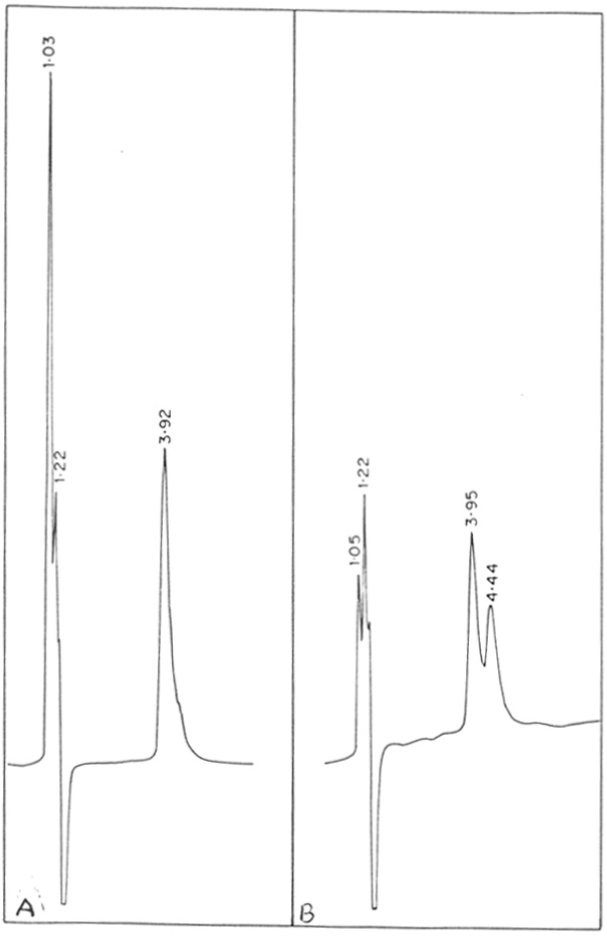
[a] : α- L rhamnosidase

[b] : β-D glucosidase

**Table 4.3** : Naringinase assay

| Fruit Component | Naringin (mg/g) |
|---|---|
| Flavedo | 2.5 |
| Albedo | 18.5 |
| Seed | 0.2 |
| Juice Sac | 0.0 |
| Juice | 0.2 |

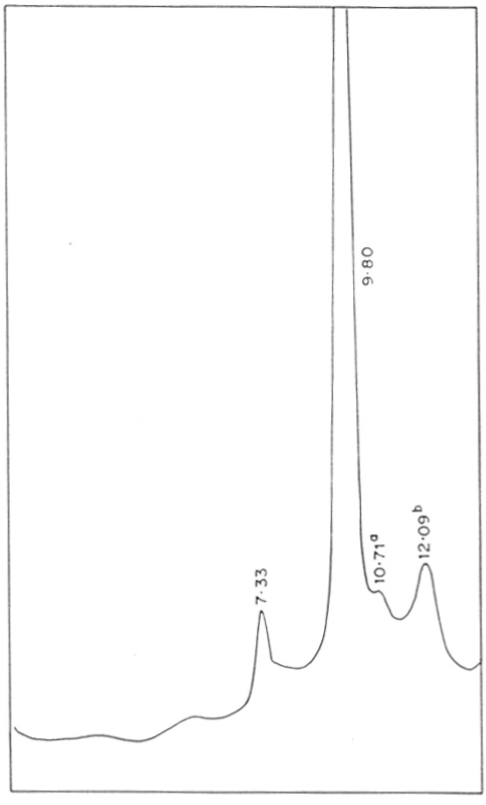
**Table 4.4** : Naringin content in different sections of grapefruit as determined by HPLC




A- Naringin standard (elution time – 3.92 min)

B- Enzyme sample

**Figure 4.3** : HPLC assay to detect substrate hydrolysis




a- Glucose (elution time 10.71 min)

b- Rhamnose (elution time 12.09 min)

**Figure 4.4 :** HPLC assay to detect hydrolysis products

## 4.4 Naringin content in local citrus varieties

The flavonoids found in grapefruit juice are naringin rutinoside, neohesperidin, hesperidin, poncirin and isosakuranetin rutinoside (Hagen *et al.*, 1965). Among the Indian citrus varieties Caliha *et al.*(1965) showed the presence of hesperidin in *Citrus limon* peel, hesperidin, neohesperidin and tangeritin in *Citrus jambhiri*, naringin and poncirin in *Citrus grandis* and hesperidin, neohesperidin, tangeritin, citrometin and 5-O- desmethyl citrometin in *Citrus reticulata*.

Of the several local citrus varieties analyzed, naringin could be detected only in grapefruit (*Citrus paradesi*). The naringin content in the different sections of grapefruit is given in Table 4.4. None of the sections of orange, lime, lemon or kinnow contained naringin. Salmah *et al.* (1990) made a similar observation where they reported the presence of naringin in pummelo and rough lime, but showed that musk lime, mandarin orange and mexican lime were devoid of naringin. Marwah *et al.* (1994) reported a naringin content of 0.25 g/L in "Kinnow" juice. The method used for naringin estimation was that given by Davis, which is non specific. In addition, Kinnow is a hybrid variety between *Citrus nobilis* and *Citrus deliciosa*, both of which do not contain naringin. Hence naringin is unlikely to be present in Kinnow.

## 4.5 Shake flask studies

Shake flask studies offer several advantages. First the ease of operation allows each set of experiments to be set up in duplicate or triplicate offering a statistical validation of the data. Secondly, several combinations of media components and physicochemical parameters can be studied, simultaneously. Moreover, the studies also prove to be more economical which is of importance at the initial screening level. Thus, shake flask studies form the basis of all preliminary optimization studies in fermentation

### *4.5.1 Naringinase production profile*

Naringinase is an extracellular enzyme similar to most hydrolytic enzymes. Glenn (1976) defined extracellular enzymes as those which are external to the cell wall and in contact with the surrounding medium. In eukaryotic microorganisms these enzymes are usually glycoproteins with disulfide bridges and high molecular weight. Physiologically these enzymes function to produce nutrients from polymers or complex macromolecules present in the medium, after the simple or easily assimable nutrients have been utilized.

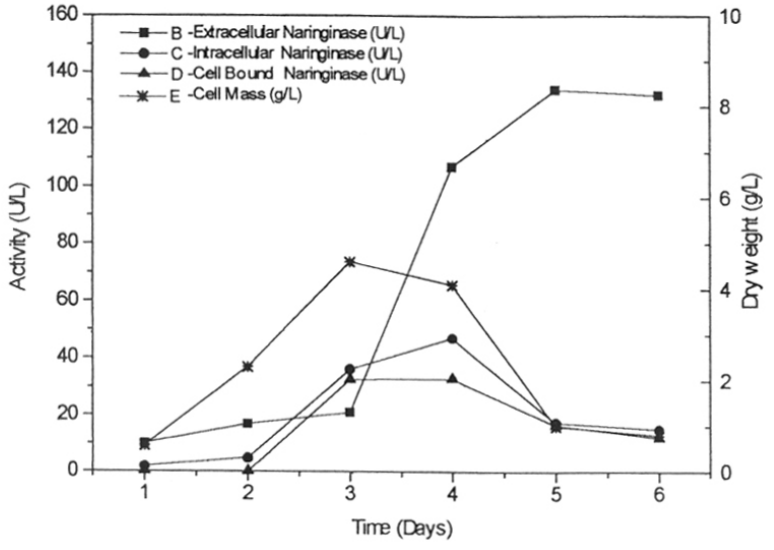
The production profile of naringinase by the present isolate is given in Fig 4.5. Intracellular, cell bound and extracellular enzyme activity was followed at 24 h interval. As seen in Figure 4.5, both cell bound and intracellular activity are growth-associated. However, secretion of enzyme is associated with the late log phase. Maximum extracellular activity was seen after 5 days and remained stable through the sixth day. It has been observed that naringinase secretion occurs in the late log phase of growth. The secretion of naringinase has been reported to occur between 3$^{rd}$ and 5$^{th}$ day of growth (Shanmugam and Yadav, 1995; Fukumoto and Okada, 1973; Bram and Solomons,1965).

### *4.5.2 Media optimization*

#### *4.5.2.1 Effect of carbon and nitrogen on naringinase production*

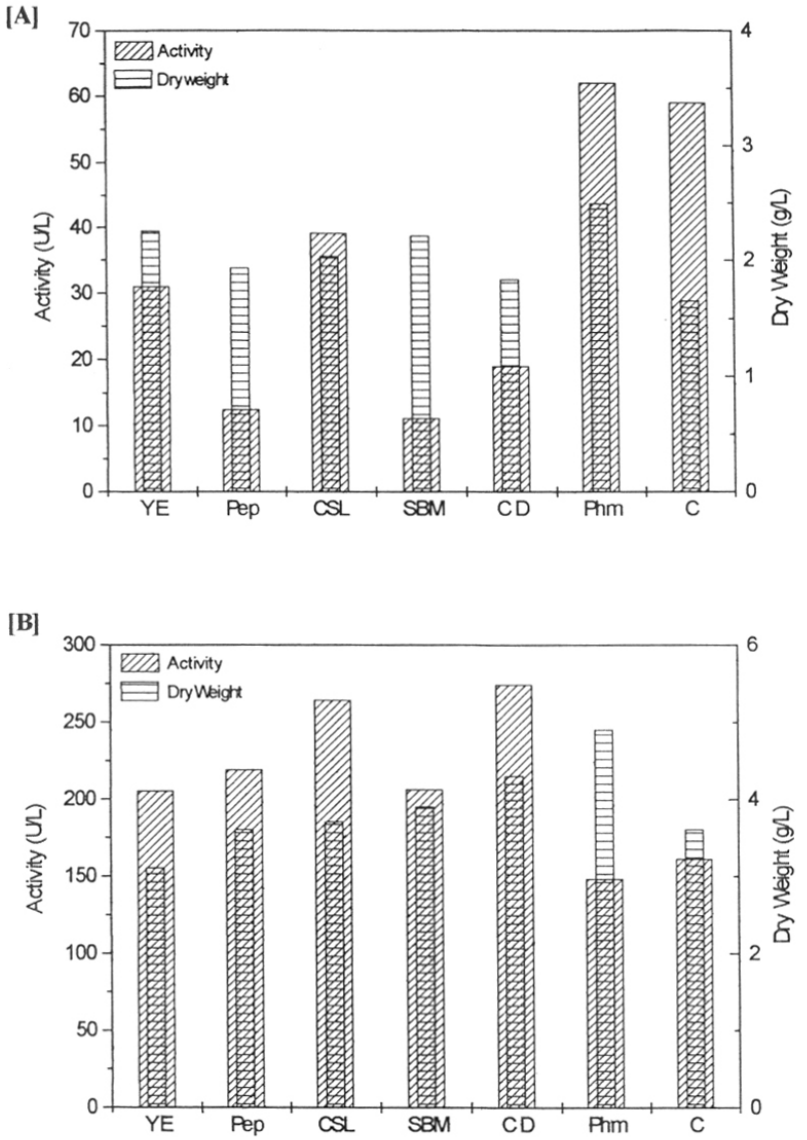
The primary consideration in formulation of a medium is that it must contain all the components required to support active cellular functions (Bridson and Brecker, 1970). In case of production of commercial enzymes it is necessary to prepare a medium with components which are easily available, cheap and reproducible over a long period of time.

Carbon in media is often provided in the form of carbohydrates. The use of pure, defined substrates like sugar often results in low enzyme yields. Hence complex substrates like agricultural byproducts are often used in commercial media formulation. Corn syrup, corn or potato starch, starch hydrolysates or ground cereals (wheat, barley, maize) are frequently




Medium composition (in g/L) : soybean digest, 10; yeast extract, 5; $KH_2PO_4$, 2; naringin, 4

**Figure 4.5** : Naringinase production pattern




YE - yeast extract; Pep - peptone; CSL - corn steep liquor; SBM - soybean meal; C D - casein digest; Phm - pharmamedia; C - control

- Medium composition for set A (in g/L) : basal medium soybean digest 10; $KH_2PO4_2$; naringin 4 with YE 1.25 / Pep 0.71 / CSL 1.33 / SBM 0.89 / CD 0.74 / Phm - 1.06
- Medium composition for set B (in g/L) : concentration of soybean digest and organic nitrogen additives in twice the quantity of set A

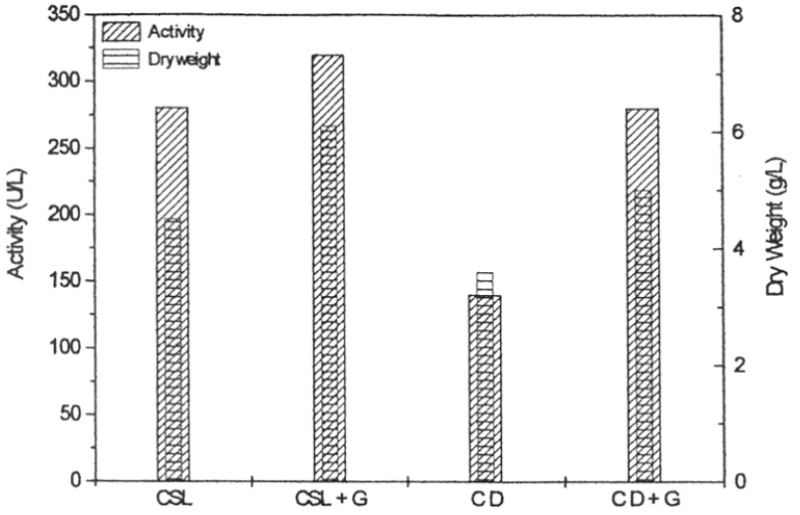
**Figure 4.6** : Effect of different organic nitrogen additives on naringinase production

employed as cheap sources of glucose. Whey can be used as a lactose source (Ratledge, 1977).

Nitrogen, the second most important nutrient in the nature, can be provided in a variety of forms ranging from simple inorganic sources such as ammonium and nitrate salts to complex mixtures such as yeast extract, corn steep liquor (CSL), cotton seed meal (CSM), peptone, soybean meal (SBM) or distillers' solubles. Phosphorus, sulphur and essential trace minerals are supplied as inorganic salts. Trace elements often appear as adventitious contaminants of water or other medium components.

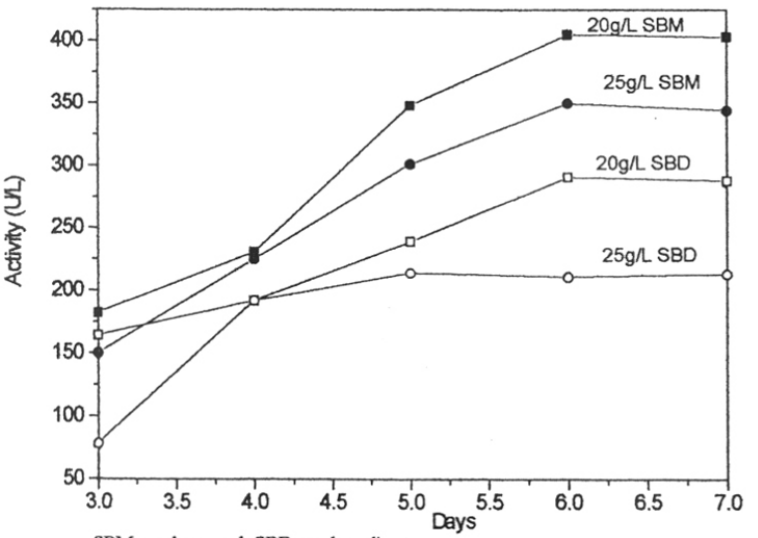
The *Penicillium citrinum* isolate showed sparse growth in the synthetic media and only trace activity was detected in media with starch as the carbon source and $NH_4H_2PO_4$, $NH_4NO_3$ and $(NH_4)_2SO_4$ as nitrogen sources. This may be due to the requirement of organic growth factors and amino acids. The other reason for poor enzyme production could be that the pH of the medium fell down from an initial value of 6 to around 3. Moreover, naringinase production shows a requirement for complex, slowly utilizable carbon and nitrogen sources (Bram and Solomons,1965). Media containing varying concentrations and combinations of soybean meal, corn steep liquor, yeast extract, wheat bran, casamino acids, rhamnolipid have been formulated for naringinase production and activity in the range of 1,000-15,000 U/L has been reported (Table 4.5). To date naringinase production in synthetic medium has been reported only by *Aspergillus niger* GrM3 with lactose and rhamnose as carbon sources (Fukumoto and Okada, 1972) and *Rhizopus nigricans* with sucrose as carbon source and nitrilo triacetic acid trisodium salt $[N(CH_2COONa)_3]$ as nitrogen source (Shanmugan and Yadav, 1995). However, enzyme production by *Rhizopus nigricans* was reported to be enhanced with the addition of rice in the medium.

Growth in media containing organic carbon and nitrogen was definitely enhanced as compared to that in synthetic media. In media containing 10 g/L of soybean hydrolysate and a supplementary organic nitrogen source at a concentration of 0.7 to 1.4 g/L (adjusted on the basis of equal nitrogen), naringinase production between 10 to 62 U/L was seen and when the concentrations of the organic nitrogen components were doubled,




CSL- corn steep liquor, CD - casein digest; G - glucose
Medium composition (in g/L) : soybean digest, 20; $KH_2PO_4$, 2; naringin, 4 with CSL, 2.7 / CD, 1.5 and G, 5

**Figure 4.7** : Comparison between corn steep liquor and casein digest with and without glucose in naringinase production




SBM - soybean meal; SBD - soybean digest
Medium composition (in g/L) : corn steep liquor, 3; glucose, 7; $KH_2PO_4$, 2; naringin 4 with SBM / SBD, 20 / 25

**Figure 4.8** : Comparison between soybean meal and digest in naringinase production

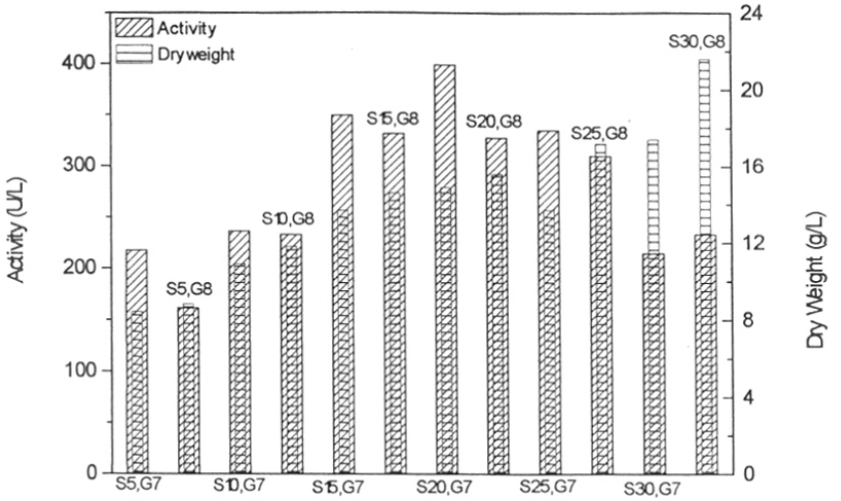
activity in the range of 150 to 275 U/L was obtained (Figure 4.6).

The addition of corn steep liquor as well as casein digest to the medium resulted in greater enhancement of activity and hence a comparison between the two was carried out. Corn steep liquor yielded higher concentration of enzyme as compared to casein digest (Figure 4.7). The addition of glucose to the medium up to a concentration of 8 g/L enhanced naringinase production. This observation is in contrast to that of Bram and Solomons (1965) where they reported inhibition of the enzyme production by glucose.

The use of soybean hydrolysate in the medium necessitates an additional step of hydrolysis of plant meal using acid or protease. Alternatively it leads to requirement of commercial preparations of hydrolysates which are quite unaffordable for commercial use. Studies on use of soybean meal vs. hydrolysate were therefore carried out. As seen in Figure 4.8, soybean meal serves as a better nutrient as against soybean hydrolysate with a 1.5 to 2 fold increase in activity.

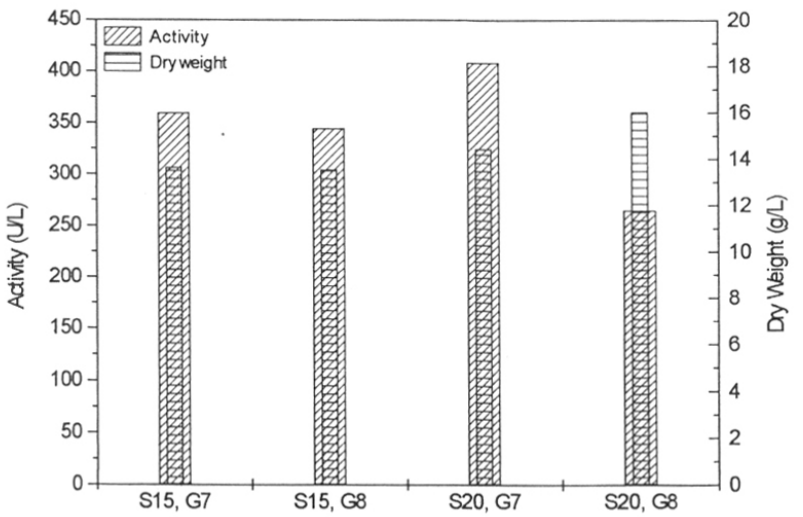
After the selection of suitable media components, their concentrations in the medium were optimized. As revealed by Figure 4.9 there was an increase in cell mass from 8 g/L to 22 g/L due to increase in soybean meal concentration from 5 g/L to 30 g/L. However, the enzyme activity increased with soybean meal only up to 20 g/L and further increase in meal did not have a significant effect on production. The cell mass increased to such a level that beyond a point, the oxygen might have become a limiting nutrient in shake flasks, particularly due to the high viscosity of the broth.

In the next set of experiments, the concentrations of meal and glucose in the medium were optimized with the omission of corn steep liquor from the medium. This step was carried out in accordance of the fact that there was no specific need for CSL and it was working as an additional nitrogen source. Also the composition of corn steep liquor is subject to variation from batch to batch. Comparable activities were obtained even in the absence of corn steep liquor and a medium with 20 g/L soybean meal and 7 g/L glucose was chosen as the optimum composition to be employed for enzyme production (Figure 4.10).




S - soybean meal; G - glucose
Medium composition (in g/L) : corn steep liquor, 3; $KH_2PO_4$, 2; naringin, 4
with soybean meal, 5 to 30; glucose, 7 / 8

**Figure 4.9** : Effect of varying concentrations of soybean meal and glucose at constant corn steep liquor concentration




S - soybean meal; G - glucose
Medium composition (in g/L) : $KH_2PO_4$, 2; naringin, 4
with soybean meal, 15 / 20; glucose, 7 / 8

**Figure 4.10** : Effect of soybean meal and glucose without corn steep liquor

#### *4.5.2.2 Effect of surfactant on naringinase secretion*

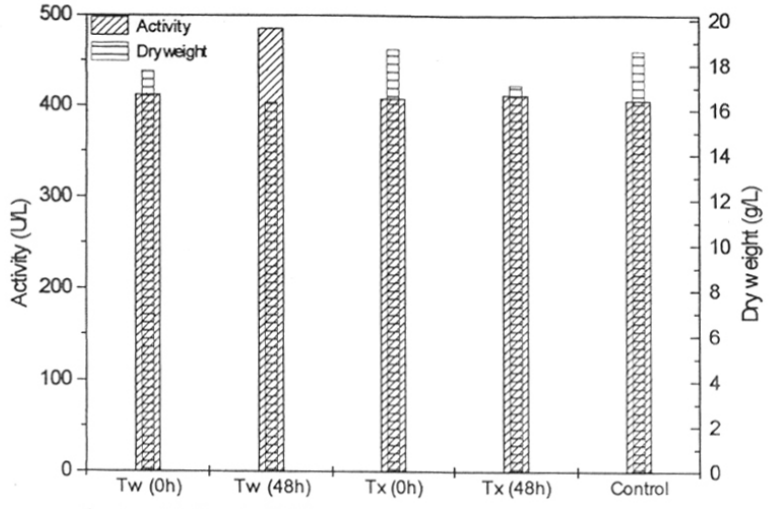
In case of extracellular enzymes an important contributing factor in medium design is the protein secretion mechanism. Certain surface active agents like non-ionic detergents result in enhanced yields of enzyme. Reese (1972) reported a significant enhancement of yield *i.e.* 15-20 fold for cellulase, invertase, β-glucosidases, xylanase, dextranase and pullulanase in the presence of 0.1% Tween 80 in the medium. Similarly, Nakamura and Nakatsu (1978) reported stimulation of inulase production from *Penicillium sp.* by nonionic surfactants. The phenomenon was also seen by Ikediobi *et al.* (1985) in case of glycohydrolase and linamarase from cultures of *Aspergillus syndowii* and *Fusarium equiseti,* respectively. Bhumibhamon (1982) showed that SDS caused enhanced protease production from *Aspergillus phaenicus*.

One of the main reasons attributed to this phenomenon is alteration in the cell membrane leading to increased permeability. Faith *et al.* (1971) offered an alternate hypothesis stating that the surfactant may offer protection to the secreted enzyme and prevent its inactivation.

The effect of surfactant on naringinase enzyme secretion was therefore studied. Figure 4.11 reveals that incorporation of surfactant at 2 g/L in the medium did not affect the growth of the fungus. Addition of Tween 80 after the growth marginally enhanced enzyme secretion. A concentration up to 4 g/L of Tween 80 was tolerated by the organism beyond which it suppressed naringinase secretion (Figure 4.12). Addition of Tween 80 in the medium increased the activity only 1.33 times and was not significantly beneficial to be incorporated into the medium.

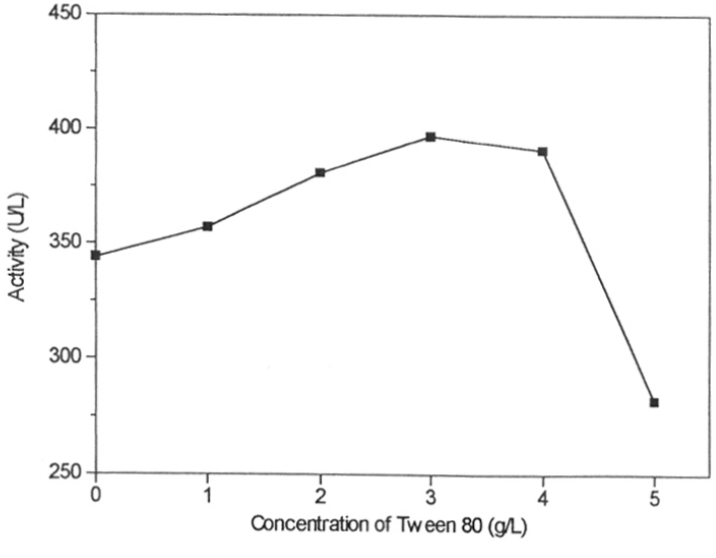
#### *4.5.2.3 Effect of naringin on enzyme production*

Many enzymes require an inducer for enzyme synthesis. The inducers are either substrates (e.g. starch for amylase, cellulose for cellulase), certain intermediate breakdown products of the substrate or substrate analogs (e.g. cellobiose in cellulase, fatty acids in lipase, isopropyl β thiogalactoside for β-galactosidase in E. coli; sophorose in cellulase).




Tw - tween 80; Tx - triton X 100
Medium composition (in g/L) : soybean meal, 20; glucose, 7; $KH_2PO_4$, 2; naringin, 4 with tween 80 / triton X 100, 2

**Figure 4.11** : Effect of Tween 80 and Triton X 100 on naringinase secretion




Medium composition : same as above with tween 80 as indicated in figure

**Figure 4.12** : Effect of varying concentrations of tween 80 on naringinase secretion

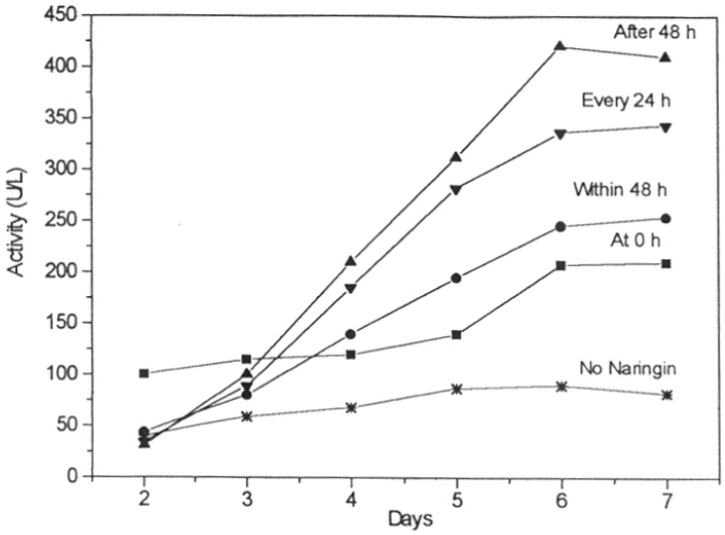
In certain cases the inducer also serves as the main carbon source like starch in case of α-amylase and pectin in case of pectinase.

Both naringin and hesperidin serve as inducers in naringinase production (Fukumoto and Okada, 1973; Bram and Solomons, 1965). Bram and Solomons (1965) reported that addition of naringin at regular intervals induced greater enzyme secretion as against a single point addition. However, no detailed study on this was reported. Figure 4.13 shows that addition of naringin within the growth phase, i.e. either at the beginning of fermentation or within 48 h had only marginal difference in enzyme activity. Addition of naringin at 24 h interval proved to yield greater activity but its addition after the growth phase at 12 h interval was most beneficial. The utilization of naringin occurs after growth phase and therefore enzyme production may be a result of requirement of use of naringin as a substrate of last preference for the fungus.

#### *4.5.2.3 Effect of temperature on enzyme production*

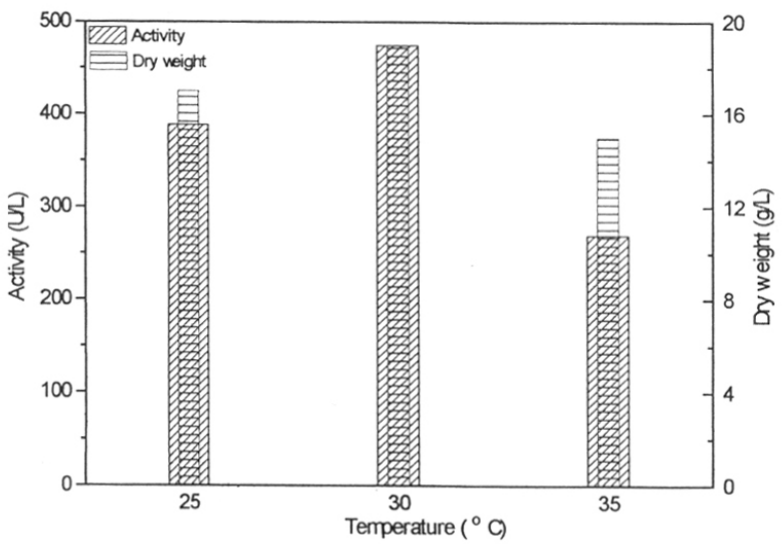
In order to minimize the batch growth time, the temperature must be maintained close to the optimum temperature. However, the optimal temperature for enzyme synthesis may be different from that for growth of the organism. Vavrova and Chaloupka (1983) showed that the maximum rate of proteinase synthesis by *Bacillus megaterium* occured at 28 °C, whereas the maximum specific growth rate was achieved at 42 °C. Similarly Grootwasink and Flemming (1980) reported a 4 °C difference in the optimum temperature for growth and synthesis of the enzyme inulase (β-fructofuranosidase).

Thus, in actual fermentations a compromise is often made and the temperature to be used is determined experimentally. In case of extracellular enzymes owing to the lability of the enzyme, the temperature may be lowered after the phase of cell growth. Tanagnu *et al.* (1981) reported a beneficial effect on cellulase production from *Trichoderma reesei* when the temperature was lowered to 28 °C after the initial growth phase at 31 °C.




Medium composition (in g/L) : soybean meal, 20; glucose, 7; $KH_2PO_4$, 2 with naringin added at a concentration of 5 in different patterns

**Figure 4.13** : Effect of varying patterns of naringin addition on enzyme production




Medium composition (in g/L) : soybean meal, 20; glucose, 7; $KH_2PO_4$, 2; naringin, 4 added after 48h

**Figure 4.14** : Effect of growth temperature on naringinase production

*Penicillium citrinum* is a mesophile and hence flask studies for optimization of temperature for enzyme production were carried out between 25-30 °C. In case of *Penicillium citrinum*, 30 °C incubation temperature yielded maximum biomass and enzyme (Figure 4.14). Higher temperature i.e. 35 °C suppressed growth and enzyme production. Production of naringinase by different organisms at different temperatures 25 °C (Tadashi and Yoo, 1970), 27 °C (Johannes *et al.* 1994) and 30 °C (Shanmugan and Yadav, 1995; Tadasa, 1988; Amano Pharmaceutical Co. Ltd. 1984; Fukumoto and Okada 1973; Bram and Solomons, 1965) has been reported.

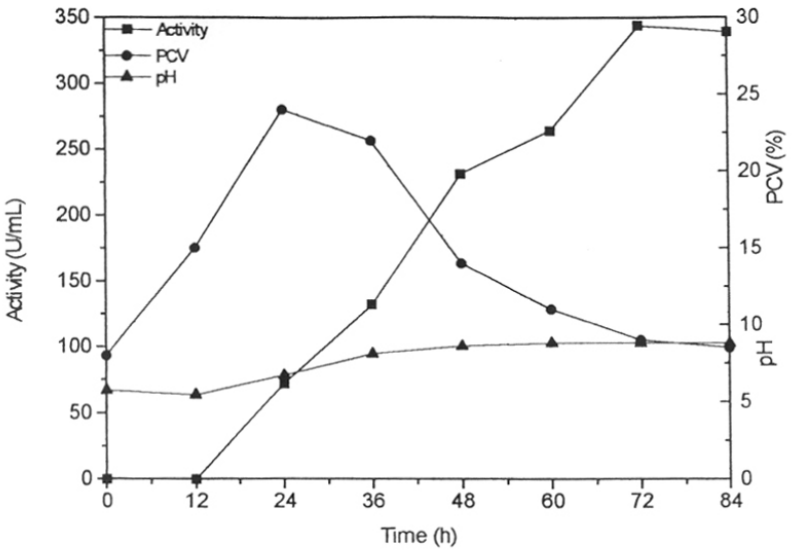
#### *4.5.2.4 Effect of medium pH on enzyme production*

The pH of the media could not be maintained during the course of fermentation in the shake flask as the change in the value exceeded the buffering capacity of the medium. Hence this study was carried out in the fermenter.

At the end of the optimization experiments in shake flask, the enzyme production was increased from a basal value of 20 U/L to 500 U/L in a medium containing 20 g/L soybean meal, 7 g/L glucose, 2 g/L $KH_2PO_4$ and 4 g/L naringin added as 1 g aliquots every 12 h after 48 h of fermentation.

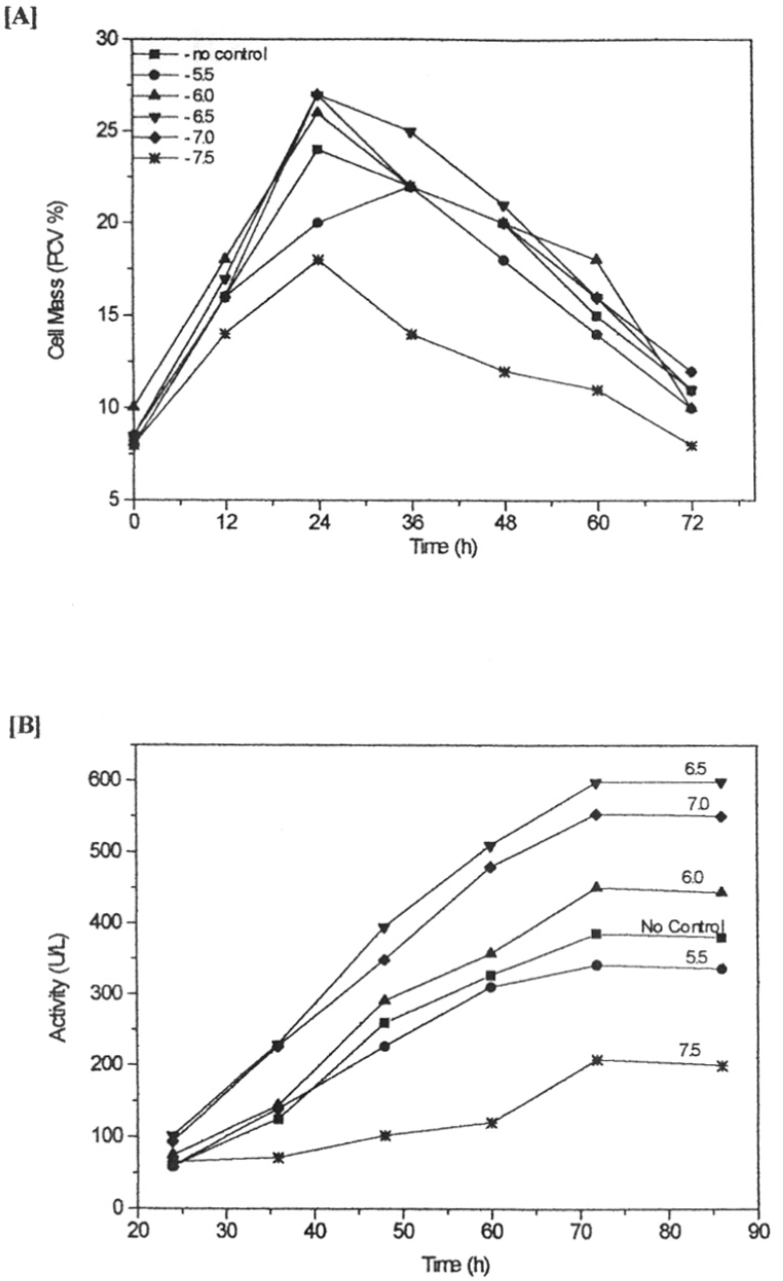
## 4.6 Fermenter studies

The basic media components, their relative proportions and physiological parameters were obtained from the flask level studies. However, in order to obtain a picture of the growth and enzyme production as the case would be in actual production it was necessary to carry out fermenter studies. Fine control and tuning of pH, temperature, aeration and agitation, are achieved only in fermenter as opposed to flask. Naringinase production was carried out in 1 L Gallenkamp as well as 14 L NBS fermenters.



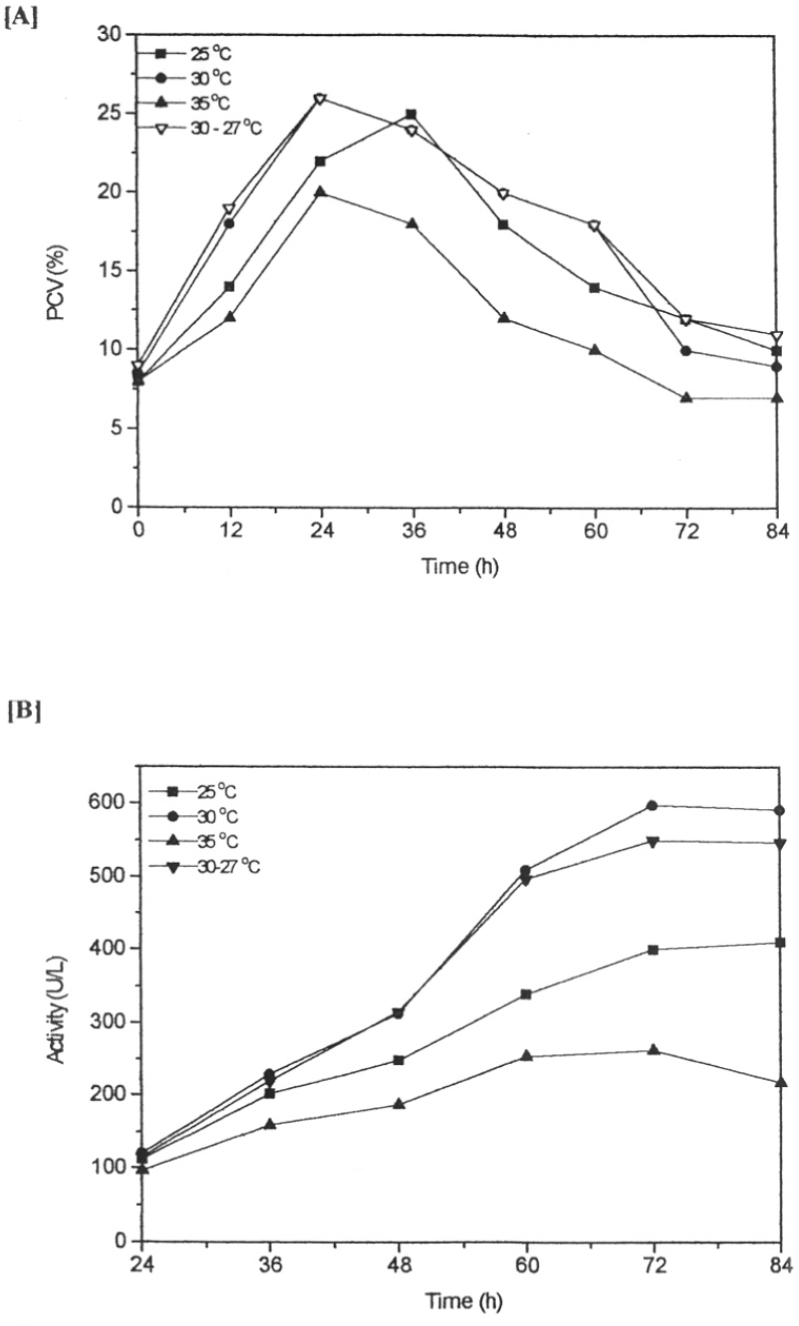

Medium composition (in g/L) : soybean meal, 20; glucose, 7; $K_2HPO_4$, 2; naringin, 4

**Figure 4.15** : Naringinase production in 1 L Gallenkamp fermenter




A - Growth at different pH values
B - Naringinase production at different pH values

**Figure 4.16** : Effect of pH on growth and naringinase production in 1 L fermenter




A - Growth at different temperatures
B - Naringinase production at different temperatures

**Figure 4.17** : Effect of temperature on growth and naringinase production in 1 L fermenter

### *4.6.1 Production of naringinase in 1 L Gallenkamp fermenter*

Optimization studies for pH and temperature were carried out in 1 L fermenter. Initially the enzyme production pattern with time was studied without pH control. Figure 4.15 gives the production of enzyme in 1 L fermenter. Similar to the shake flask studies, enzyme secretion was associated with the late log phase with a peak at 72 h and the value stabilized after that. The cell mass in terms of packed cell volume (PCV) increased upto 24 h. There was a dip of 0.3 pH units after 12 h of growth and a rise of 1 unit after 24 h of growth. The pH increased further and the final pH of the broth after 72 h was around 8.9. In the fermenter runs with controlled pH, it was controlled with 2 N NaOH or 2 N HCl at the required value to minimize volume changes due to acid or alkali addition. As seen in Figure 4.16 (A) the pH did not affect growth in the range of 6 to 7. A pH value of 5.5 showed a decrease in growth as well as a delay in reaching maximum cell mass whereas at 7.5 the decrease was substantial. The enzyme activity was maximum at pH of 6.5 yielding 600 U/L [Figure 4.16 (B)]; at pH 7.0 about 550 U/L were obtained and at 6.0 the yield was 440 U/L.

Temperature studies were carried out at 25°, 30°, 35 °C. In one run it was controlled at 30 °C for initial 36 h and then decreased to 27 °C. In all these runs the pH was maintained at 6.5. Similar to the shake flask experiments, growth at 25 °C and 30 °C was comparable whereas at 35 °C it was suppressed. The enzyme activity was maximum at 30 °C. A decrease in the temperature to 27 °C after growth did not cause any enhancement of enzyme activity (Figure 4.17).

### *4.6.2 Production of naringinase in 14 L NBS fermenter*

The enzyme production in the 14 L fermenter was carried out at a pH of 6.5 and temperature was maintained at 30 °C (Figure 4.18). Both the aeration and agitation were altered to maintain dissolved oxygen tension above 40% of saturation. Figure 4.19 shows the DO and agitation profiles of the fermentation as well as the cell mass and enzyme

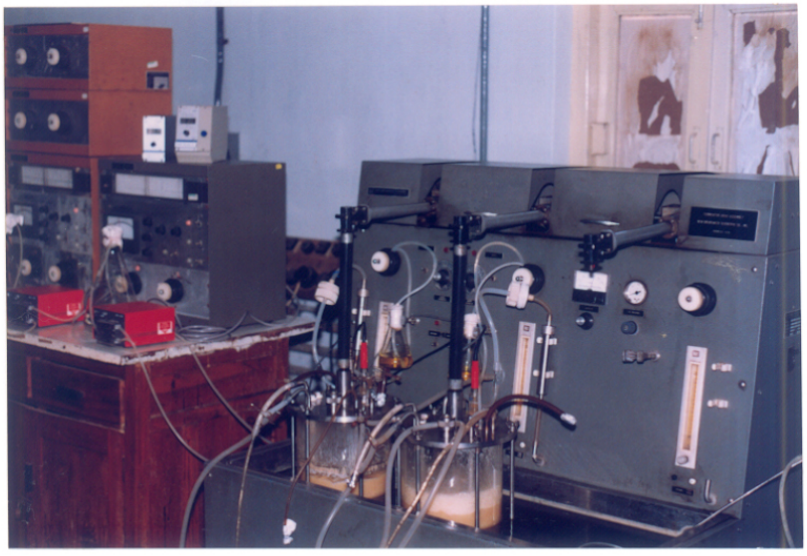
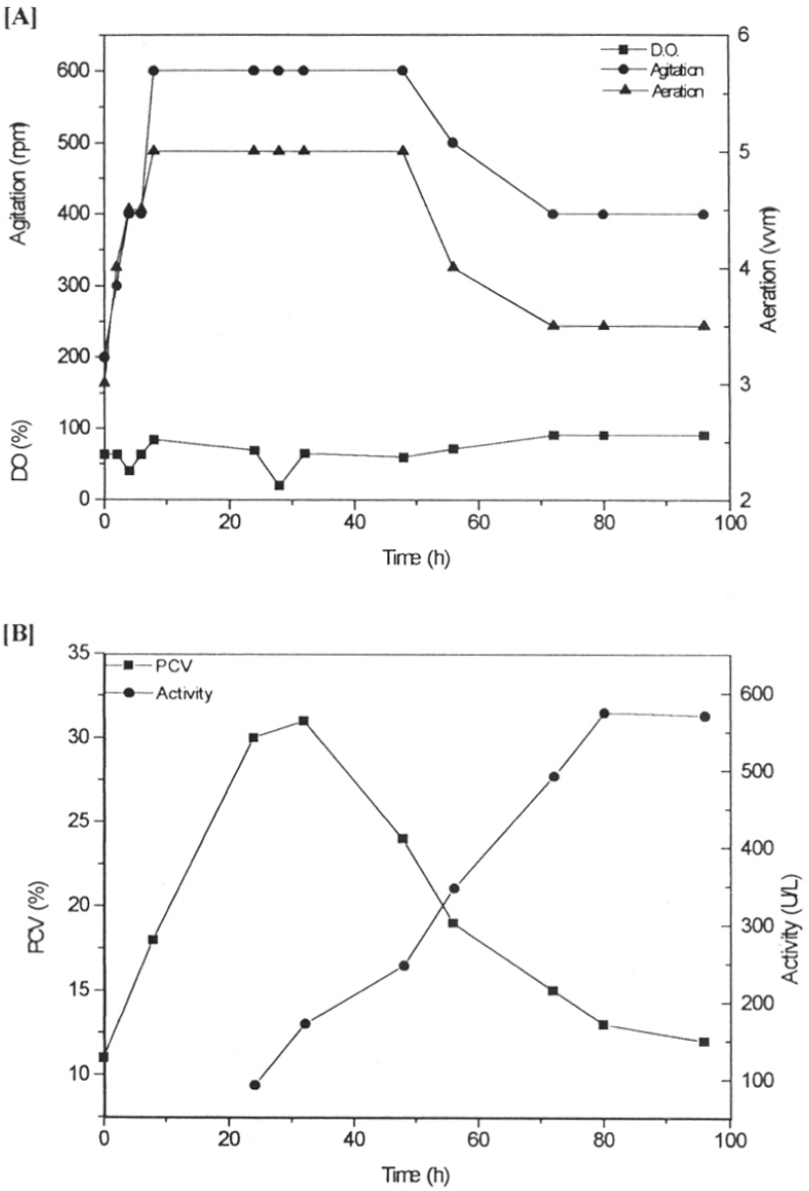


**Figure 4.18** : Photograph showing fermentation for naringinase production in 14 L NBS fermenter




A - aeration, agitation and DO pattern
B - naringinase production pattern

**Figure 4.19** : Naringinase production in 14 L NBS fermenter

| Culture | Medium and Culture Conditions | Activity (U/mL) | Reference |
|---|---|---|---|
| *Aspergillus niger* NRRL 72-4 | a) Shake flask<br>CSL 40, Y.E. 40, $KH_2PO_4$ 2, $CaCO_3$ 5, naringin 4, pH 5-5.5, 30 °C for 5 days | 440 | Bram and Solomons, 1965 |
| *Aspergillus niger* NRRL 72-4 | b) 10L Fermenter<br>medium same as in flask but with 1 g/L naringin added continuously throughout fermentation, 500 rev/min, 1vvm air, 5 days | 550 | Bram and Solomons, 1965 |
| *Aspergillus niger* GrM3 | raw hesperidin 20, SBM 20, CSL 25, $KH_2PO_4$ 2 , $CaCl_2$ 0.1, $MgSO_4$ .$7H_2O$ 0.1; 30 $^0$C for 5 days | 10 - 15 | Fukumoto and Okada, 1973 |
| *Rhizopus nigricans* | sucrose 40, rice 100, ammonium tartarate 8, $N(CH_3COONa)_3$ 1.5, $CaCl_2$ 1, $MgSO_4$. $7H_2O$ 3, $KH_2PO_4$ 2, trace elements | 2.2 with pNPR | Shanmugan and Yadav, 1995 |
| *Penicillium* sp.<br>DSM 6825<br>DSM 6826 | rhamnolipid or alkyl ester of rhamnolipid (concentrated culture filtrates of *Pseudomonas aeruginosa*) 3, $KH_2PO_4$ 1, $(NH2)_2PO_4$ 0.5, $MgSO_4$. $7H_2O$ 0.1, $CaCl_2$ 0.1, Cas amino acids 0.1, pH 5.5, 300 rev/min, 0.6 vvm , 27 °C, 5-10 days | 5 | Johannes et.al. 1994 |
| *Penicillium citrinum* | SBM 20, glucose 7, $KH_2PO_4$ 2 , naringin 5, pH 6.5, 30 $^0$C, 5 days | 0.6 | Present investigation |

CSL - corn steep liquor, YE - yeast extract, SBM - soybean meal

**Table 4.5** : Comparison of naringinase production

| Step | Medium Composition (g/L) | Activity or Activity range (U/L) |
|---|---|---|
| Screening | SBD 10; Y.E 4.; dried citrus peel 2; $K_2HPO_4$ 2 | 20 |
| ↓ | | |
| Organic Nitrogen - (a) single strength | SBD 10; $K_2HPO_4$ 2; naringin 4. | 10 - 60 |
| - (b) double strength | YE; SBM; CSL; CD; Pep; Phm added in the range of 0.7 to 1.4 on the basis of equal N. The concentration of these components and SBD were doubled in (b) | 160- 275 |
| ↓ | | |
| CSL Vs CD with Glucose | SBD 10; $K_2HPO_4$ 2; naringin 4 with CSL 2.7 or C.D 1.5 and glucose 5 | 150 - 325 |
| ↓ | | |
| SBM Vs SBH | CSL 3; glucose 7; $K_2HPO_4$ 2; naringin 4 with SBM or SBD 20 / 25 | 405 |
| ↓ | | |
| Different concentrations of SBM and Glucose with constant amount of CSL | CSL 3; $K_2HPO_4$ 2; naringin 4; with SBM 5 to 30; glucose 7 / 8 | 160 - 400 |
| ↓ | | |
| Different concentrations of SBM and Glucose without CSL | 2 $K_2HPO_4$; 4 naringin; 7 / 8 glucose; 15 / 20 SBM | 300 - 401 |
| ↓ | | |
| Surfactant | SBM 20; glucose 7; $K_2HPO_4$ 2; naringin 4 with Tween80 or Triton X 100 2 | 485 |
| ↓ | | |
| Effect of Naringin | SBM 20; glucose 7; $K_2HPO_4$ 2 with varying patterns of naringin addition | 421 |
| ↓ | | |
| Fermenter - 1 L | SBM 20; glucose 7; $K_2HPO_4$ 2; naringin 5 | 602 |
| - 14 L | | 585 |

CSL - corn steep liquor, CD - casein digest, Pep - peptone, Phm - pharmamedia
SBM - soybean meal, SBD - soybean digest, YE - yeast extract

**Table 4.6** : Yield enhancement of naringinase through optimisation steps

activity profile. Similar to the 1 L vessel, the activity reached its peak value after 72 h of fermentation and a yield of 600 U/L of enzyme was obtained. The studies performed in 14 L fermenter with a 10 fold volume showed nearly identical fermentation profile and enzyme production. This indicates the possibility of furthur scaleup of naringinase production in semi-pilot, pilot and production scale fermenters. The published yields of naringinase along with their production conditions are summarised in Table 4.5 along with the results obtained with *Penicillium citrinum*. As seen in the Table only Bram and Solomons (1965) report an activity as high as 400 U/mL. This may be due to the non specific assays available at that time for the estimation of the reaction products. Subsequent studies, however, have always obtained enzyme titres much less in contrast.

The work presented thus, shows that using a wild isolate of *Penicillium citrinum*, hetherto not reported to produce naringinase, the enzyme production was carried out in shake flasks, 1 L and subsequently 14 L fermenters. The temperature, pH and media components were optimized to get about 600 U/L of enzyme with simple, cheap and locally available media components (Table 4.6).

## 4.7 Purification of naringinase

Naringinase purification is reported by several workers as revealed by Table 4.7. However, most of them report purification of commercially available enzyme preparations either in order to determine the amino acid composition of the enzyme or to separate its subunits as glycopeptides or isoenzymes. In a recent report on naringinase purification from fermentation broth (Johannes *et al.*, 1994), the authors used a series of chromatographic techniques like ion exchange, FPLC and gel filtration, to get a homogenous enzyme preparation with a molecular weight between 60-100 kD.

Although the naringinase from *Penicillium citrinum* in the present investigation was purified using conventional chromatographic techniques, the matrices used were characteristically different. All workers so far have used agarose based resins. However, owing to poor elution and resolution on the conventional matrices, acrylate based matrices were used in this study.

| Step | Activity (U/mL) | Protein (mg/mL) | Specific activity (U/mg) | Fold purification | Recovery % |
|---|---|---|---|---|---|
| Cell free supernatant | 0.16 | 4.6 | 0.034 | 1 | |
| Ultrafiltered broth | 0.86 | 16.2 | 0.053 | 1.6 | 100 |
| IEC | 1.98 | 10.0 | 0.198 | 5.8 | 69 |
| HIC | 0.65 | 0.56 | 1.16 | 34.1 | 52 |
| GPC | 0.95 | 0.02 | 47.5 | 1397.1 | 21 |

**Table 4.7** : Purification of Naringinase

### *4.7.1 Ultrafiltration of the filtered broth for enzyme concentration*

The cell-free supernatant was subjected to microfiltration using a tubular ceramic membrane. An average flux of 70 mL/min was achieved. The retentate was then subjected to ultrafiltration through a polysulfone hollow fibre module with a molecular weight cut-off of 20 kD. The membrane fouling was minimal and distilled water flux could be regained by normal washing procedures. The entire enzyme activity was retained in the retentate with 100% recovery of the enzyme.

### *4.7.2. Ion exchange chromatography*

The initial binding of naringinase was examined on DEAE Sepharose. In the batch studies a pH of 6.5 was found to be the suitable pH for binding of enzyme to the matrix and an ionic strength of 0.3 M of NaCl was the minimum required concentration for elution of enzyme from the matrix. Thus, a column of DEAE Sepharose was packed wherein the enzyme was bound at pH 6.5 and elution performed with a gradient of 0-0.5 M NaCl in buffer. However only 10% of the enzyme was recovered from the matrix and the elution was seen as a broad peak distributed over a large number of fractions instead of a sharp peak.

The naringinases reported so far are glycoproteins. The matrix was carbohydrate based (cross linked agarose) and therefore the enzyme might have a strong affinity for the matrix itself. Hence it was not eluted by salt.

On account of poor recovery from DEAE-Sepharose, a non-carbohydrate based material with the same functional group *i.e.* DEAE-methacrylate was successfully used for ion exchange. The elution pattern with the linear gradient of NaCl is given in Figure 4.20. A fold purification of 5.82 with 69% recovery was achieved. This supports our assumption that the present naringinase may also be a glycoprotein. Further work needs to be done to confirm it.

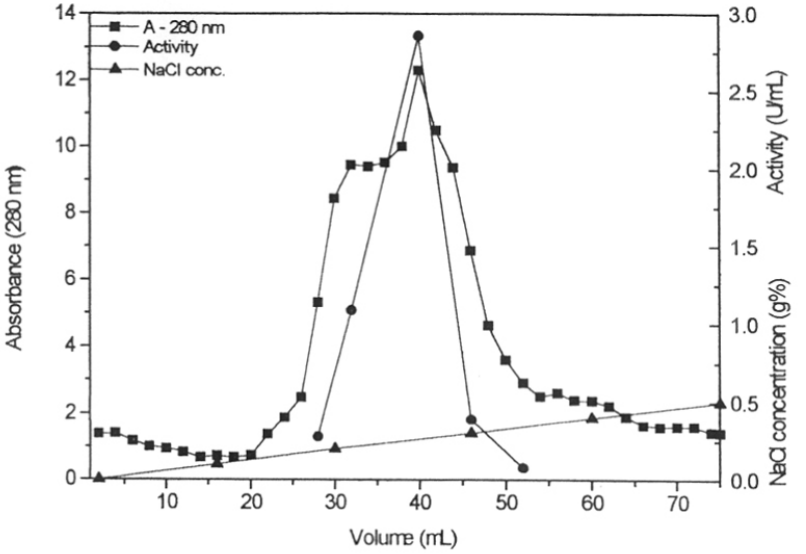



**Figure 4.20** : Ion exchange chromatography on Macroprep DEAE

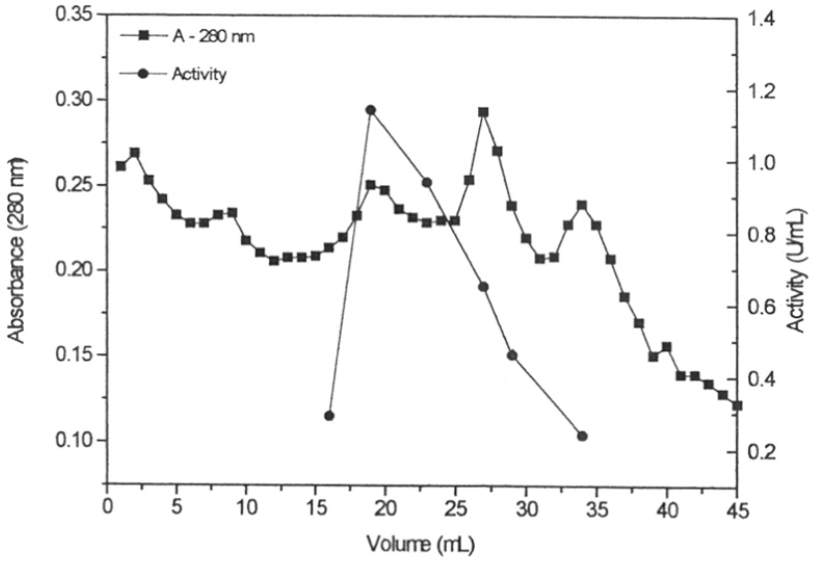



**Figure 4.21** : Hydrophobic interaction chromatography on Macroprep methacrylate support

### *4.7.3. Hydrophobic interaction chromatography*

As seen in the case of use of sepharose based IEC matrix; even sepharose based hydrophobic materials like Phenyl Sepharose yielded poor recoveries of enzyme with trailing and diffused elution of protein. Hence the methacrylate based support from Bio-Rad was used for HIC. A fold purification of 34.1 with a recovery of 52% was obtained as seen in Figure 4.21.

### *4.7.4. Gel permeation chromatography*

Naringinases from *Penicillium* sp. are reported to be glycoproteins with molecular weights ranging from 85-110 kD (Hsieh and Tsen, 1991; Gunata *et al.* 1991; Young *et al.* 1989; Johannes *et al.* 1994). A column of Sephacryl S-200 with a resolution range from 5 to 25 kD was used for molecular sieving of the enzyme. As revealed by Figure 4.22 naringinase eluted as a sharp peak with a recovery of 21% and a fold purification of 1397.

Naringinase was thus, successfully purified using the following sequence of steps:

1. Cell removal by filtration and clean-up by microfiltration
2. Concentration of enzyme in broth by ultrafiltration
3. Ion exchange chromatography using a Macroprep DEAE-methacrylate anion exchanger
4. Hydrophobic interaction chromatography on a Bio-Rad methacrylate column
5. Gel filtration chromatography on a Sephacryl S-200 column.

The results of the enzyme purification are summarized in Table 4.8. The purified enzyme had a specific activity of 47.5 U/mg protein (protein estimation in final step was carried out by measuring absorbance at 280 nm with BSA as standard). The total recovery was 21% with a fold purification of about 1397.

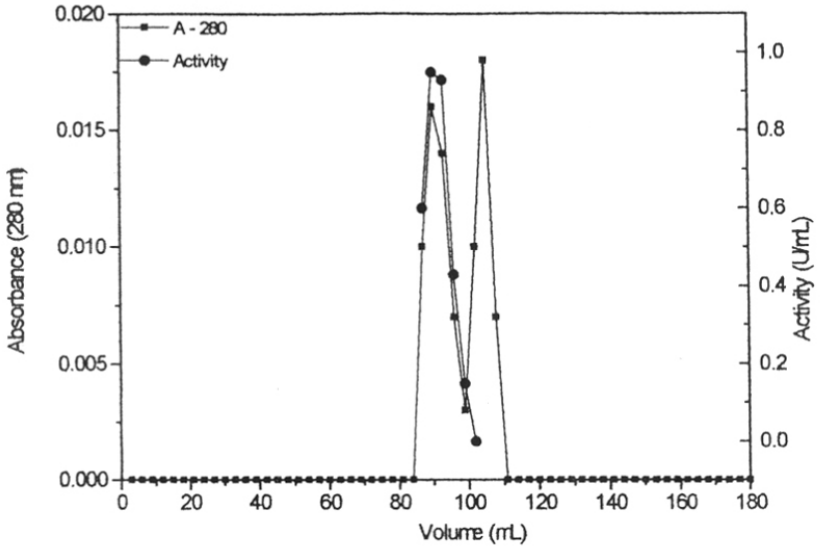



**Figure 4.22** : Gel filtration through Sephacryl S - 200

## 4.8 Characterization of naringinase

### *4.8.1 Determination of molecular weight by SDS PAGE*

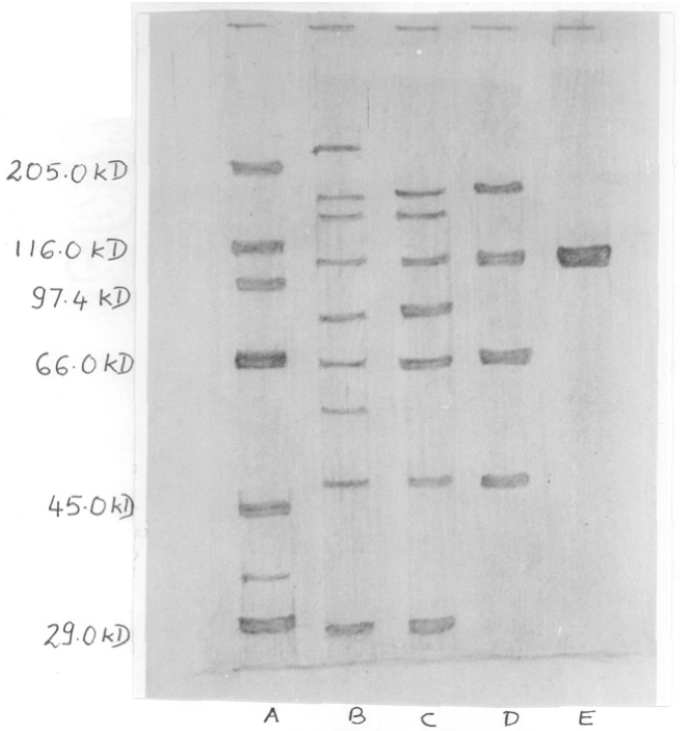
The molecular weight of *Penicillium citrinum* naringinase was approximately in the same range as those reported for the *Penicillium* and *Aspergillus* naringinases. The *Aspergillus* naringinases have molecular weights ranging from 87 kD (Margin, 1994) to 95 kD (Roitner *et al.*, 1984). The enzyme from *Penicillium* sp DSM 6826 has a molecular weight between 60-100 kD (Johannes *et al.*, 1994) whereas that from *Penicillium decumbens* shows a weight of 97 kD by SDS PAGE, 88 kD by GPC, 52 kD by size exclusion HPLC and 41 kD for the deglycosylated enzyme (Young *et al.*, 1989).

Molecular weight markers and purified naringinase were loaded on the gel and after electrophoresis the molecular weight was calculated to be 105 kD. Figure 4.23 shows the purified naringinase as revealed by a single band on the gel.

### *4.8.2 Effect of pH on naringinase activity*

Enzymes possess many ionizable groups and those involved in the structure of the active site must be in proper ionic form to maintain the conformation of the active site. This is essential for both the catalytic action as well as for substrate binding. Moreover, the substrate too may contain ionizable groups and only one ionic form may bind to the enzyme or react. Therefore, the pH influences the rate of an enzyme catalyzed reaction. The effect of both can be reversible, when the activity of the enzyme is restored to it's original value after a completed pH cycle or irreversible when the secondary or tertiary structure of the enzyme is altered in the course of the pH change. Thus, the pH effect on the stability of the enzyme must be taken into account when studying the pH dependence on enzyme catalysis. The three important effects seen are:

- A reversible change in active site conformation thereby causing a change in $V_{max}$.
- A change in the enzyme /substrate binding affinity thus altering $K_m$.




Lane A: Molecular weight markers, B: Ultrafiltered broth, C: IEC fractions, D: HIC fractions E : GPC fractions

**Figure 4.23** : Molecular weight determination of naringinase by SDS PAGE

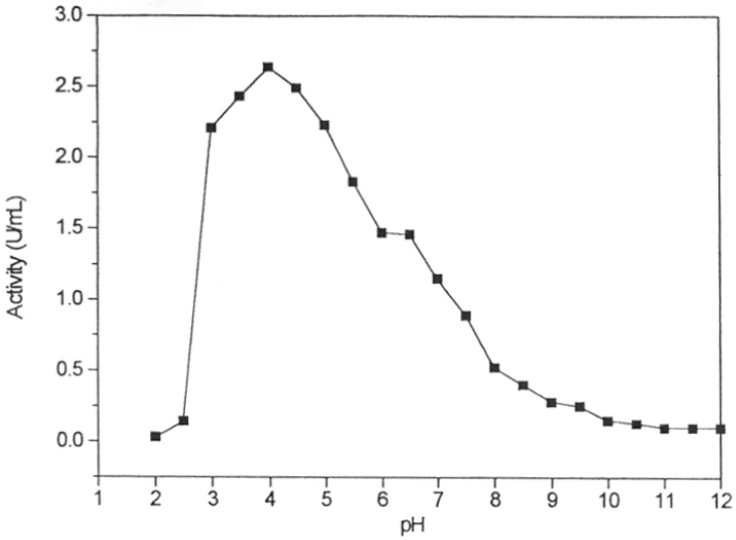
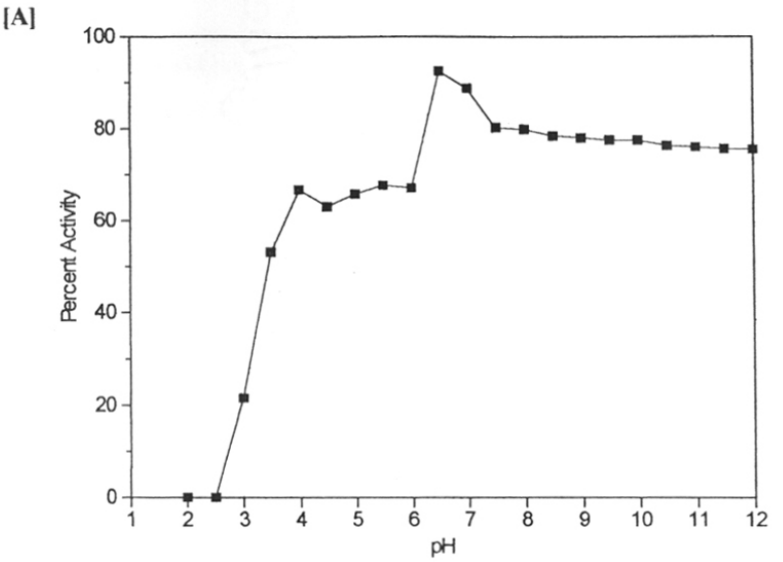
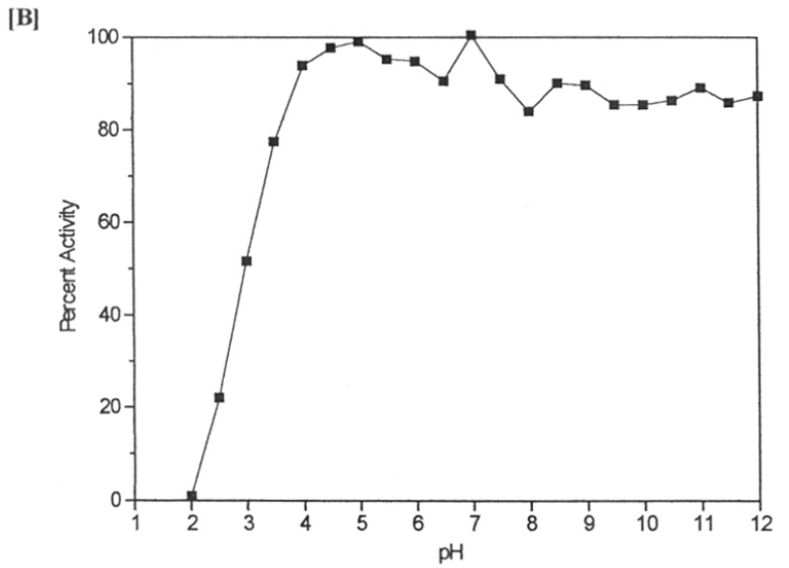



**Figure 4.24** : Effect of pH on naringinase activity




A - Stability at 40 °C
B - Stability at 4 °C

**Figure 4.25** : Effect of pH on stability of naringinase

- An irreversible change in the stability of the enzyme i.e. denaturation, which occurs at extreme pH values on both sides of the pH optimum. As this is a rate process, both $K_m$ and $V_{max}$ are altered.

All the effects mentioned above can also occur simultaneously. By superimposition of the pH activity and the stability curves, one can get a better understanding of the pH dependence.

The enzyme had a pH optimum between 4 and 4.5 (Figure 4.24). However, an activity of more than 90% was seen in the range from 3.5 to 5.0. The enzyme showed no activity at pH 2.0 and retained only 10% activity in the range of 9-12. Most citrus juices have pH values ranging from 3 to 4.5. Hence it is extremely beneficial to have an enzyme preparation most active in this range. *Penicillium decumbens* naringinase has a pH optimum of 3.7 (Hsieh and Tsen, 1991) whereas for *Penicillium* sp. DSM 6826 the optimum lies between 5 and 5.5 (Johannes *et al.*, 1994). *Aspergillus* sp. also have their optimum at a higher pH range; 4.5 for *A. niger* (Hsieh and Tsen, 1991) and 5.5 to 6 for *A. aculeatus* (Mutter, 1994).

The enzyme showed a very high stability over a wide pH range. It retained more than 65% of original activity over a range from 4 to 12 at 40 °C for 24 h with maximum stability between 6.5 and 7.0. Below pH 3, the enzyme rapidly lost its activity. When the enzyme was incubated at 4 °C for 24 h, the enzyme retained more than 90% activity in the range of 3.5 to 12 with maximum stability at pH 7.0 (Figure 4.25).

### *4.8.3 Effect of temperature on naringinase*

Similar to the chemical reactions, the rate of enzyme catalyzed reactions increases with temperature. The tertiary structure of the enzyme molecule is primarily maintained by weak non-covalent bonds which can be easily disrupted when the enzyme molecule absorbs too much energy. Denaturation of the enzyme or loss of catalytic activity is a reaction which competes with the enzyme catalysis. As the temperature increases the rate of denaturation is accelerated eventually dominating the catalytic activity. Hence a plot of

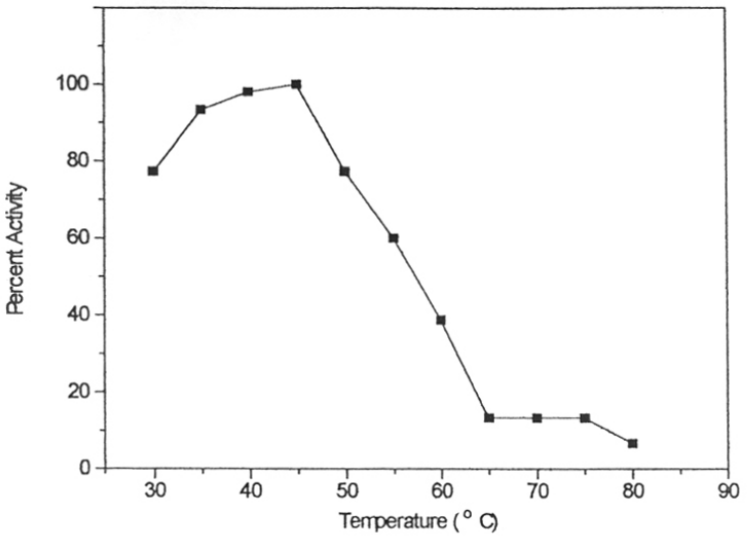



**Figure 4.26** : Effect of temperature on naringinase activity

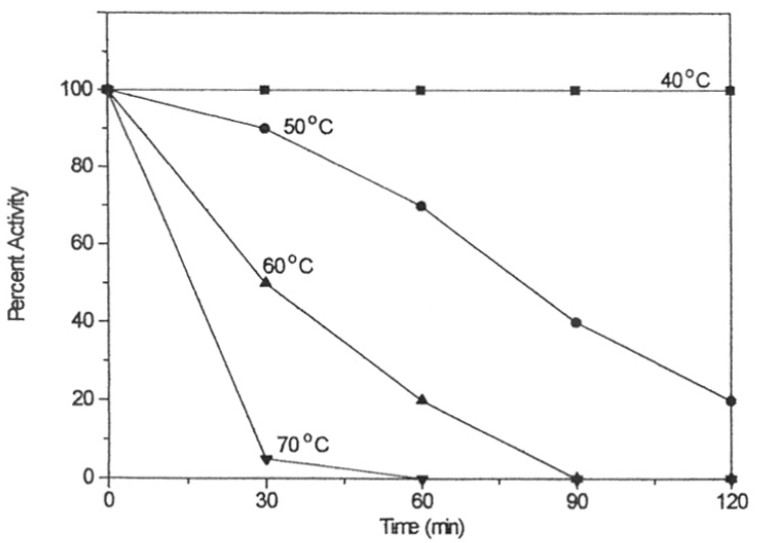



**Figure 4.27** : Effect of temperature on stability of naringinase

catalytic activity Vs temperature usually exhibits a maximum called as the $T_{opt,}$ the position of which depends on the time of the measurement of the reaction rate. The shelf stability of a biocatalyst is determined by a deactivation measurement which involves high temperature preincubation combined with low temperature activity measurement. However, the operational stability of the enzyme that is under the actual reactor conditions is of primary importance for the design and operation of the bioreactor.

Naringinase from *P. citrinum* was found to have a temperature optimum between 40 and 45 °C. It retained more than 90% activity in the range from 30-50 °C. Above 60 °C the enzyme rapidly lost its activity (Figure 4.26). The temperature optimum of the enzyme is close to that reported for the *Aspergillus* enzyme which has an optimum of 40 °C (Park and Chang, 1979). The *Penicillium decumbens* naringinase has a higher optimum of 50 °C (Tsen and Yu, 1989; Puri *et al.* 1996).

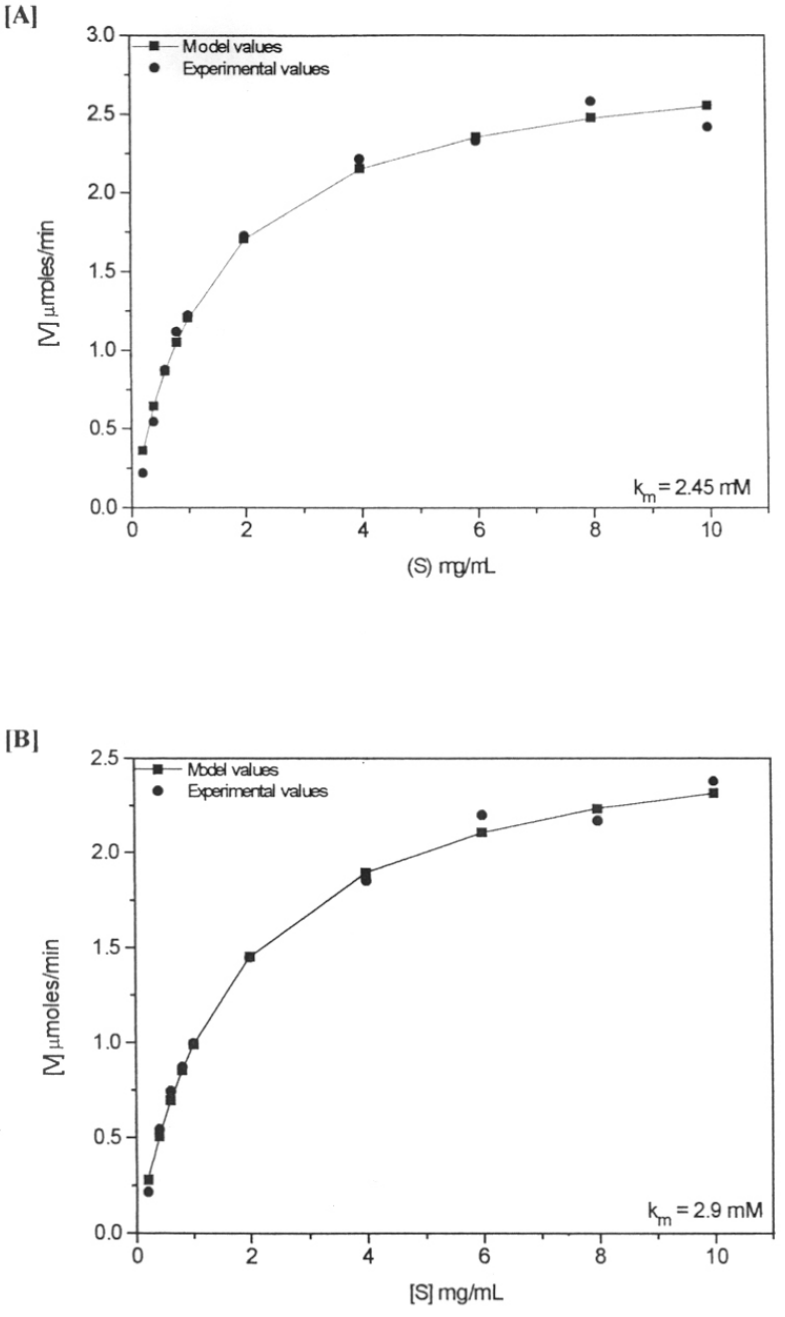
The enzyme stability experiment reveals that naringinase from *P. citrinum* is quite stable retaining more than 90% activity at 40 °C for 24 hr. The enzyme retains about 65% activity at 50 °C for 1 hour but it loses most of its activity at temperatures greater than 50 °C within an hour (Figure 4.27).

The excellent pH and temperature stabilities of the present enzyme are very useful characters considering its application in fruit juice debittering processes and several other applications found in literature.

### *4.8.4 $K_m$ and $V_{max}$ of naringinase*

Kinetic studies on the enzyme were carried out to determine the kinetic constants $K_m$ and $V_{max}$. The values for the kinetic constants were determined by DNSA assay and HPLC. Naringinase was found to have a $K_m$ of 2.45 mM and $V_{max}$ of 145.5 U/mg protein by the former method and a $K_m$ of 2.9 mM and $V_{max}$ of 138 U/mg protein by the latter. The values determined by the two methods show a good correlation (Figure 4.28).

The *Penicillium citrinum* isolate has an enzyme with lower $K_m$ than that reported for *Penicillium* sp. Earlier Romero *et al.* (1985) reported a $K_m$ value of 7 mM and $V_{max}$ of




A - Kinetic studies by DNSA
B - Kinetic studies by HPLC

**Figure 4.28** : Kinetic studies of naringinase

150 U/mg for naringinase from *Penicillium decumbens* (Sigma preparation). In addition they determined the kinetic values for α-L-rhamnosidase with a synthetic substrate pNPR and the values obtained were 1.52 mM and 10.7 U/mg respectively. Furthermore studies carried out by Tsen *et al.*(1989) and later by Puri *et al.* (1996) have expressed $K_m$ values of 7.8 mM and 8.4 mM, respectively.

*Aspergillus* naringinase, on the other hand, has lower $K_m$ values. Tsen (1984) reported value of 0.84 mM for naringin, while Ono *et al.* (1977) obtained a value of 0.64 mM. Park and Chang (1979) also got a value of 0.86 mM.

In specifying and characterizing an enzyme $K_m$ is a useful parameter. It is the value of the substrate concentration which gives an initial velocity equal to half the maximum velocity at that enzyme concentration. It is independent of the enzyme concentration and is a true characteristic of the enzyme under defined conditions of temperature and pH. Although $K_m$ values have a wide range, for most industrially used enzymes they lie in the range 100 to 0.01 mM when acting on substrates under normal conditions. It can be considered as the measure of the affinity of an enzyme for a particular substrate. A low value denotes a high affinity and vice versa. This concept of affinity of the enzyme for its substrate is useful in context of enzyme inhibiting and competing substrates. The $K_m$ values are used in applied enzymology to determine the most useful substrate concentration ranges for fast conversion.

### *4.8.5 Effect of sugars on naringinase activity*

As seen in Table 4.8, citrus juices have a total sugar content ranging from 3.1 g% in lemon to 9.3 g% in orange. Sugars are present as a combination of glucose, fructose and sucrose. Glucose is an end product of the enzyme reaction and thus the enzyme may be inhibited by this sugar commonly found in citrus fruits. Therefore, inhibition of naringinase activity by these three sugars at varying concentrations was tested. As given in Table 4.9 none of these sugars had any effect on the enzyme activity at concentrations present in juice as well as even at those far higher than normally found. This is a very important finding as it is relevant to the use of the enzyme in actual debittering of juice and in the

| Fruit | Glucose (g%) | Fructose (g%) | Total Reducing Sugar (g%) | Sucrose (g%) | Total Sugar (g%) |
|---|---|---|---|---|---|
| Orange | 2.0 | 2.5 | 4.5 | 4.8 | 9.3 |
| Grapefruit | 1.7 | 1.8 | 3.5 | 2.6 | 6.1 |
| Tangerine | 1.1 | 1.5 | 2.6 | 6.5 | 9.1 |
| Lemon | 1.4 | 1.4 | 2.7 | 0.4 | 3.1 |
| Lime | na | na | 3.5 | 0 | 3.5 |

na - data not available

**Table 4.8** : Sugar content in some citrus juices

| Concentration | Percent activity remaining in the presence of | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Glucose | | Fructose | | Sucrose | | Rhamnose | |
| | S | P | S | P | S | P | S | P |
| 0 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 0.5 | | | | | | | 78 | 92 |
| 1 | 75 | 100 | 62 | 100 | | | 42 | 87 |
| 1.5 | | | | | | | 25 | 85 |
| 2 | 70 | 100 | 40 | 100 | 90 | 100 | 20 | 85 |
| 4 | 65 | 100 | 22 | 100 | 85 | 100 | | |
| 6 | 60 | 100 | 18 | 100 | 80 | 100 | | |
| 8 | | | | | 75 | 100 | | |

S- Naringinase from *Penicillium decumbens* (Sigma Chemical Co. Ltd.)
P- Naringinase from *Penicillium citrinum* (present study)

**Table 4.9** : Effect of sugars on naringinase activity

efficiency of debittering. Moreover, this is the first ever report of an enzyme almost free of inhibition by juice sugars. Tsen *et al.* (1988) reported $K_i$ values of 178 mM, 68 mM and 12 mM for glucose, fructose and rhamnose, respectively. Rhamnose is the most potent competitive inhibitor for *Aspergillus* and *Penicillium* enzymes. The naringinase from *Penicillium citrinum* retains as high as 85% of its activity in the presence of even 2% w/v rhamnose whereas at the same concentration, the commercially available *Penicillium* enzyme shows only 20% of the original activity. Thus, the enzyme from our isolate shows great commercial potential in debittering of grapefruit juice. It will also be useful in rhamnose preparation.

## 4.9 Immobilization of naringinase

Naringinase has been immobilized on a variety of matrices like DEAE-Sephadex, chitin, porous glass beads, hollow fiber, cellulose triacetate fiber and calcium alginate gel as summarised in Table 2.8 of literature review. Most of the preparations face constraints in their functioning due to inhibition by juice components or clogging of the reactor, diffusion limitations, or poor mechanical strength of the matrices under operating conditions, thereby hampering prolonged use. Immobilization of naringinase onto chitin and controlled pore glass has been reported. The chitin bound enzyme faces the problem of inactivation by juice components thereby making it unsuitable for field use. Porous glass needs several derivatisation and activation steps prior to binding of enzyme.

Attempts to immobilize naringinase by covalent binding, entrapment as well as adsorption were carried out in this study. As seen in Table 4.10 the enzyme got bound to several matrices but expression of the bound enzyme in most cases was rather poor. As the enzyme showed maximum expression of 40% on Matrex PAE Silica 1000L it was chosen for further immobilization studies.

### *4.9.1 Stabilisation of the immobilized enzyme using glutaraldehyde*

The naringinase bound to Matrex PAE Silica 1000 L expressed about 40% of the activity of the total bound enzyme. However, with the passage of time there was a release of the enzyme into the solution leading to loss of activity of the immobilized enzyme. The immobilized matrix effected 100% removal of naringin in the first run with a subsequent decrease to 16% in the sixth run and no debittering action in the subsequent runs. A stabilisation step was thus required to retain the bound enzyme on the matrix. Glutaraldehyde, a dialdehyde, is often employed in covalent linkage of enzyme to matrices such as chitin and porous glass (Tsen and Tsai, 1988). It binds to the amino group of the enzyme as well as the matrix forming a Schiffs base. Thus, as seen in Figure 4.29 glutaraldehyde treatment caused cross linking of naringinase to Matrex PAE Silica. A concentration of 1.5% for a cross-linking period of 1 h yielded optimum results (Figure 4.30 and Figure 4.31).

## 4.10 Effect of immobilization on the kinetics and properties of enzymes

Upon immobilization many properties of the enzyme undergo changes. The following factors play a significant role in contributing to the change

- Conformational effects : These are the cumulative effects of conformational change in the enzyme at the active site due to modification of the active site amino acids, change in the entire protein and change in the charge of the enzyme.
- Steric effects : This is a result of an altered interaction between substrate and enzyme due to steric hindrance.
- Partitioning effects : These are related to the chemical nature of the supporting material which may cause electrostatic or hydrophobic interactions between the matrix and the low molecular weight species present in the reaction mixture leading to a modified microenvironment.

| METHOD | MATRIX | % ENZYME BOUND | % EXPRESSED |
|---|---|---|---|
| COVALENT | GMA - DVB 10 | 11 | 0 |
| | 30 | 75 | 0 |
| | 50 | 94 | 0 |
| | 60 | 100 | 0 |
| | 200 | 29 | 0 |
| | | | |
| | GMA - PETA 50 | 0 | 0 |
| | 75 | 18 | 0 |
| | 100 | 64 | 0 |
| | | | |
| | GMA - TRIM 50 | 83 | 1.8 |
| | 75 | 80 | 2.0 |
| | 100 | 92 | 1.9 |
| | | | |
| | GMA - TRIM + ES 50 | 43 | 20 |
| | 75 | 52 | 21 |
| | 100 | 74.5 | 15 |
| ENTRAPMENT | CTA - Fiber | 41 | 13 |
| | Membrane | 100 | 2 |
| | Coating on precast membrane | 100 | 15 |
| | | | |
| ADSORPTION | XAD -2 | 30 | 0 |
| | XAD - 4 | 10 | 0 |
| | XAD - 7 | 78 | 7 |
| | NPA - 1 | 25 | 0 |
| | SILICA | 12 | 0 |
| | PAE SILICA 1000L | 100 | 40 |

GMA - glycidyl methacrylate, DVB divinyl benzene, TRIM - trimethalol propane trimethacrylate, PETA - penta erythritol triacrylate, CTA - cellulose triacetate

**Table 4.10** : Immobilization of Naringinase by different methods

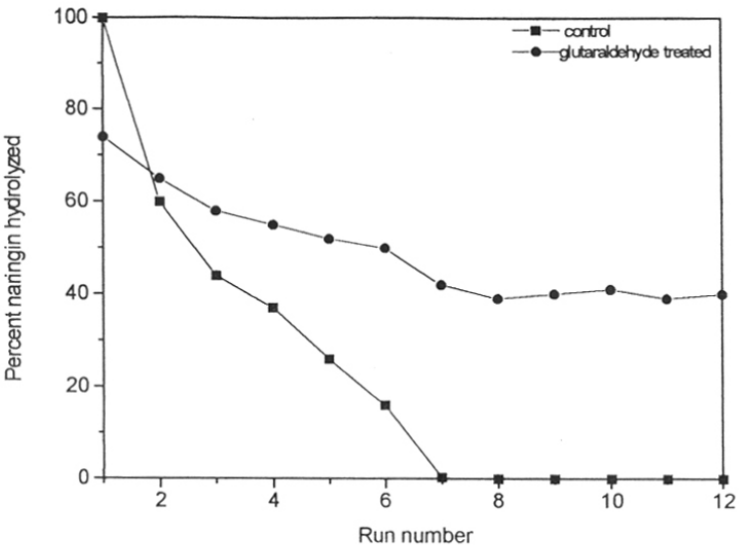



**Figure 4.29** : Effect of glutaraldehyde treatment on stabilisation of immobilised enzyme

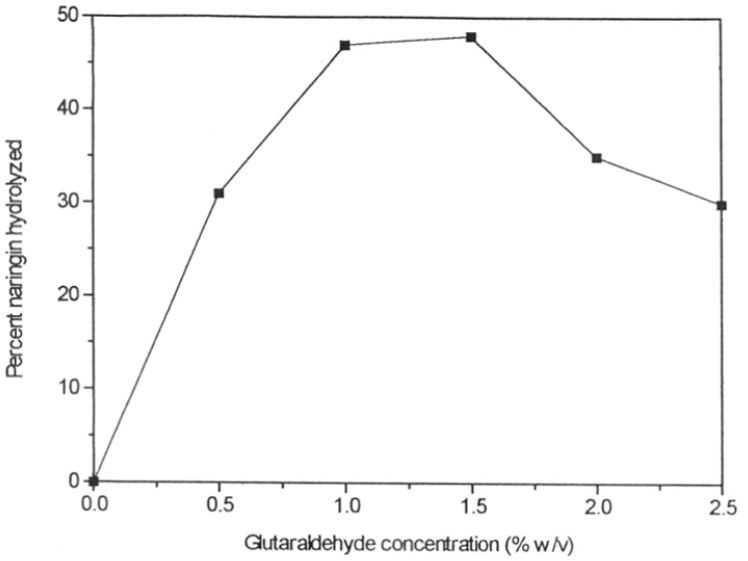



**Figure 4.30** : Effect of glutaraldehyde concentration on stabilisation of immobilised naringinase

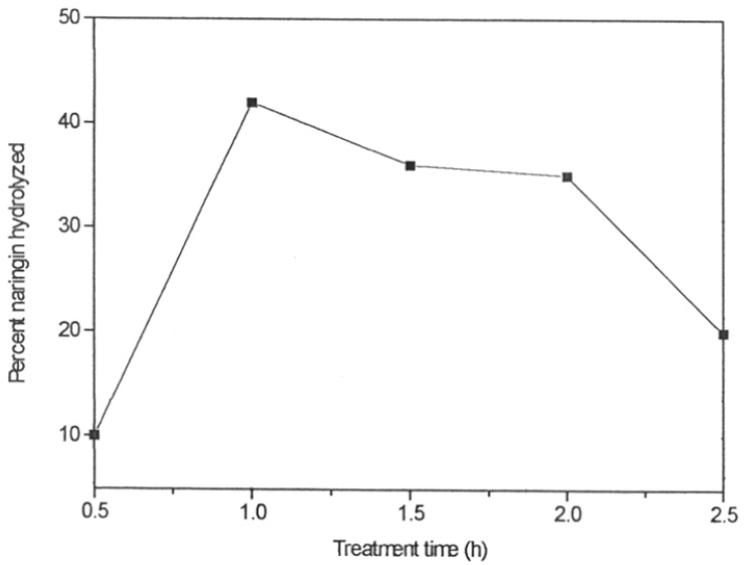



**Figure 4.31** : Effect of glutaraldehyde treatment time on stabilisation of immobilised naringinase

• Mass transfer or diffusional effects: These effects refer to diffusional resistance to the transport of the substrate from the bulk solution to the active site of enzyme and the diffusion of the products back into solution. The resistance is either internal or intraparticle when the enzyme is located in a porous medium, external or interparticle which occurs between the bulk solution and surface of the enzyme matrix particle.

### *4.10.1 Effect of pH on immobilized naringinase*

Enzymes are affected by environmental conditions and especially by pH of the aqueous media. The changes in the optimum pH as well as the pH activity curve depend upon both the charge of the support as well as that of the enzyme. In case of charged matrices, the change in kinetic behavior of the enzyme may be attributed to the partition effects. These result in different concentrations of charged species like $H^+$ ions, $OH^-$ ions, substrates or products in the microenvironment of the immobilized enzyme and in bulk solution owing to the electrostatic interactions with the fixed charges on the support. One of the consequences of these partition effects is a shift in the optimum pH with a displacement of the pH activity profile towards more alkaline or acidic pH values for negatively or positively charged matrices respectively. The pH stability of the immobilized enzyme may be enhanced due to partition effects, internal diffusion effects and/or conformational effects.

The immobilized enzyme in the present study has a pH optimum between 3 and 3.5, one pH unit to the acid side of that shown by the free enzyme (Figure 4.32). The enzyme also showed greater than 60% activity in the pH range from 3 to 5. However, it was inactive below pH 3 and above pH 8.0. The decrease in the optimum pH is beneficial for juice debittering owing to the fact that citrus juice has a pH between 3 and 4.5. A decrease in pH optimum from 4.5 to 4 was also seen in case of *Aspergillus* enzyme (Ono *et al.*, 1977). This shift was attributed to the redistribution of the hydrogen ions between the positively charged carrier and the surrounding aqueous solution. In case of the enzyme from *Penicillium decumbens* the pH optimum remained unaltered at 3.7 (Tsen *et al.*, 1989).

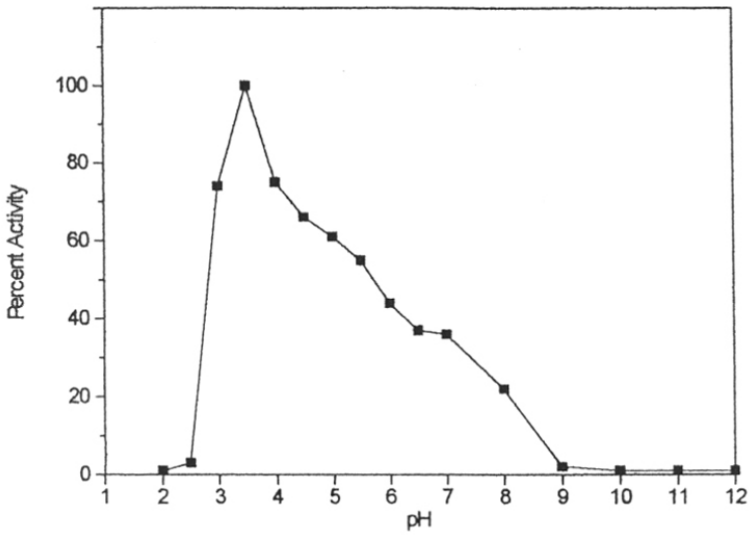



**Figure 4.32** : Effect of pH on activity of immobilised naringinase

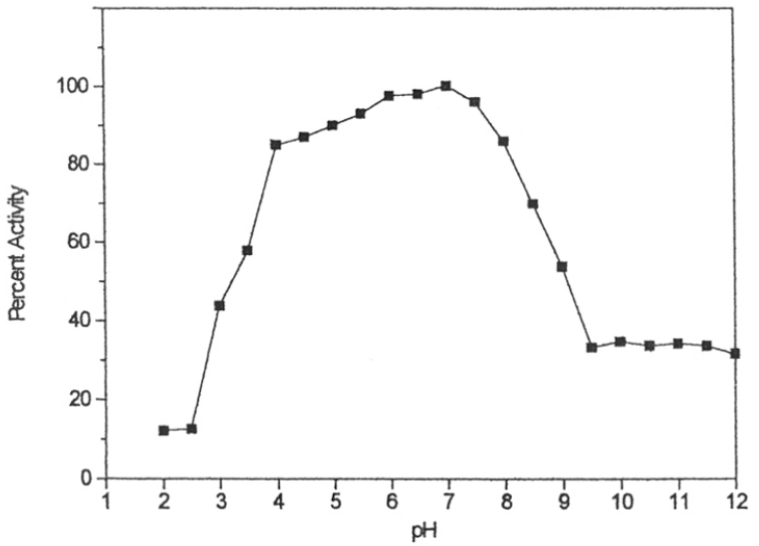



**Figure 4.33** : Effect of pH on stability of immobilised naringinase at 40 °C

The immobilized enzyme showed a wide range of pH stability retaining greater than 70% activity in the range from 4 to 8.5 at 40 °C for 24 h. However, above pH 9, the enzyme retained only 30% of its activity (Figure 4.33).

### *4.10.2 Effect of temperature on immobilized naringinase*

Immobilization changes the temperature optimum in most enzymes. In entrapped enzymes as well as those bound by ionic or covalent linkage the optimum becomes higher than that for the native. This increase is attributed to the diffusional effects in case of entrapment and binding on porous supports, which protects the enzymes against heat denaturation. Conformational changes and steric hindrances also play important role in the increased tolerance to heat denaturation.

The immobilized enzyme showed a temperature optimum between 50 and 55 °C that is 10° higher than that shown by the free enzyme (Figure 4.34). The reason that could be attributed to this is probably a higher conformational stability due to immobilization. An increase in heat stability and temperature optimum was also reported for both the *Aspergillus* and *Penicillium* naringinase (Park *et al.*, 1979; Ono *et al.*, 1977; Puri *et al.*, 1996).

### *4.10.3 Kinetic parameters*

The kinetic parameters for immobilized enzymes are often different from those for the native enzyme as a cumulative effect of conformational changes, steric hindrances, and partitioning effects.

The change in the $K_m$ of the immobilized enzyme depends on the method used for immobilization. The $K_m$ value reflects the affinity between the enzyme and substrate which can either increase or decrease due to immobilization. The $K_m$ of immobilized naringinase as determined by DNSA reducing sugar estimation was 1.12 mM and that determined by HPLC was 1.42 mM (Figure 4.35). The $K_m$ of the enzyme thus remained unaltered. The *Aspergillus* naringinase showed a decrease in $K_m$ (Ono *et al.*, 1977; Park and Chang,

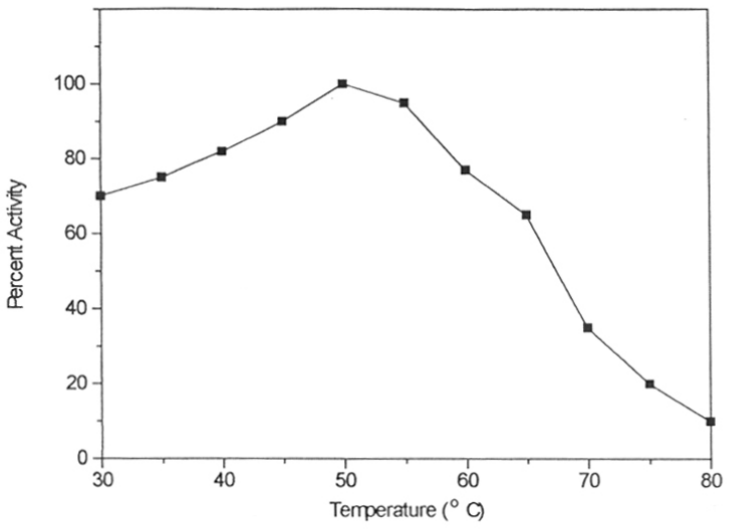
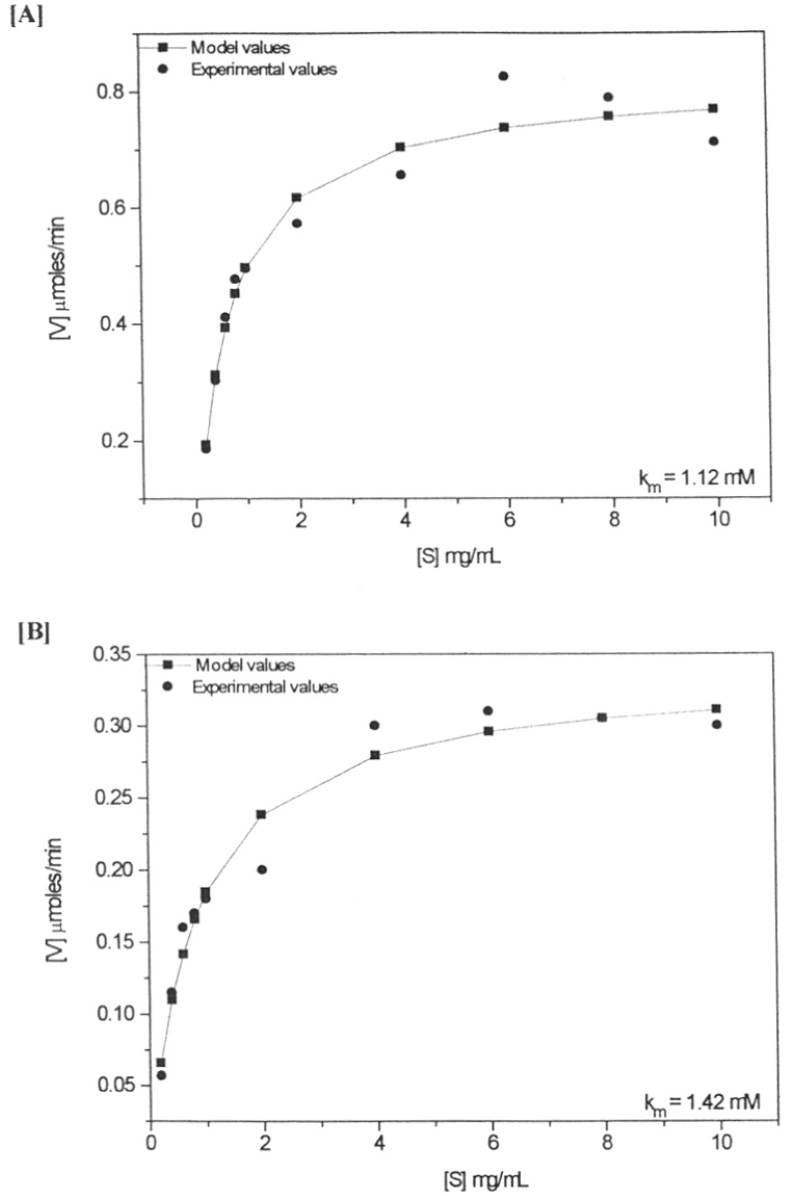



**Figure 4.34** : Effect of temperature on activity of immobilised naringinase




A - Kinetic studies by DNSA
B - Kinetic studies by HPLC

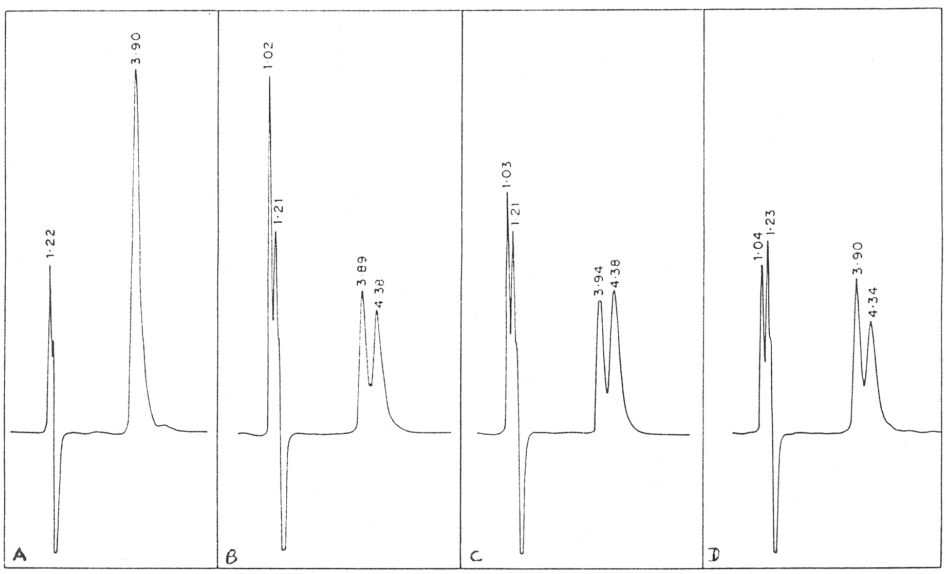
**Figure 4.35** : Kinetic studies of immobilised naringinase

1979) whereas in case of naringinase from *Penicillium decumbens* immobilization resulted in increased $K_m$ values (Tsen *et al.*, 1989; Puri *et al.*, 1996).

The $V_{max}$ of the immobilized naringinase from Penicillium citrinum was found to be 0.82 U/g of matrix by DNSA method and 0.34 U/g of matrix by HPLC method.

### *4.10.4 Effect of sugars on immobilized enzyme*

Figure 4.36 reveals that as in case of the free enzyme, the immobilized enzyme also was not inhibited by any of the sugars present in the natural juice at concentrations prevalent in juice. In addition, the sugars did not affect the enzyme activity at considerably higher concentrations too. Moreover, rhamnose, which showed slight inhibition in case of the free enzyme, had no effect on the immobilized naringinase at concentrations upto 2% w/v. This is the first ever report of an immobilized naringinase preparation free from inhibition by sugars. Both the immobilized preparations from *Penicillium decumbens* and *Aspergillus niger* are inhibited by all four sugars irrespective of the method used for immobilization (Ono, 1977; Park and Chang, 1979; Tsen, 1984; Tsen and Tsai, 1988; Puri *et al.*, 1996). Naringinase from *Aspergillus niger* and *Penicillium decumbens* are inhibited by all sugars. At concentrations prevalent in natural grapefruit juice the *Aspergillus* naringinase faces a 30% inhibition by glucose, 40% by fructose and 20% by sucrose whereas the *Penicillium* enzyme activity is inhibited to about 30%, 60% and 15% by glucose, fructose and sucrose, respectively (Tsen and Tsai, 1988). Owing to this shortcoming these preparations face a limitation in debittering of natural fruit juices and hence cannot be used on a commercial scale. Naringinase from *Penicillium citrinum* in the present investigation thus, shows a potential for commercial use.



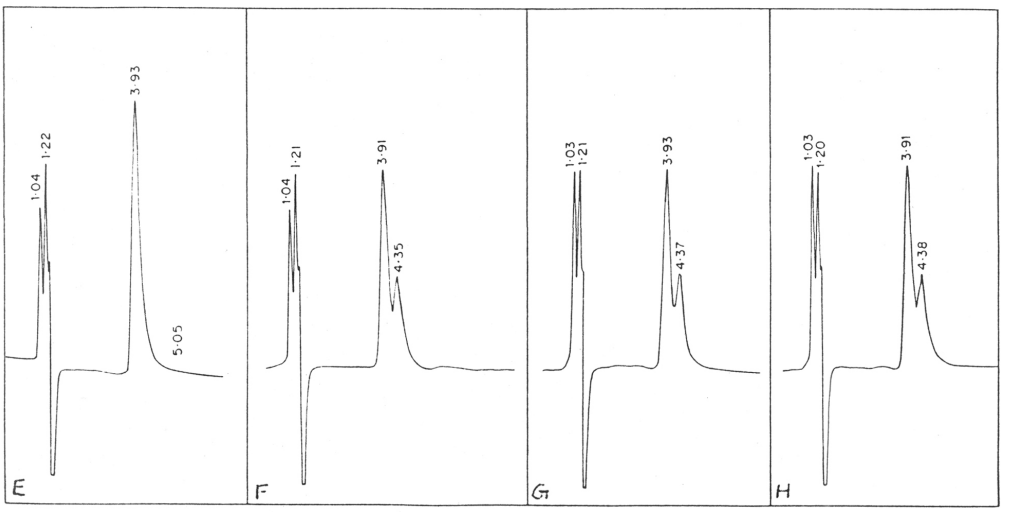

A- Naringin standard (elution time – 3.90)

B- Control (no sugar)

C- 4% glucose

D- 2% fructose

**Figure 4.36 a**: Effect of sugars on immobilised naringinase




E – Naringin standard (elution time – 3.93 min)

F – Control (no sugar)

G – 6% sucrose

H – 2% rhamnose

**Figure 4.36 b** : Effect of sugars on immobilised naringinase

Immobilization of naringinase resulted in a few changes in some of the properties of the enzyme. The kinetic constant $K_m$ remained unaltered as a consequence of immobilization. The pH curve of the immobilized preparation shifted slightly to the acidic side. As citrus juice is acidic with a pH ranging from 3 to 4.5, this decrease is a beneficial property. The enzyme also showed an increase in its temperature optimum, probably due to enhanced conformational stability. Both the free and the immobilized preparations were free from inhibition by glucose, fructose, sucrose and citric acid, all of which are major components of citrus juice (Table 4.11).

The naringinase from *P. citrinum* has a few similar and a few unique properties in comparison with the commercial naringinase preparations.

- The molecular weight (105 kD) is close to that reported for the enzyme from *Penicillium decumbens* (Sigma Chemical Co.).
- The kinetic constant $K_m$ is far lower than that reported for Sigma naringinase. Furthermore, there is a decrease in $K_m$ upon immobilization.
- The enzyme from *P. citrinum* has a very broad range of pH stability similar to that of Sigma naringinase.
- The temperature optimum of the enzyme is lower than the *Penicillium decumbens* enzyme and is close to the *Aspergillus* naringinase preparation. However, the optimum increases to that of the *Penicillium decumbens* preparation upon immobilization. The enzyme is also temperature stable at 40 °C for a period of at least 24 h.

The most unique property of *P. citrinum* naringinase is that it is free from inhibition by the sugars glucose, fructose, sucrose and citric acid, the main components of natural citrus juice.

| Property | Free enzyme | Immobilized enzyme |
|---|---|---|
| $K_m$ - DNSA | 2.45 mM | 1.12 mM |
| - HPLC | 2.90 mM | 1.42 mM |
| pH Optimum | 3.5 - 4.0 | 3.0 - 3.5 |
| pH Stability - 40°C | > 65% between 4.0 - 12 | |
| - 4°C | > 90% between 3.5 - 12 | > 70% between 4 - 8.5 |
| Temperature Optimum | 40 - 45 °C | 50 - 55 °C |
| Effect of Sugars - glucose | 100% activity upto 6% | 100% activity upto 6% |
| - fructose | 100% activity upto 6% | 100% activity upto 6% |
| - sucrose | 100 % activity upto 10% | 100% activity upto 10% |
| - rhamnose | 85% activity at 2% | 100% activity upto 2% |

**Table 4.11** : Comparison between properties of the free and immobilized naringinase

This is the first ever report of its kind, since both the commercial naringinases are inhibited by all the sugars, either in a competitive or noncompetitive manner.

The immobilized preparation can thus be used for debittering of grapefruit juice.

## 4.11 Debittering of grapefruit juice in a fluidized bed reactor

The most common equipment used for immobilized enzyme catalysed reactions are of two types, the packed bed reactor (PBR) and the continously stirred tank reactor (CSTR). The fluidized bed reactor (FBR) shares features with both these two types.

In an ideal CSTR, the contents of the reactor are uniformly mixed. Consequently, all the elements of the reactor have the same composition which is same as the composition of the outflow. Therefore, the rate of the reaction is determined by the composition of the exit stream from the reactor. The open construction of the CSTR permits ready replacement of the immobilized enzyme catalyst. It also facilitates easy control of temperature and pH. CSTRs have mainly been used for particulate enzyme systems. CSTR systems require a means of retaining the supported enzyme particle within the reactor which can be achieved by providing a filter at the outlet of the reactor or by a separate settling stage.

In a PBR there is a steady movement of the substrate across a packed bed of immobilized enzyme in a chosen spatial direction. If the fluid velocity profile is perfectly flat over the cross section, the reactor is said to operate as a plug flow reactor (PFR) under ideal conditions. Several immobilized enzyme systems operate as PBRs where the enzyme is attached to porous glass beads, beads of ion exchange resins, polyacrylamide gel disks, sheets or plates of enzyme containing polymers, chips of collagen membranes, etc.

In a FBR the substrate solution is passed upward through the immobilized enzyme bed at a velocity high enough to suspend the particles. However, the velocity is not enough to sweep away the particles from the reactor itself. The fluid flow pattern provides

a degree of mixing that falls between complete as in a CSTR and no backmixing as in a PFR. FBR finds application in supported enzyme catalysis where viscous, particulate substrates are to be handled. The individual particles in a FBR are kept in motion by the continous flow of the substrate.The pressure drop of the fluid flow essentially supports the weight of the bed. The reactor thus, provides for the free movement of the catalyst particles throughout the bed. Small catalyst particles can be used in this type of reactor. Hence, the yield limiting diffusion associated problems, as seen in conventional fixed bed reactors can be overcome (Padmini *et al.*, 1995). FBRs offer good solid liquid mixing and minimal pressure drops as against fixed bed reactors. Gueguen *et al.* (1997) showed that for the same amount of immobilized enzyme, the reaction time was 6 h 15 min when operated as a FBR and 16 h 40 min with a PBR. Some of the applications of FBRs are summarised in Table 4.13. The advantages of using Matrex PAE silica 1000L immobilized preparation as a FBR are

- The fine particulate matrix (50 μm particle size) offers minimal diffusion limitations.
- Fluidization prevents precipitation of the insoluble end product naringenin that otherwise would block the pores of matrices leading to diffusional problems in subsequent runs.
- The reactor can handle certain amount of suspended and fine particles.
- It helps in easy settling and resuspension in a single reactor.
- The power requirement is less as compared to CSTR.

Figure 4.37 shows the experimental setup of the fluidized bed reactor. As seen in Figure 4.38 the fluidized bed reactor stably debittered both naringin solution and simulated grapefruit juice. The column resulted in removal of 90% of the original naringin in a period of 3 h. Moreover, the efficiency of debittering was not affected by time and the reactor was stable for at least 7 runs over a period of 7 days.

Puri *et al.* (1996) has reported 82% naringin removal in 7 h. However, their efficiency dropped to 56% in the first 24 h. Fibre entrapped naringinase by Tsen *et al.* (1989) resulted in 80% removal of naringin in 2 h, an improvement over chitin immobilized enzyme which effected only 40% removal in the same period. Olson *et al.* (1979) and

| Reaction catalysed | Enzyme | Reference |
|---|---|---|
| Starch hydrolysis | amylase | Barker *et al.*, 1971 |
| Hydrolysis of lactose in cheese whey | β galactosidase | Coughlin *et al.*, 1973 |
| Esterification of geraniol with lauric acid | lipase | Pyle *et al.*, 1990 |
| Glucose isomerisation | glucose isomerase | Vos *et al.*, 1993 |
| Solubilization/liquefaction of bituminous coal | hydrogenase and cytochrome c oxidase | Kaufman *et al.*, 1995 |
| Hydrolysis of ricebran oil | lipase | Padmini *et al.*, 1995 |
| Clotting of skimmed milk | pepsin | Rejikumar *et al.*, 1995 |
| Aroma enhancement of Muscat wine | β glucosidase | Gueguen *et al.*, 1997 |

**Table 4.12** : Applications of fluidized bed reactors

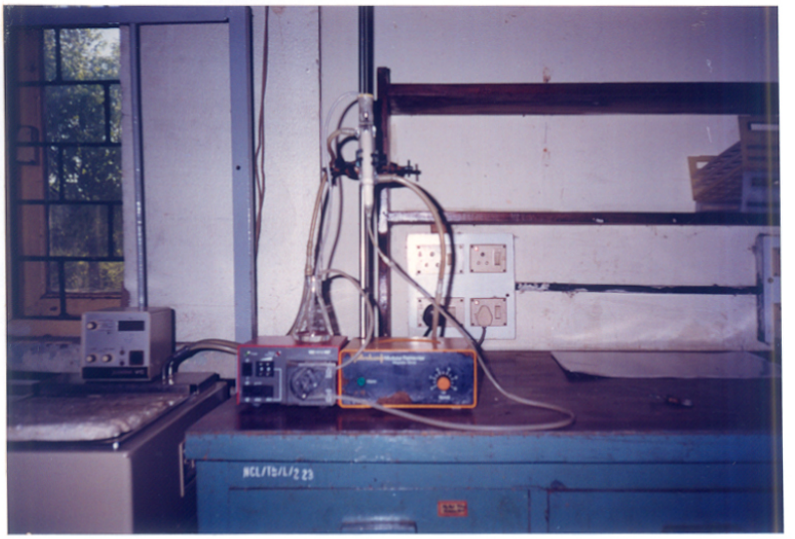


**Figure 4.37** : Photograph showing experimental setup of fluidized bed reactor

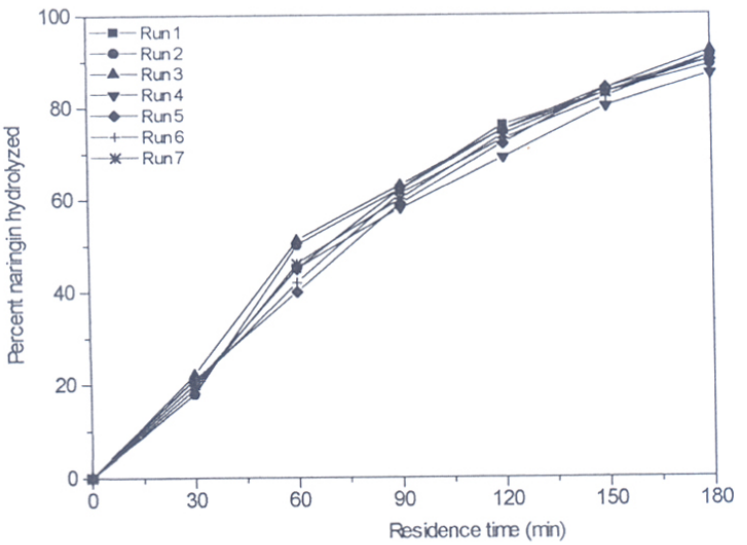



**Figure 4.38** : Debittering of simulated grapefruit juice in a fluidized bed reactor

Gray *et al.* (1981) achieved 40% and 22% removal in 3 h, respectively, by immobilizing the enzyme in hollow fibre cartridges. However, the major drawback faced by all the immobilized preparations so far is that the efficiency of the reactor falls with time owing to the fact that these enzymes are inhibited by the components of the juice itself. In the present investigation however, the efficiency of debittering remained stable for sufficient length of time.

# 5. CONCLUSIONS

A fungus, identified as *Penicillium citrinum*, that produced naringinase was isolated from garden soil. The enzyme activity was estimated spectrophotometrically by reducing sugar assay and by HPLC giving activity as total naringinase as well as in terms of α-L-rhamnosidase and β-D-glucosidase.

Analysis of citrus varieties in India revealed a naringin content of 0.2 mg/mL in grapefruit juice. No naringin could be detected in sweet lime, lemon, orange and kinnow.

A detailed study of enzyme production and media optimisation was carried out in shake flask. Naringinase is an extracellular enzyme produced during late log phase. Enzyme yield was greater in organic media containing soybean meal and incorporation of glucose in the medium enhanced enzyme production. Addition of naringin as aliquots after the growth phase induced maximum enzyme production. The physiological conditions like temperature and pH were standardized in shake flask as well as in 1 L Gallenkamp fermenter. A pH of 6.5 and a temperature of 30 °C was found to be most suitable for growth and enzyme production. Thus, at the end of media optimisation the activity was enhanced from 20 U/L to 600 U/L.

The enzyme was purified using a series of steps including concentration by ultrafiltration, ion exchange chromatography, hydrophobic interaction chromatography and gel permeation chromatography. The enzyme was purified to homogeneity as revealed by a single band on PAGE. A fold purification of 1397 was achieved with the pure enzyme having a specific activity of 47.5 U/mg. The enzyme was characterized as a protein with a molecular weight of 105 kD as determined by SDS-PAGE. It was stable over a wide pH range from 4 to 12 retaining more than 65% activity for 24 h at 40 °C and more than 90% activity at 4 °C. The optimum pH was found to lie between pH 3.5 and 4 and the optimum temperature between 40 and 45 °C. The kinetic constants, $K_m$ and $V_{max}$, of naringinase as determined by DNSA assay were found to be 2.45 mM and 145.5 U/mg protein. These values as determined by HPLC were 2.9 mM, and 138 U/mg protein, respectively. The naringinase preparation did not show any inhibition by glucose, fructose, sucrose and citric acid, all natural components of the juice. Rhamnose effected only 20 % inhibition at a concentration as high as 2 % w/v.

The enzyme was immobilized on Matrex PAE-Silica 1000L by adsorption. The preparation was stabilized by glutaraldehyde treatment at a concentration of 1.5 % w/v for a period of 1 h. The immobilized enzyme had a pH optimum between 3 and 3.5, i.e. 0.5 units to the acid side of the free enzyme and was stable over a pH range of 4 to 8.5 retaining more than 70% activity at 40 °C for 48 h. The temperature optimum of the immobilized preparation was between 50 and 55 °C, 10 ° higher than that of the free enzyme. The $K_m$ and $V_{max}$ values for the immobilized naringinase were found to be 1.12 mM and 0.82 U/g of matrix with the DNSA assay. A $K_m$ value of 1.42 mM and $V_{max}$ value of 0.34 U/g of matrix was obtained with the HPLC assay. As in the case of the free enzyme even the immobilized preparation was not inhibited by glucose, fructose, sucrose and citric acid at various concentrations. Furthermore rhamnose at a concentration of 2% w/v also did not cause any inhibition.

The immobilized preparation was packed in a column and run as an expanded bed reactor for debittering of naringin and simulated juice. Thirty mL of either naringin solution or simulated juice at a flow rate of 2 mL/min was debittered to achieve 90% removal of naringin after 2 h at 40 °C. The column retained 100% activity even after 7 runs over a period of 7 days. Thus, the naringinase from *Penicillium citrinum* was found to have great industrial potential owing to its stability at juice pH, temperature stability and lack of inhibition by various juice components - a feature unique to the enzyme from this isolate.

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