Studies on Limonin Biotransformation by *Pseudomonas putida*

A THESIS

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT

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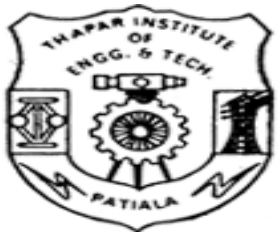
MASTER OF SCIENCE (BIOTECHNOLOGY)



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I feel lacunae of words to express my deepest sentiments to my parents for their sustained encouragement all through.

In the end, I am thankful to the Almighty for blessing me to complete this work.

(RACHNA SHARMA)

CANDIDATE'S DECLARATION

I hereby declare that work which is being presented in dissertation entitled "**Studies on Limonin Biotransformation by *Pseudomonas putida"*** in partial fulfillment of requirements for the award of the degree of Master of Science in Biotechnology, Department of Biotechnology and Environmental Sciences, Thapar Institute of Engineering and Technology, Patiala is an authentic record of my work during a period of 5 months from January 2003 to May 2003 under the supervision of Dr. Geetanjali Bansal, Lecturer, Department of Biotechnology and Environmental Sciences, Thapar Institute of Engineering and Technology, Patiala. I have not submitted the matter embodied in this dissertation for any other degree or diploma.

Place: (Rachna Sharma)

Date:

This is to certify that the above statement made by the candidate is correct and true to the best of my knowledge.

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ABSTRACT

Kinnow, which is the hybrid of *Citrus nobilis* and *Citrus delicosa*, was developed through lengthy hybridization process. The main problem in acceptability of Kinnow fruit juice is the development of bitterness with time. Two principle components responsible for the development of bitterness in Kinnow juice are naringin (immediate bitterness) and limonin (delayed bitterness). Several efforts have been made abroad to process citrus juice that is free from bitterness. Though all these methods showed certain results, considerable success has not been found in debittering technology. Further, most of the research has been restricted to grapefruit juice and other citrus fruit juices. Very little work has been done on Kinnow fruit juice. The present work aimed to address this problem using *Pseudomonas putida*.

Firstly, optimum growth conditions for *Pseudomonas putida* were studied where in different parameters like pH, temperature, duration, source of limonin, and type of growth medium were optimized for maximum limonin biotransformation. Further, an attempt was made to identify the reaction products so formed. Maximum limonin biotransformation along with optimum growth could be achieved by growing *Pseudomonas putida* at a temperature of 35^{o}C and pH 5 for 120 hours. Results of thin layer chromatography indicated the presence of deoxylimonin and deoxylimonoic acid in the culture medium. Future work needs to be done to confirm the metabolites of limonin as well as to purify and characterize the bacterial enzymes involved in the pathway.

List of Abbreviations

DVB	divinyl benzene styrene
HPLC	High Performance Liquid Chromatography
mg	mili gram
ml	mili litre
MPA	Mega Pascal
MS	Mass Spectroscopy
MT	Metric Tons
nm	nano meter
NMR	Nuclear Magnetic Resonance
p values	Probability values in student t test
ppm	parts per million
rpm	revolution per minute
RDA	Recommended Daily Allowance
SEM	Standard error of means
TLC	Thin Layer Chromatography
TSS	Total Soluble Solids
µg	micro gram
µl	microlitre

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INTRODUCTION

India holds third rank in respect of production of citrus fruits in the world. The citrus group includes sweet orange, mandarin, lemon, acid lime, pumello, grapefruit etc. Citrus fruits are native of Southeast Asia and are cultivated in warm climate. The commercial cultivation of citrus is concentrated in the northern, eastern and western parts of India. Maharashtra is very famous for its 'Mausami' and 'Nagpuri santra'. Punjab is known for its Kinnow and Malta. Citrus fruits are considered as one of the nutritious fruits because these are the rich source of β-carotene (vitamin A), ascorbic acid (vitamin C) and folic acid. All these vitamins provide protection against fatal diseases like cancer, heart ailments, and neural tube defects. Doctors recommend orange juice for infants as a supplementary source of vitamin C.

Kinnow is indigenous to Pakistan, the largest grower of Kinnow in the world. Cultivation of Kinnow has assumed a considerable significance during recent years in northern India. This has emerged as one of the main crops of Punjab and accounted for 45% of its total fruit production (Statistical Abstracts, Punjab, 1991).

We produce nearly four million MT per annum of citrus fruits. However, due to our poor post-harvest infrastructure facilities, the wastage of citrus fruits amounts to nearly one million MT per annum. There is a need to utilize this wastage to improve the nutritional and health status of people as also to provide remunerative prices to the farmers during seasonal glut. Further, despite the high volume of production, waste occurs thus want suitable preservation methods to produce non-bitter citrus juice. Hence, the major impediment for the increased consumption of fresh citrus juices and their products is the development of bitter taste in the juice after extraction. Potential areas for expansion of citrus fruits such as Kinnow, orange, etc. in north India is associated with several other problems like lack of pre-cooling and cold storage infrastructure facilities, low prices due to seasonal glut, stem puncturing followed by *penicillium* rotting, etc. The preservation of citrus juices is also very difficult because the citrus juice becomes highly bitter within four hours of extraction.

Scientific mention of bitterness in literature was first made during 1857 in Java. Later two classes of chemical compounds namely flavonoids and limonoids were found responsible for bitterness in citrus juices. However, there is a difference between flavonoid and limonoid bitterness. The fruits containing high flavonoids are bitter even when consumed as fresh. The peel (rind) of the citrus fruit contains very high amount of flavonoids like naringin, neohesperidine, etc. making it highly bitter. The limonoids are present initially in the form of non-bitter compound (limonoate-A-ring lactone), which is converted to bitter limonin and other bitter limonoids in the presence of enzyme limonoate-D-ring lactone hydrolase on storage. Hence, the fresh citrus juice does not taste bitter but turns highly bitter on storage. Several other factors like storage temperature, acidic medium of the juice, etc. also play a vital role in the development of bitterness. This is known as "delayed bitterness".

Ever since naringin and limonin were discovered in citrus fruit juices in 1965 and found to contribute significantly to its bitterness, several efforts have been made abroad to process citrus juice free from bitterness. Various physico-chemical and biotechnological approaches such as use of polyamides for adsorptive debittering, treatment with ethylene, debittering by passage through polystyrene-DVB resins and β-cyclodextrin, use of immobilized microbial mass as well as immobilized enzymes have been employed for this purpose.

Though all these methods show certain results, success has not been achieved in debittering technology in India. In India, CFTRI, Mysore, IARI, New Delhi, and other state agricultural universities have conducted a good amount of research on this aspect, yet not a single commercial unit has been established so far in the country. The National Horticultural Board (NHB) has sanctioned one research project to the Dr Y.S. Parmar University of Horticulture and Forestry, Solan (HP), to establish a pilot scale debittering plant. Further, most of the research has been restricted to grapefruit juice and other citrus fruit juices. It is clear that very little work has been done on Kinnow fruit juice till now. The present study was designed to address this problem.

Further, microbial whole cells as well as enzymes have been studied for their limonin biotransformation activity in citrus juices by various investigators in the past. Some enzymes have been purified and characterized from different bacteria. But, work specifically on Kinnow juice has been reported only by two investigators- one that has employed immobilized *Arthrobacter globiformis* (Premi *et al*., 1995) cells and the other using immobilized *Rhodococcus fascians* (Marwaha *et al*., 1994) for debittering Kinnow juice. Even in these studies, metabolites of limonin have not been identified, nor has an attempt been made to isolate and characterize the enzyme(s) involved in the pathway.

The simplest and the most convenient way to solve this problem would be direct addition of a limonin biotransforming enzyme to the raw juice. For this, the enzyme must have sufficient stability and catalytic activity at pH 3-4 (pH of the juice) to convert limonin efficiently to a nonbitter metabolite. Keeping this in mind, the present study was designed to achieve the following aims and objectives:

- Optimization of the growth conditions of *Pseudomonas putida* where maximum limonin biotransformation is achieved
- Identification of the metabolites of limonin biotransformation by *Pseudomonas putida*

REVIEW OF LITERATURE

2.1 Kinnow, The Fruit

Kinnow, is the hybrid of *Citrus nobilis* and *Citrus delicosa.* Late H.B.Frost first evolved it at the University of California, Regional Fruit Station USA through a lengthy hybridization process (Khurdiya and Lotha, 1994). It was introduced in India during early 1940's at fruit experimental station of Punjab Agricultural College and Research Institute, Lyallpur by S.Bahadur Lal Singh (Singh, 1978). Kinnow is known for its superior characters such as heavy bearing, wide adaptability and fruit quality. It contains potassium, vitamin C and vitamin A. Kinnow has the following features that differentiate it from other citrus varieties.

- Easy Peel: The rind containing numerous oil glands, comes off easily with bare hands since it is loosely bound compared to other types of citrus fruit. Therefore, Kinnow can just be peeled off and enjoyed anywhere, anytime.
- High Juice Content and Rich Flavor: Kinnow consists of several easily separated sections and has high juice content. Its full-bodied flavor and its juice content combine to make it a most satisfying and healthy fruit.
- Vitamin C: One Kinnow provides sufficient Vitamin C to fulfil your need for a day. It is the ideal health choice.
- Use of Pulp: Use the pulp to make delicious desserts, jams and sauces.
- Skin: The skin of the fruit can be used to make cosmetics and essence.

Table 1: Nutritional Value Comparison of orange and Kinnow juices:

	(%RDA)	
	Orange	Kinnow
Vitamin A	2%	3%
Vitamin C	130%	130%
Calcium	6%	4%
Phosphorous	2%	3%
Iron	2%	4%

Cultivation of Kinnow has assumed a considerable significance during recent years in northern India. This has emerged as one of the main crops of Punjab

and accounted for 45% of its total fruit production (Statistical Abstracts Punjab, 1991). This is due to its adaptability to wide range of agro-climatic conditions (Khurdiya and Lotha, 1994). It is a seasonal fruit available from January to April.

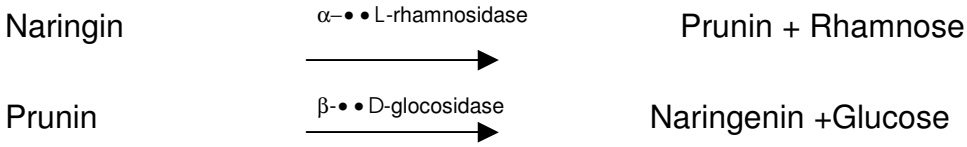
2.2 The Problem

As compared to Kinnow production, the fruit processing industry did not progress simultaneously due to lack of consumer awareness, uncertainty of market response, problems in debittering technology, entrepreneurial hazards arising from variety of sources, including the extension of farm services and marketing of fruit juice. The processing of Kinnow juice faced formidable problems in terms of ”bitterness” and delayed ”bitterness”, thereby affecting its consumer acceptability.

2.3 Mechanism of Bitterness

The major problem encountered by the citrus fruit industry is that of development of bitterness in Kinnow juice with time. Bitterness in Kinnow juice is basically of two types-one that is present initially when juice is extracted and the other that develops with time called delayed ‘’bitterness”. Two different compounds are responsible for these two types of bitterness: naringin for ‘’bitteness” and limonin for ‘’delayed bitterness.”

Naringin is the primary bittering component of citrus fruit. It is a water-soluble component found in fruit membrane and albedo, and which becomes extracted into fruit juices. It is the 7-β–neohesperidoside of naringenin, the flavanone glucoside (Fisher and Wheaton, 1976). Its concentration is a degree of fruit maturity (Yousof *et.al.,* 1990). It is present in least amount in ripened fruits. As the fruit matures, naringin is hydrolyzed by alpha-L-rhamnoside to prunin and L-rhamnose. Prunin which is 33% as bitter as naringin, in turn is further hydrolyzed to naringenin and D-glucose. This conversion reduces the bitterness due to naringin.



The presence of naringin and the route of its degradation in Kinnow have already been established by HPLC analysis. Therefore, this is not a big problem.

2.3.1 Delayed Bitterness

Navel oranges, in general, do not taste bitter if eaten fresh or if juice is squeezed from the fruit and consumed immediately. However, the juice becomes bitter within a few hours after juicing at room temperature. This gradual development of bitterness is termed as delayed bitterness. This delayed bitterness differentiates limonoid bitterness from flavanone neohesperidoside bitterness, which occurs in citrus cultivars, related to pummelo.

Limonoids are a group of chemical substances found in the rutaceae and meliaceae families, which includes fruits like oranges, lemon and pumello. Twenty-nine limonoids have been isolated from citrus and citrus hybrids so far. They are highly oxidized triterpenoids that contains a furan moiety substituted at three position of D-ring lactone. Among 36 limonoid aglycones found in citrus and its hybrids, only six are bitter. Limonin was the first compound to be characterized from this group. It is the major limonoid found in most citrus fruit juices and is also the major cause of delayed bitterness. Nomilin is also involved, but its role is minor. Other bitter limonoids are not found in significant concentrations in the commercial citrus juices (Arigoni *et.al.,*1960; Emerson,1948; Higby,1938; Kefford,1959). Biosynthetic pathways of limonoids have been proposed based on radioactive tracer research by Hasegawa *et al.* (1992*).*

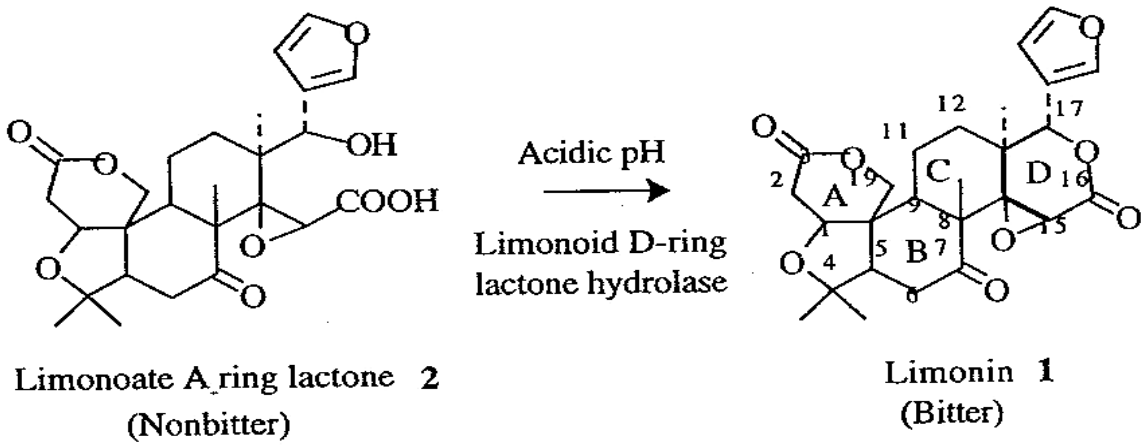


Figure 1. Mechanism of delayed bitterness

Limonin is present in fruit body in very low amount of less then 6ppm. However, its non-bitter precursor, limonate-A-ring lactone (LARL), is present endogenously in the cell cytoplasm at neutral to slightly alkaline pH in membranous sacs (Maier and Beverly, 1968; Maier and Margileth, 1969). This non-bitter precursor, LARL, is converted to limonin under acidic pH of juice when these sacs rupture during juice processing (Maier and Beverly, 1968). This conversion is accelerated by the action of limonoate D-ring lactone hydrolase that has been shown to be present in citrus fruits (Maier *et.al.,*1969). This enzyme catalyses the closure of the D-ring to form limonin that causes bitterness in juice. As bitterness results after some time of processing, it is termed as ‘’delayed bitterness” (Joslyn and Pilnik, 1961; Kefford 1959). The development of bitterness i.e. conversion of limonoate-A-ring lactone to limonin gets completed in 7h of juice extraction at 12^{o}C and in 4h when kept at 25^{o}C (Premi *et.al.,* 1995) .

2.4 Chemistry of Limonoids

Limonoids are present in citrus in three chemical forms:

1) limonoid monolactone (open D-ring aglycones) such as limonoate A-ring lactone

2) limonoid dilactones (D-ring closed aglycones) such as limonin

3) limonoid glucosides (such as limonin 17-D-glucopyranoside).

Limonoid monolactones (acidic) are soluble in water and some polar solvents; limonoid dilactones (neutral) are hydrophobic; the limonoid glucosides are all 17-?-D-glucopyranoside esters of the acidic aglycones, and are soluble in water and alcohol, but insoluble in non polar solvents.

Structures of some of the common and important limonoids are illustrated below:

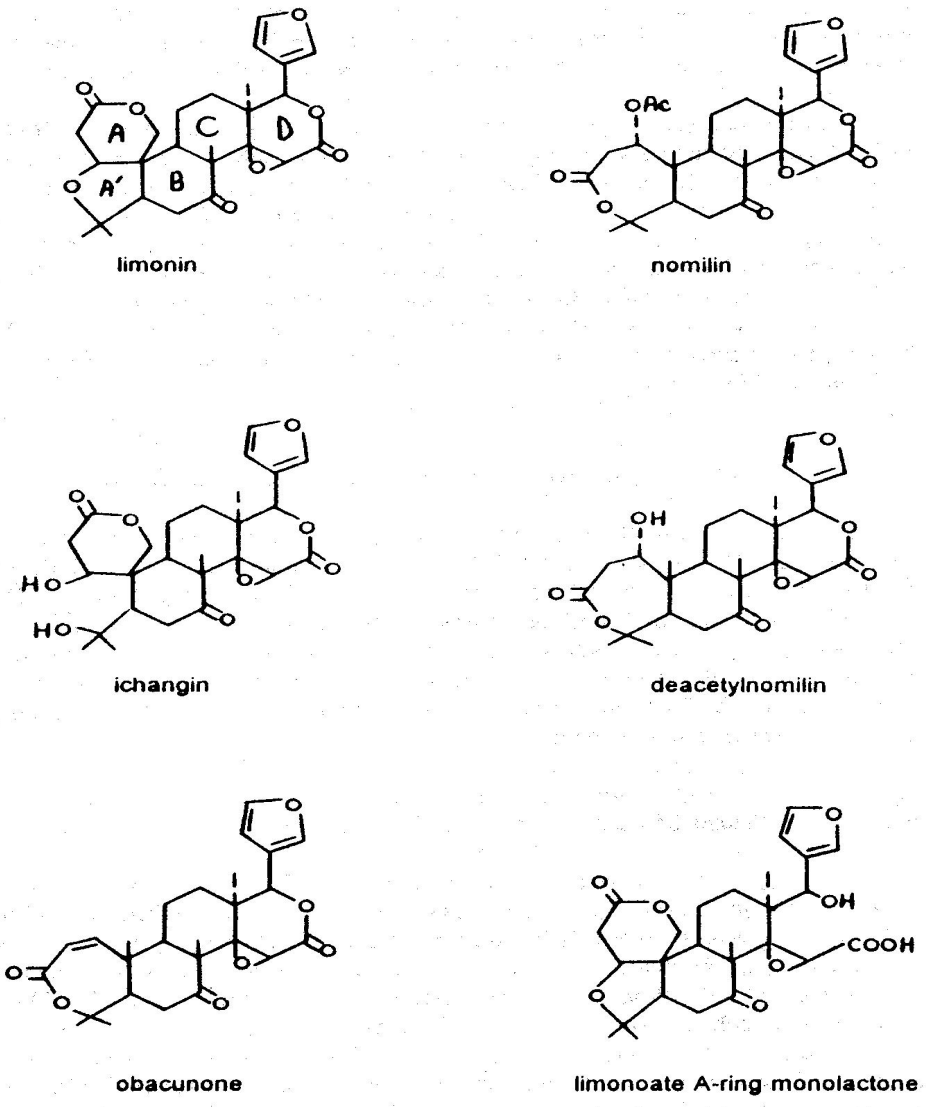
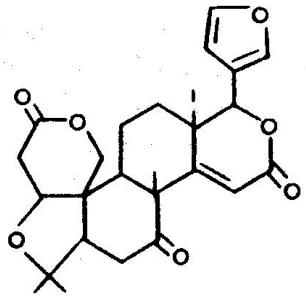
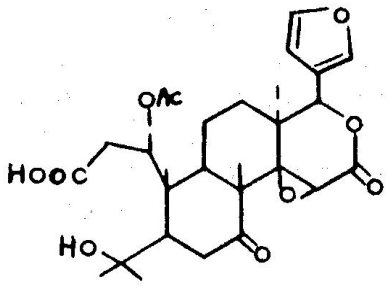


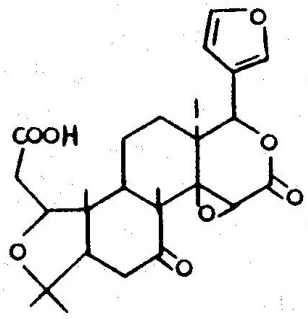
Fig. 2 *Structures of limonin, nomilin, ichangin, deacetylnomilin, obacunone, limonoate ring monolactone, deoxylimonin, nomilinic acid, isoobacunoic acid, and limonol.*



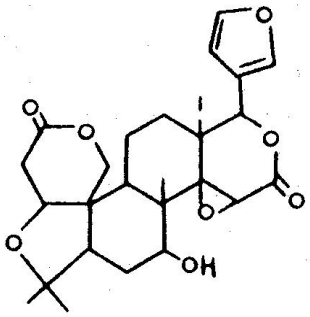
deoxylimonin



nomilinic acid



isoobacunoic acid



limonol

Figure 2 Continued.

2.5 Analysis of Limonoids

The accumulation of limonin and naringin in Kinnow juice needs to be determined precisely in order to establish the contributions of these compounds to immediate and delayed bitterness potential (Melntosh, 1987).

Dreyer made several initial significant contributions to the field of limonoid analysis, including TLC analysis for limonoids detection and the use of NMR for determination of limonoid structure. He isolated deacetynomilin and deoxilimonin using these methods (Dreyer, 1965). Hasegawa and Bennett used above two methodologies to isolate thirty additional limonoid aglycones and twenty limonoid glucosides from citrus and its allied species such as limonol from *Arthrobacter globiformis*. The major analytical techniques for the detection and quantitative

analysis of limonoids are HPLC introduced by Fisher, radioimmunoassay introduced by Mansell and Weiler and recently HPLC-MS by Manners and Hasegawa (1975). Most of these methods (except radio immuno assay) require sample preparation protocols using organic solvent extraction, partitioning and solid phase extraction. HPLC has become the most widely used method as it is accurate and reproducible; both normal phase and reverse phase methods have been developed using isocratic and gradient development protocols.

While thin layer chromatography (Tatum and Berry, 1973) is a versatile tool for detection of bittering components, limonin is better and more sophisticatedly assayed using spectrophotmetry (Wilson and Crutchfeild, 1968), radio immuno assay (RIA) (Weiler and Mansell, 1980), enzyme linked immunosorbant assay (EIA) (Jourdan *et.al.,* 1984), gas chromatography (GC) (Krunger and Colter, 1972), HPLC (Rouseff and fisher, 1980), and reverse- phase HPLC (Widmer, 1991). Among these techniques, EIA and HPLC are most widely used. While EIA has the advantage of specificity and simultaneous analysis of several samples, thereby imparting the rapidity of analysis desired in periodic monitoring of an industrial scale debittering process, HPLC affords accuracy and reproducibility in the quantitative assay, especially desired in quality assurance.

Naringin too may be assayed using the above-mentioned techniques. However, spectrophotometry (Davis, 1947) and HPLC (Yousof *et al*., 1990) provide rapidity and reliability in its quantitative determination.

2.6 Previous Attempts for Debittering Citrus Juices

Bitterness due to limonin in citrus fruits is one of the major problems in citrus juice industry and has a significant economic problem. Therefore various physicochemical, chemical and biotechnological approaches has been employed to find out a solution to this problem.

2.6.1 Physicochemical Approaches

The use of polyamides to selectively adsorb significant quantities of limonin has been successfully explored (Chandler and Kefford, 1968; Griffith, 1969). Various other adsorbents such as cellulose acetate, nylon based matrices, porous polymers and ion exchangers have been used to reduce bitterness (Johnson and Chandler, 1988). Various degrees of debittering have been achieved with individual matrices and their combinations with cellulose acetate and two porous polymers exhibiting intense affinity for limonin.

Treatment of grapefruit juice with activated magnesium silicate (florisil) in a batch mode significantly reduced limonin, naringin and total acids without adversely affecting nutritional quality (Barmore *et.al.,* 1986). Application of ultra-filtration along with adsorption efficiently removed limonin from grapefruit juice at pilot-plant scale (Fernandez *et.al.,* 1992) where the efficiency of removal was independent of temperature between 13-48^{o}C.

β–cyclodextrin polymer has been used to reduce 58% of initial bitterness from grapefruit juice**,** lyo orange etc. (Shaw and Wilson, 1983). This reduced bitterness was due to formation of an inclusion complex between κ-cyclodextrin and naringin or limonin. This information led to utilization of β–cyclodextrin polymer in a batch/continuos column mode to remove limonin and naringin from their aqueous solutions, oranges and grapefruit juice (Shaw and Wilson, 1983).

In batch operations, cross-linked DVB resins reduced naringin and limonin in grapefruit juice by 80-90% (Puri, 1984). Processing navel juices with these resins have no effect on minerals, acid, and amino acid content of juice (Kimball and Norman, 1990). Several commercial processes comprise of centrifugation of orange juice to separate suspended pertinacious solids (clarification of juices) followed by passage of clarified juice through divinyl benzene resins (Wethern, 1991).

2.6.2 Chemical methods

Treatment with ethylene(20μg/ml) for 3h to accelerate ripening in navel oranges, lemons, and grapefruits with a concomitant reduction in bitterness have been reported(Maier *et.al.,*1973).

Using CO_2 at pressures of 21 to 41 MPA at 30°C-60°C for 1h resulted in an average removal of 25% of the limonin from navel orange juice. By extending the treatment to 4h, 60% of the limonin was removed (Kimball, 1987).

Limitations and drawbacks

These methods alter the chemical composition of the juice, through either chemical reactions or removal of nutrients, flavor, color etc thus affecting the palatability. The methods are nonspecific in nature and therefore, inherently inefficient, introducing batch-to-batch variations due to undesirable or non-monitorable changes. Moreover, the chemicals used cannot be fully recycled, thereby, rendering disposal of pollutants costly. The methods were cumbersome, gave less reproducibility, lower yield and cause partial loss of desired nutrients while removing bitter components. So, debittering by these methods is achieved at the cost of nutritional quality, texture, flavor, color or stability. Hence, biotechnological approaches were thought of.

2.6.3 Biotechnological Approaches :

2.6.3.1. Use of Immobilized Microbial Cells

A survey was made of microorganisms capable of metabolizing limonin to non-bitter compounds. Many investigators have found that several species of bacteria and fungi obtained from soil grow well on a medium containing limonin or limonoate as a single carbon source.

Nomura (1966) reported that *Aspergillus niger* and two species of *Penicillium* were capable of producing enzymes, which metabolize limonin and nomilin.

Activities of these enzymes were, however, very low and no metabolites could be isolated. In citrus fruits, limonoid content was found to decrease with advancing maturity (Higby, 1938; Maier *et.al.,* 1973, Scott, 1970). An isolate of *Arthrobacter globiformis*, grown on sodium-limonoate rich medium, secreted intracellular limonoate dehydrogenase (Hasegawa *et.al.,* 1972). It catalyzed the reversible conversion of limonoate to 17-dehydrolimonoate in presence of NAD.

A limonoid-metabolizing bacterium was isolated from soil and designated as *Pseudomonas* 321-18. This organism was seen to metabolize limonoate mainly through deoxylimonin. A limonoid-metabolizing enzyme, deoxylimonin hydrolase was isolated from cell-free extracts of these bacteria. This enzyme catalyses hydrolysis of deoxylimonin to form deoxylimonic acid and apparently attacks only the closed D ring of deoxylimonin (Hasegawa *et.al.,*1974b). The cell-free extracts also contained considerable amount of limonoate dehydrogenase activity, indicating the presence of another pathway involving 17-dehydrolimonoate. This enzyme was characterized as limonoate-NAD oxidoreductase (Hasegawa *et.al.,*1974).

A soil bacterium *Acientobactor* species was isolated by enrichment culture. It was capable of using limonin as a sole carbon source. Two non-bitter metabolites were isolated from the reaction mixture and identified as deoxylimonin and deoxylimonoic acid. The bacteria were immobilized in dialysis sacs and used for debittering early season juice. A total of 120 mg of bacteria was found to convert 1L of bitter early season orange juice to drinkable juice (Vaks and Lifshitz, 1981). A pathway was proposed for debittering limonin by the bacteria:

Limonin ⟶ Deoxylimonin ⟶ Deoxylimonoic acid

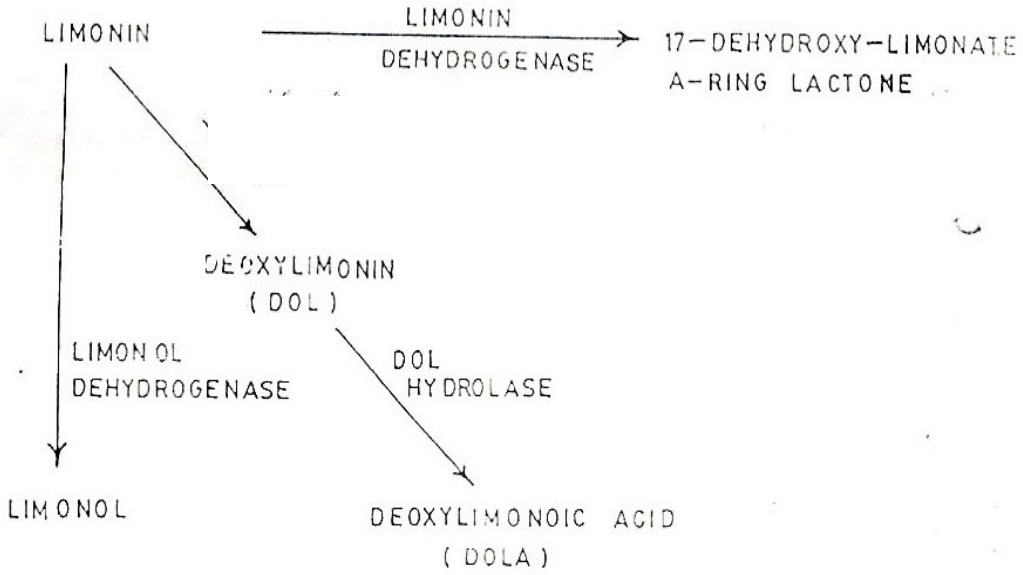


Fig. 3: Limonin metabolizing pathways in microorganisms

Another species of bacterium, capable of metabolizing limonin, was isolated from soil. This organism was isolated by enrichment of 3-furoic acid and identified as *Corynebacterium fascians* (Hasegawa and King, 1982). It metabolizes limonoids mainly through the 17-dehydrolimonoid pathway, but also utilizes a trans-19-hydroxybacunoic acid pathway (Hasegawa and Bennett, 1982).

Arthrobacter globiformis II, has been reported to metabolize bitter limonin of citrus juice to another non-bitter metabolite, limonol, when the juice is treated on a column packed with immobilized cells. On the basis of this information, a biological process that uses immobilized cells of this bacterium in acrylamide gel was developed for the reduction of the limonin bitterness of citrus juice sera (Hasegawa *et.al.,* 1983).

Another microorganism *Rhodococcus fascians* having constitutive enzymes was found to degrade limonin (Martinez-Madrid *et.al.,* 1989). The microorganism was entrapped in different matrices and evaluated. The gels were coated either with polyethylamine or cross linked with glutaraldehyde in lieu of coating. The coated system showed lower efficacy, doubtful stability, and unexpected diffusional

limitations vis-a-vis the cross-linked system. *Rhodococcus fascians* cells entrapped in κ-carrageenan have also been used for limonin degradation (Manjon *et.al.,* 1991). Their ability for continuous degradation of limonin as a function of pH showed optimal limonin degradation when pH was changed from 4.5 to 5. Recently, process parameters for the debittering of Kinnow mandarin juice by *R. facians* have been optimized by Marwaha *et.al.* (1994).

The comparative biochemical studies of limonoid metabolism in bacteria and citrus have led to substantial advances in the understanding of limonoid metabolism in citrus. Radioactive tracer technique showed the presence of both the limonoate dehydrogenase (Hasegawa *et.al.,*1974a) and limonin epoxidase (E-3) (Hasegawa *et.al.,*1980) activities in navel orange albedo tissues and calamonding leaves, respectively.

2.6.3.2 Enzymes Involved in Limonin Biotransformation

Limonin D-ring lactone hydrolase: It was found to be present in citrus by Maier *et.al.* (1969) as also in *Pseudomonas* 321-18 (Hasegawa,1976) and *A. globiformis* (Hasegawa *et.al.,*1982). This hydrolase catalyses the reversible lactonization-hydrolysis of open and closed D-ring of limonoids.

Limonate dehydrogenase: This enzyme is involved in the first step of 17-dehydrolimonoid metabolic pathway. It attacks OH group at 17-C of limonoid molecules and converts them to non-bitter 17-dehydrolimonoids (Hasegawa *et.al.,* 1972).

Limonin epoxiases: These enzymes catalyze the conversion of limonin to deoxylimonin. This enzyme only attacks the D-ring of limonoid (Hasegawa *et.al.,* 1974)

Deoxylimonin hydrolase: These enzymes catalyze the addition of water molecule to deoxilimonin, and has been isolated from *Pseudomonas* 329-18 (Hasegawa *et.al.,* 1974 b)

Deoxylimonic acid A-ring lactone hydrolase: This enzyme is involved in the third step of deoxylimonoid pathway, catalysing the hydrolysis of A-ring lactone of deoxilimonoate (Hasegawa *et.al.,* 1980).

Table 2: Microorganisms and various pathways involved in limonin Biotransformation

Microorganisms	**Pathways**	**Fruits**	**References**
Arthrobacter globiformis	17-dehydrolimonoids	Sodium limonoate (synthetic media)	Hasegawa *et.al.,* 1972
Pseudomonas 321-18	Deoxylimonoids, 17-Dehydrolimonoids	Grape fruit juice	Hasegawa *et.al.,* 1972, 1974
Bacterium 342-152-1	Deoxylimonoids, 17-Dehydrolimonoids	Grape fruit juice	Hasegawa and Kim 1975
Acinetobactor sp	Deoxylimonoids,	Orange juice	Vaks and Liftsitz, 1981
Arthrobacter globiformis11	Deoxylimonoids, 7-Hydrolimonoids	Orange juice	Hasegawa *et.al.,* 1983
Corynebacterium fascians	17-Dehydrolimonoids, trans-19-HBA	Citrus fruits	Hasegawa and King 1983 ; Hasegawa *et.al.,* 1985
Rhodococcus facians		Orange juice	Manjon *et.al.,* 1991; Martinez-madrid *et.al.,* 1989.

2.6.3.3 Immobilized Enzymes for Debittering

Naringinase, from *Asperigillus niger,* immobilized on copolymers of styrene and maleic anhydride has been used to hydrolyze naringin (Goldstein *et.al.,* 1971). In another case, naringenase immobilized on tannin aminohexylcellulose showed half life of 198 days at 25^{o}C and 88 days at 37^{o}C after debittering of citrus natsudaidai juice (Ono *et.al.,* 1978).

Naringinase from *Penicillium* sp. entrapped in cellulose triacetate showed higher K_m values than its soluble form (Tsen *et.al.,* 1989). The entrapped enzyme was used for hydrolysis of naringin, initially in a simulated fruit juice and subsequently in a real grapefruit juice.

2.6.3.4. Work done on Kinnow fruit in India

The quality of Kinnow juice is affected by the methods of juice extraction; five different methods of juice extraction as hydraulic pressing, hand reaming, using screw type juice extractor (SE), crushing without peel and crushing with peel have been evaluated. The juice yield was found to be in the range of 36.36 to48.40%. The Crushing with Peel method yielded high amount of juice and had the highest values for TSS, ascorbic acid, pectin, cloudiness, viscosity, and pulp content. The juice obtained from crushing with peel method was the most bitter and from hydraulic pressing was least bitter (Lotha and Khurdiya; 1994).

Table 3: Effect of methods of juice extraction on the physico – chemical composition of Kinnow mandarin juice (Lotha and Khurdiya; 1994).

Extraction Methods	TSS %	pH	Acidity %	pectin %	Viscosity CPU	density g/ml	ascorbic acid Mg/100ml	reducing sugars %	sucrose %	Total caro-tenoids mcg/100ml	b- carotene mcg/ 100ml
Hydraulic Pressing	10.45	3.50	0.79	0.34	4.84	1.00	18.48	4.38	3.70	6.38	2.16
Hand reaming	10.90	3.39	0.79	0.54	6.80	1.00	16.97	4.68	3.57	19.23	8.26
Using screw Type juice Extractor	10.70	3.54	0.74	0.97	19.05	1.02	18.30	4.18	3.99	18.07	6.01
Crushing Without peel	12.00	3.67	0.71	1.52	89.00	1.03	20.27	4.65	4.07	20.14	11.34
Crushing With peel	12.25	3.85	0.54	3.56	600.00	1.03	26.50	4.83	3.69	31.70	10.56

Premi *et al.* (1995) have studied the effect of five different debittering techniques on removal of bitter principle and quality of Kinnow juice, namely, raising pH, addition of sweetening agents, addition of β-cyclodextrin monomer for forming inclusion complexes of limonin, passing through adsorbent XAD-16 and conversion of bitter principles to non-bitter components in the juice by the action of immobilized bacteria (*Arthrobacter globiformis*). All these techniques led to reduction of limonin contents of treated juices as compared to control. The highest reduction in limonin and naringin content were recorded in juices treated with adsorbent XAD –16.

Though a lot of literature pertaining to the problem of bitterness in citrus juices is available, it is clear that not much work has been done on finding biotechnological solutions to the problem of bitterness in Kinnow juice till now. The present study was designed to address this problem specifically.

For this, *Pseudomonas putida* was the organism of choice. Since limonin is the component that is responsible for delayed bitterness in Kinnow juice, the study was carried out to see its transformation by *P. putida*. Also, since the ultimate objective is to remove limonin from Kinnow juice, Kinnow juice serum containing limonin and a host of other compounds was taken as a source of limonin in the growth medium of bacteria and limonin levels were monitored there in.

MATERIALS AND METHODS

3.1 Materials Required

Fresh, clean, healthy mid season Kinnows were bought from fruit shops of Patiala. Freeze-dried cultures of *Pseudomonas putida* (MTCC 1072) were bought from Institute of Microbial Technology, Chandigarh.

All solvents (chloroform, ethyl acetate, dichloromethane, and benzene) were from Merck. Limonin was from Sigma Chemical Company, USA. Silica gel GF245 and 4- dimethyl aminobenzaldehyde was from Hi Media Laboratory Limited, Mumbai. All other chemicals like (sodium bicarbonate, iron sulphate, potassium dihydrogen orthophosphate, etc) were of analytical grade.

3.2 Extraction of Juice:

Fresh Kinnows were cleaned and washed with water and fed as a whole in FMC extractor and juice was extracted and stored in screw capped reagent bottles. Autoclaved screw capped Scott Duran bottles were used for storing and processing the juice.

Immediately after extraction, juice was pasteurized by heating it at 95^{o}C for 5 min in boiling water bath and then cooling it rapidly at 0^{o}C. Juice pulp and juice supernatant (serum) was separated by centrifugation at 5000 rpm for 5 min. Pulp was discarded and serum was stored in aliquots of 500 ml at 4^{o}C till further use.

3.3 Analyses of Juice:

pH of juice (unpasteurized and pasteurized) was measured using digital pH meter and limonin content of juice was analysed by the method of Vaks and Liftshitz (1981).

3.3.1 Limonin Estimation:

Stock solution (1mg/ml) of limonin was prepared in either acetonitrile or chloroform. For preparing standard curve, different dilutions of limonin (0µl –

200µl) were drawn from limonin stock and volume was raised to 1ml using chloroform. To this, was added 1ml Ehrlich's reagent (Annexure – I).

After thoroughly mixing by vortexing, the reaction mixture was incubated at 27^{o}C for 30 min. The reddish pink colour so developed was read at 503nm using spectrophotometer and graph was plotted.

For estimation of limonin in juice serum, 1ml chloroform was added to 1ml of juice serum. Chloroform layer was separated and to this was added, 1ml of Ehrlich's reagent. The mixture was vortexed and incubated at 27^{o}C for 30 min. The reddish pink colour so developed was read at 503nm using spectrophotometer. Limonin content in the unknown sample was estimated using the standard curve.

3.4 Bacterial Cultures

For preparing the inoculum, *Pseudomonas putida* was grown in Luria broth at 35^{o}C with shaking at 150^{o}C for 24 hours. Subsequently, this culture was used as 1% inoculum in further experiments. Glycerol stocks of bacteria were also prepared by adding 135µl of glycerol to 865µl of *Pseudomonas putida* grown overnight and were stored at -80^{o}C.

3.5 Comparison of Different Carbon Sources Used in the Medium

Either glucose or limonin was used as the sole carbon source in the growth medium for *P. putida* and growth and limonin levels in the medium were monitored at different time points. For this, three flasks containing 30ml mineral salt medium- one with 30ppm limonin dissolved in chloroform, second with 0.2% glucose, and third flask which did not contain any carbon source, were inoculated with overnight grown *P.putida* cultures. These flasks were incubated at 35^{o}C at shaker speed of 130rpm. Then the comparison was drawn by monitoring the growth at 660nm, spectrophotometrically at different time intervals and growth curve was plotted. Also residual limonin was estimated in growth medium at these time intervals using method of Vaks and Liftshitz (1981) as described

earlier. Since limonin is soluble in organic solvents, a control containing same volume of either acetonitrile or chloroform was also put up and growth of bacteria was monitored.

3.6 Comparison of Different Sources of Limonin in the Growth Medium

Pure limonin and Kinnow juice serum were tested as a source of limonin in the growth medium. Growth as well as limonin levels in the medium were evaluated. To this end, three flasks containing 30ml mineral salt media- one flask having 30ppm limonin dissolved in acetonitrile, second flask containing 30ppm limonin dissolved in chloroform and third flask having juice serum containing equivalent amount of limonin were inoculated with 1% inoculum of overnight grown *P.putida*. These flasks were incubated at 35^{o}C at shaker speed of 130rpm. The growth was monitored at 660 nm spectrophotometrically, at different time intervals and growth curve was plotted. Also residual limonin was estimated as described earlier.

3.7 Optimization of Growth Conditions of *Pseudomonas putida* Showing Maximum Limonin Biotransformation

Using a typical experimental set up optimized the different growth conditions for maximum limonin biotransformation such as temperature, pH, and duration of time. 30 ml of mineral salt medium with juice serum containing 30ppm of limonin was inoculated with 1% inoculum grown for 24 hours at a speed of 130rpm. Growth as well as limonin content in terms of residual limonin content of growth medium was monitored as a function of time keeping other parameters constant.

3.7.1 Effect of Temperature:

To four flasks each containing 30ml mineral salt media with juice serum added 1% inoculum of overnight grown *P.putida* culture. These flasks were then incubated at four different temperatures ranging from (25^{o}C-40^{o}C) with a five-degree increment. Then by monitoring the growth at 660 nm. spectrophotometrically drew comparison at different time intervals and growth

curve was plotted. Residual limonin was also estimated by the method described above(Vaks and Liftshitz, 1981).

3.7.2 Effect of pH:

Culture of *P. putida* was grown in mineral salt media containing juice serum at four different pH ranging (3.0,5.0,7.0,9.0) at 35^{o}C at shaker speed of 130rpm. Then comparison was drawn by monitoring the growth at 660 nm. spectrophotometrically at different time intervals growth curve was plotted. Also residual limonin was estimated using the method given by Vaks and liftshitz (1981).

3.7.3 Comparison of Type of Growth Medium

Two different types of media- one a rich medium containing standard sources of carbon and nitrogen and other is mineral salt medium were compared for growing *P.putida* to monitor limonin biotransformation. For this 1% inoculum of *P. putida* was added to 30ml of both media 30ppm limonin. These cultures were incubated at 35^{o}C at 130rpm. Then comparison was drawn by monitoring the growth at 660 nm. spectrophotometrically at different time intervals growth curve was plotted. Also residual limonin was estimated using the method given by Vaks and Liftshitz (1981).

3.7.4 Effect of Time:

All the parameters discussed above were studied for different time points ranging (0, 12, 24, 48, 72, 96, 120). Then growth and limonin content is estimated at these time points and graphs were plotted according to methods given above.

3.5 Identification of Metabolites Formed:

Using silica gel G plates for TLC did identification of metabolites

For TLC first of all sample preparation was done.

Sample Preparation

500ml culture was centrifuged to remove cells and supernatant was taken for sample preparation for TLC. After adjusting pH to2 with 1N HCl, it was boiled in boiling water bath for 5min. Then this sample was taken in separating funnel and extracted two times with 300ml chloroform used each time. This chloroform layer was pooled completely and evaporated at 40^{o}C by rotary vacuum evaporator. The completely dried sample was re-dissolved in 100ml dichloromethane (neutral fraction) and taken in separating funnel and was extracted with 50ml $KHCO_3$. Then dichloromethane layer was separated from potassium bicarbonate layer. Both the layers were proceeded separately. Dichloromethane layer was given washing with distilled water so as to remove acid. Then anhydrous calcium chloride was added to remove moisture in the layer, and let it stand for 10min. After keeping it for some time the solution was filtered with pre-wetted filter paper and then evaporated at 35^{o}C by rotary vacuum evaporator.

Potassium bicarbonate layer was taken in another tube and pH was adjusted to 2 with 1N HCl. Then this layer was further extracted with 100ml Ethyl acetate (acidic fraction). Ethyl acetate layer was given washing with distilled water to remove acid. Then anhydrous calcium chloride was added to remove extra moisture and let it stand for 10min. After keeping it for some time the solution was filtered with pre-wetted filter paper and then evaporated at 35^{o}C by rotary vacuum evaporator. The two layers are ready to perform Thin Layer Chromatography.

Thin Layer Chromatography

Glass TLC plates were coated with silica gel GF245. Preparative TLC was performed on 0.5mm silica gel coated plates. Analytical TLC was carried out using 0.2mm silica gel coated plates. Plates were activated at 90^{o}C for half an hour. Samples were spotted on the plate with help of micropipette and dried for some time before developing. Preliminary studies were done with cyclohexane-ethylacetate (50:50). Plate was run until the solvent was about one centimeter

below the top of the plate. The spots were revealed by visualizing the plates under short wave U.V. light followed by spraying with Ehrlich's reagent and exposing to warm HCl gas. Distance of spots from origin was noted and R_f value of each was calculated by dividing it by the distance of the solvent from origin.

The compounds, which were visualized with preparative TLC were isolated from TLC plates and checked for purity with analytical TLC using 0.2mm silica gel coated plates. To identify the compounds, these were subjected to TLC in different solvent systems:

1. Cyclohexane-Ethylacetate (50:50).

2. Ethanol

3. Benzene-ethanol-water-acetic acid (200:47:15:1)

U.V. spectra of these compounds were also taken. For this 2mg of sample was dissolved in 2ml of solvent and spectrum was taken from 190nm to 400nm.

RESULTS AND DISSCUSION

Kinnow is the hybrid of *citrus nobilis* and *citrus delicosa*. It is known for its superior characters such as heavy bearing, wide adaptability and fruit quality. Cultivation of Kinnow has assumed a considerable significance during recent years in northern India. This has emerged as one of the main crops of Punjab. Although it has lots of advantages yet its processing industry has not made much progress.

The major problem encountered by the industry is that of development of bitterness in Kinnow juice with time. Bitterness in Kinnow juice is basically of two types-one that is present initially when juice is extracted and other that develops with time called ‘’delayed bitterness”. Two different compounds are responsible for these two types of bitterness: naringin for ‘’bitterness” and limonin for ‘’delayed bitterness.”

Several methods have been tried to reduce bitterness in juices from citrus fruits such as raising pH of juice (Renote and Bains, 1982), suppression of bitterness by addition of sweetening agents (Guadagni *et.al.,*1974).addition of β-cyclodextrin monomer for forming inclusion complexes of limonin (Konno *et.al.,* 1981), use of adsorbent XAD-16 (Wilson *et.al.,*1989), and conversion of bitter principles to non-bitter components in the juice by the action of immobilized bacteria (Hasegawa *et.al.,* 1983). All these studies are directed towards solving problem of bitterness in grapefruit juice, orange juice and other citrus juices. Though Puri *et.al.,* (1996) have tried immobilized *R. fascians* cells for the purpose, to the best of our knowledge, no other study has been reported using bacterial enzymes for debittering Kinnow juice,. The study in hand is an attempt to fill this lacuna.

Towards this end, *P.putida* (MTCC 1072) was the microorganism of choice as other *Pseudomonas* species have been shown to metabolize limonin and have been proposed to degrade limonin present in citrus juices other than Kinnow juice earlier. But no report is available in literature showing the use of *P. putida* (MTCC 1072) for debittering Kinnow juice.

Biotransformation of limonin was studies in two ways- first using pure limonin as the sole carbon source in the growth medium and secondly using Kinnow juice serum as source of carbon and limonin. The first part was necessary to understand the exact metabolic pathway by which limonin is transformed by *P.putida,* while the second part was carried out to see if the pathway is still applicable to Kinnow juice as a whole and not limonin alone.

4.1 Analysis of Juice

4.1.1 pH of Juice :

pH of unpasteurized and pasteurized juice was analyzed by using pH meter. Not much difference was observed in pH of pasteurized (3.58) and unpasteurized (3.25) juice.

4.1.2 Limonin Content:

To determine the effect of time on limonin content and maximum limonin levels in juice serum stored at $4^{o}C$, Limonin content was monitored at different time intervals. Table 4 and Fig.4 summarize the data showing development of bitterness in juice with time in terms of its limonin content in ppm. Limonin level in 0 hours was found to be 2.5ppm. It increased rapidly in first 72 hours after juice extraction till it reached a maximum of 60ppm after which it becomes nearly constant. Since the maximum increase in limonin level occurred with in first 12 hours after extraction and reached 30ppm, this was the limonin content taken in growth medium in all-subsequent experiments.

4.2 Comparison of Different Carbon Sources Used in Growth Medium

To determine the influence of the type of carbon source in the medium on the growth of *P.putida* and limonin biotransformation by *P. putida*, $O.D._{660}$ as well as residual limonin levels in the growth medium were monitored at different time points with either glucose or pure limonin as a sole carbon source. One set of experiment was also put up without any carbon source. Since limonin is soluble

Table 4: Limonin content in juice

Time (hours)	Limonin (ppm)
0	2.5
1	6
3	15
6	22
12	30
24	41
48	53
72	60
96	58
120	59
144	60
168	59
2 weeks	58.9
4weeks	59.8
8 weeks	58
12 weeks	60

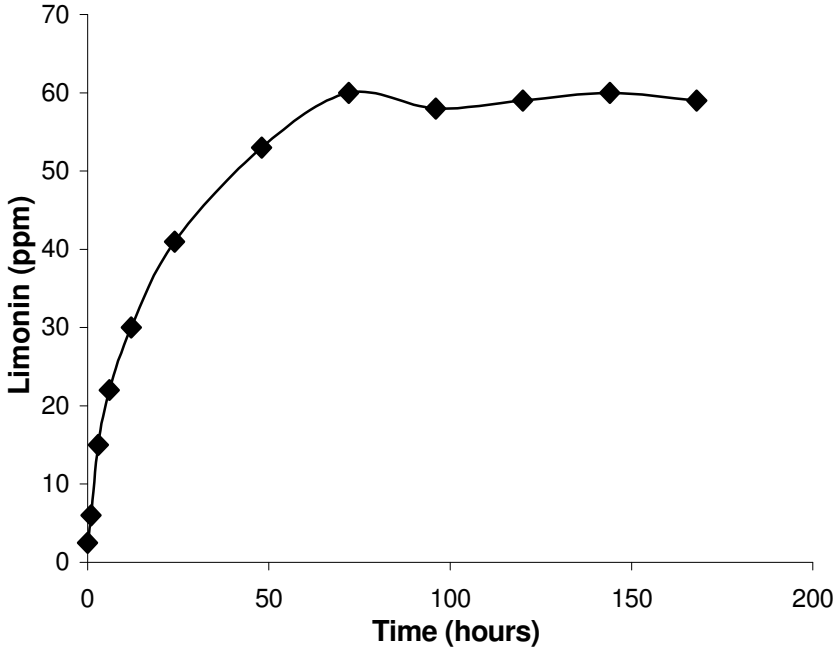


FIG. 4: Limonin content in juice

maximally in acetonitrile followed by chloroform, therefore, to see the effect of these organic solvents on the growth of *P. putida*, another set up with medium containing either of these organic solvents along with glucose as the carbon source was also used.

Effect on growth: Table 5 and Fig. 5 show the growth of *P. putida* after different time intervals with different carbon sources. As expected, negligible change in $O.D._{660}$ was observed in case of *P. putida* grown in mineral salt medium containing no carbon source. Growth of *P. putida* was observed to follow a typical growth curve involving lag phase, log phase and stationary phase in mineral salt media containing limonin dissolved in chloroform or acetonitrile and mineral salt medium containing 0.2% glucose as a sole carbon source. Maximum absorbance at 660nm was 1.113 in mineral salt media containing 0.2% glucose followed by mineral salt media containing limonin dissolved in chloroform or acetonitrile. Comparison of growth of *P. putida* in medium containing 0.2%glucose with or without solvent showed lesser $O.D._{660}$ in case of growth media containing an organic solvent (chloroform or acetonitrile) as shown in fig.6. Growth of *P. putida* was observed to follow a typical growth curve involving lag phase, log phase and stationary phase when no solvent was added in growth medium containing 0.2% glucose.

Transformation of limonin as a function of carbon source: Standard curve was prepared by taking varying conc. (0 μg to 200 μg) of standard limonin solution (1mg/ml in chloroform or acetonitrile). From this standard curve values of samples were determined. Table 6 and Fig. 7 summarize the data on residual limonin levels in the growth medium with different carbon sources at different time intervals. Limonin levels were found to be negligible in mineral salt medium without any carbon source and in that containing 0.2% glucose.

In case of *P.putida* grown in mineral salt medium with limonin dissolved in chloroform, a gradual and significant reduction in limonin level in growth medium was observed with time. Maximum reduction in limonin levels was seen to be

Table 5: Growth of *Pseudomonas putida* in mineral salt medium containing different carbon sources

Time (hours)	O.D. 660			
	No carbon source	Glucose	Limonin in chloroform	Limonin in acetonotrile
0	0.032	0.032	0.034	0.008
12	0.023	0.077	0.038	0.02
24	0.023	0.272	0.034	0.045
48	0.023	0.618	0.085	0.056
72	0.023	0.696	0.101	0.08
96	0.023	0.718	0.103	0.128
120	0.023	1.113	0.06	0.101

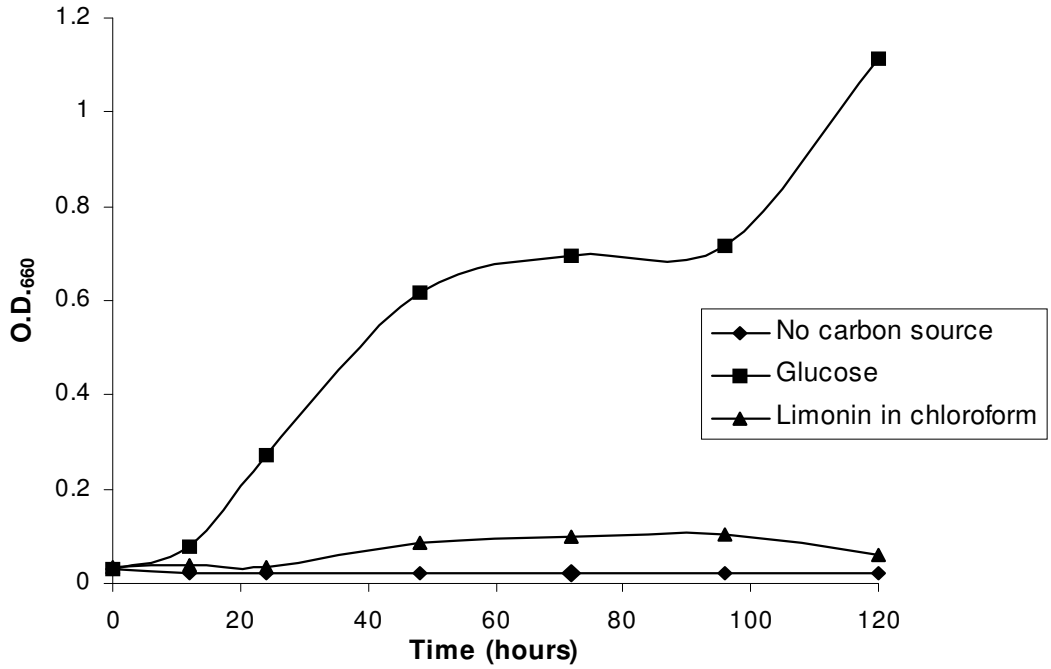


FIG.5:Comparison of growth of *P.putida* in mineral salt medium with different carbon sources

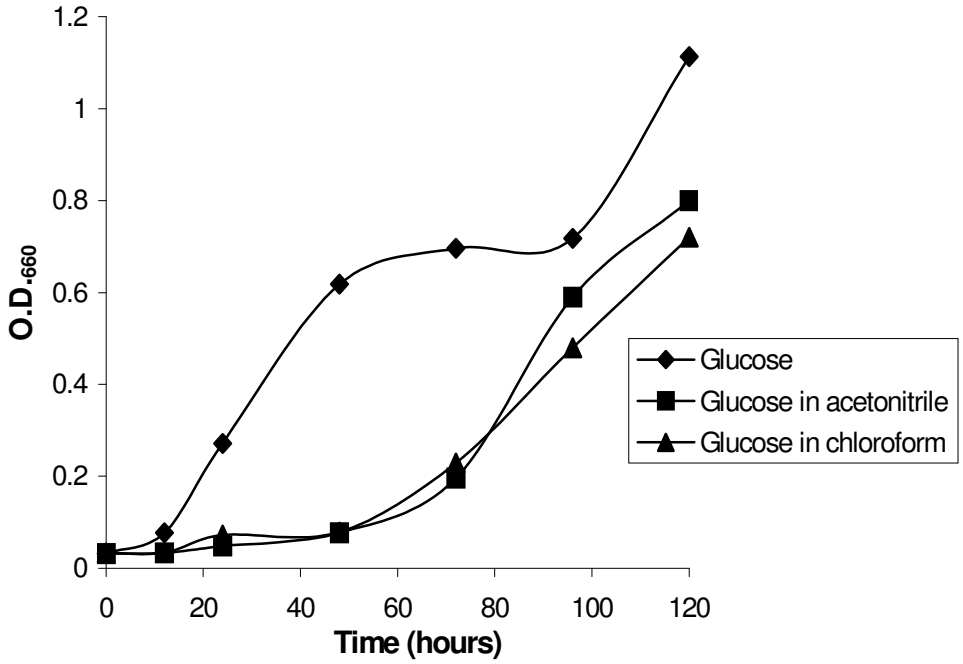


FIG.6 Comparison of growth of *P.putida* in glucose alone or with either chloroform or acetonitrile

Table 6: Limonin biotransformation activity of *Pseudomonas putida* in mineral salt medium containing different carbon sources

Time (hours)	Residual Limonin (ppm)			
	No carbon Source	Glucose	Limonin in chloroform	Limonin in acetonitrile
0	0.005	0.005	28.12	30.4
12	0.004	0.005	28	25
24	0.005	0.004	25	28
48	0.004	0.005	17.5	26.1
72	0.005	0.005	15.31	29.1
96	0.005	0.004	11.18	27.2
120	0.005	0.004	6.83	28

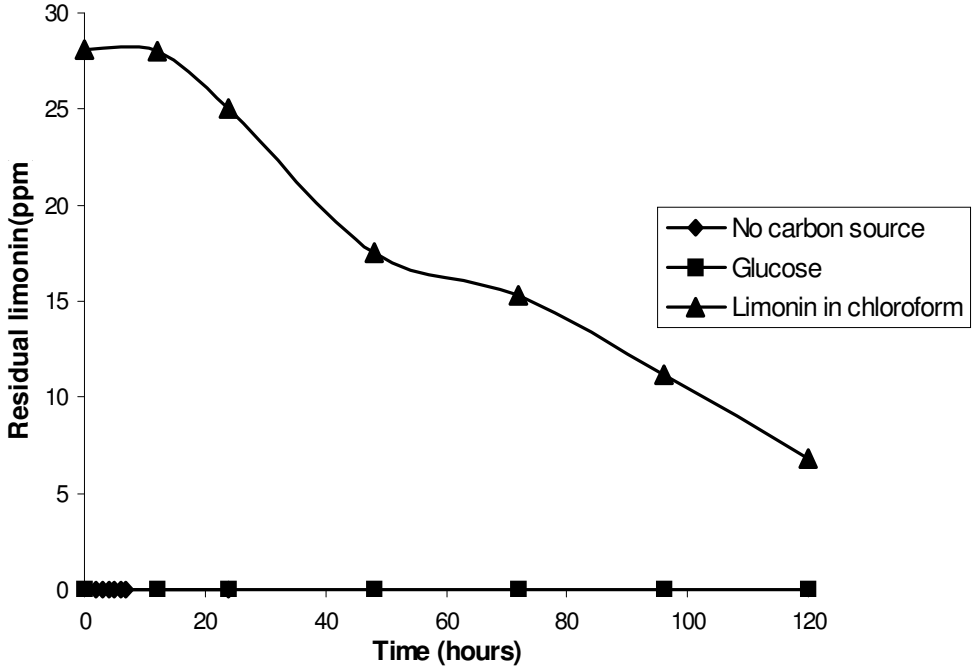


FIG. 7 : Comparison of residual limonin in medium containing different carbon sources

Table 7: Growth and limonin biotransformation activity of *Pseudomonas. putida* in mineral salt medium containing Juice serum.

Time (hours)	$O.D._{660}$	Residual limonin	% reduction in
0	0.059 ± 0.0005	25.2 ± .057	0
12	0.4 ± 0.038	23.6 ± 0.038	6.3
24	1.35 ± 0.025	19.46 ± 0.025*	23
48	1.99 ± 0.142	16.61 ± 0.07***	34.08
72	1.97 ± 0.27	14.64 ± 0.11***	41.9
96	1.974 ± 0.026	13.3 ± 0.037***	47.22
120	1.945 ± 0.26	10.09 ± 0.03***	59.96

Values are Mean ± SEM of triplicates.
* P< 0.05, *** p< 0.001

approximately 75% after 120 hours of treatment. In case of mineral salt medium with limonin dissolved in acetonitrile, residual limonin levels remained close to the initial levels observed in this case. This showed a correlation between growth and limonin transformation activity of cells under these conditions. It could be that acetonitrile being a strong organic solvent affects the viability of cells, consequently adversely affecting limonin biotransformation activity of cells. These results also show that *P. putida* is able to utilize limonin as the carbon source in the growth medium.

4.3 Comparison of Different Sources of Limonin in Growth Medium

The effect of source of limonin on the growth and limonin biotransformation activity of *P. putida* was investigated. The growth as well as residual limonin levels in the growth medium were monitored either in growth medium with pure limonin (30 ppm) or in medium with juice serum containing equivalent amount of limonin.

Effect on growth: Tables 5 and 7 and Fig. 8 show the growth of *P. putida* in medium containing either limonin dissolved in chloroform or juice serum. Negligible change in $O.D._{660}$ was observed in case of medium with limonin dissolved in chloroform. On the other hand, growth of *P. putida* was observed to follow a typical growth curve involving lag phase, log phase and stationary phase in medium containing juice serum. A maximum absorbance of 1.805 was observed after 72 hours.

Transformation of limonin: Tables 6 and 7 and Fig.9 summarize the data on residual limonin levels in the growth medium containing different sources of limonin from 0 hours to 120 hours. Residual limonin levels show significant reduction in medium containing limonin dissolved in chloroform as well as in case of medium with juice serum ($p<0.001$). Maximum reduction in limonin (75%) was observed with pure limonin dissolved in chloroform whereas approx. (60%) Values are Mean ± SEM of triplicates.

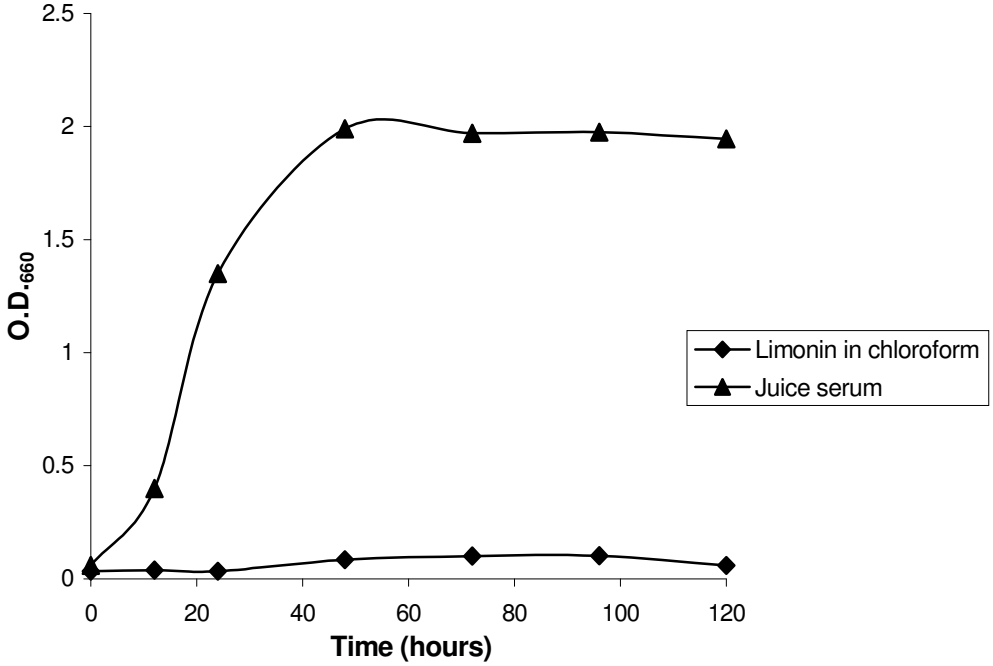


FIG. 8 : Comparison of growth of *P.putida* in mineral salt medium containing different source of limonin

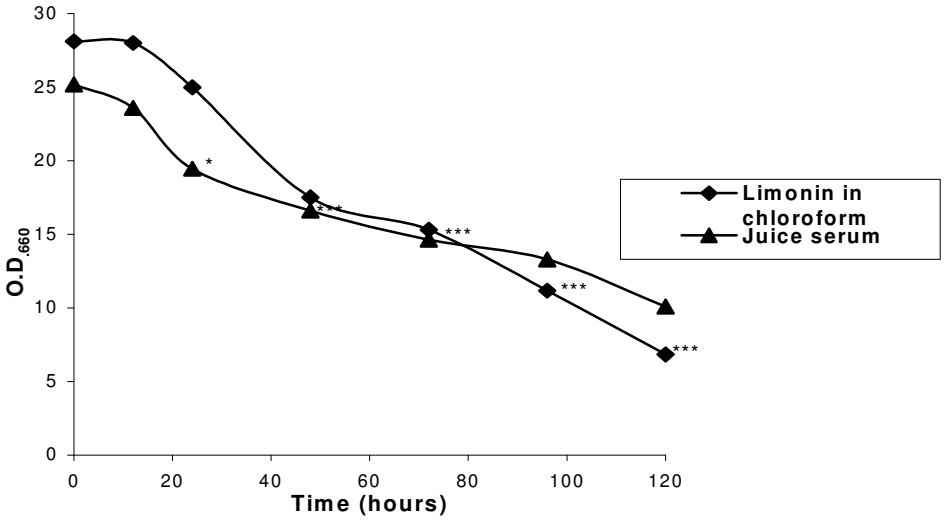


FIG.9: Comparison of limonin biotransformation activity of *P.putida* in mineral salt medium containing different sources of limonin

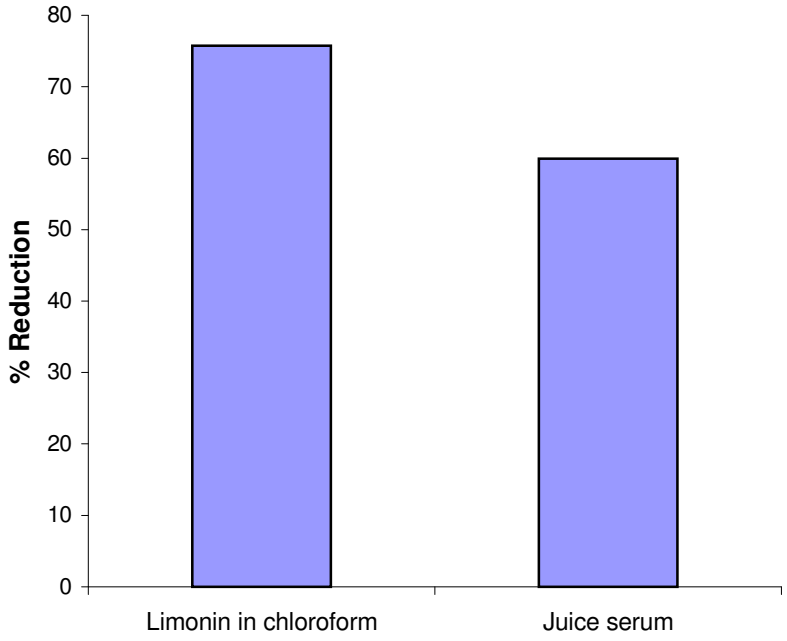


FIG.10: Comparison of % reduction of limonin in mineral salt media with different sources of limonin

Table 8: Growth and limonin biotransformation activity of *Pseudomonas putida* at pH 3.

Time (hours)	$O.D._{660}$	Residual limonin(ppm)	% reduction in limonin levels
0	0.233	30.5	0
12	0.236	30	1.63
24	0.236	29	4.91
48	0.242	30	1.63
72	0.24	29	4.91
96	0.242	28.7	5.91
120	0.25	30.1	1.31

reduction was achieved after 120 hours in case of medium with juice serum (Fig. 10).

A comparison of growth of *P.putida* in the two media- one containing pure limonin in chloroform, and the other containing juice serum having equivalent amount of limonin revealed that the growth of *P.putida* was better in medium containing juice serum. Juice serum contains a host of carbon sources apart from limonin, which might be responsible for better growth. On the other hand, organic solvent might be adversely affecting the viability of bacterial cells. Moreover, since ultimately a solution is to be found for limonin present in juice serum, therefore, juice serum was a preferred source of limonin in the subsequent experiments.

4.4 Effect of pH

To determine the influence of pH on the growth and limonin biotransformation activity of *P. putida*, $O.D._{660}$ as well as residual limonin levels in the growth medium were monitored over the pH range (3.0, 5.0, 7.0, 9.0). Optimum pH was considered to be the one at which maximum limonin biotransformation was observed. The study at higher pH than 9.0 was not possible because of precipitation in the growth medium at pH higher than this.

Effect on growth: Tables 8 to11 and Fig.11 show the growth of *P. putida* and residual limonin levels in the growth medium after different time intervals at different pH. Negligible change in $O.D._{660}$ was observed at pH 3.0. Growth of *P. putida* was observed to follow a typical growth curve involving lag phase, log phase and stationary phase at pH 5.0, 7.0 and 9.0. The absorbance at 660nm was maximum at pH 9.0 followed by pH 5.0.

Transformation of limonin as a function of pH: Tables 8 to11 and Fig.12 summarize the data on residual limonin levels in the growth medium at different pH from 0 hours to 120 hours. Residual limonin levels did not decrease appreciably at pH 3.0. This correlates well with the $O.D._{660}$ values at this pH. However, significant reduction in limonin levels was observed in the middle of the

9: Growth and limonin biotransformation activity of *Pseudomonas putida* at pH 5

Time (hours)	$O.D._{660}$	Residual limonin(ppm)	% reduction in limonin levels
0	0.059 ± .0005	25.2 ± .057	0
12	0.4± 0.038	23.6 ± 0.038	6.3
24	1.35 ± 0.025	19.46 ± 0.025*	23
48	1.99 ± 0.142	16.61 ± 0.07***	34.08
72	1.97 ± 0.27	14.64 ± 0.11***	41.9
96	1.974 ± 0.026	13.3 ± 0.037***	47.22
120	1.945 ± 0.26	10.09 ± 0.03***	59.96

Values are Mean ± SEM of triplicates.
* P< 0.05, *** p< 0.001

Table 10: Growth and limonin biotransformation activity of *Pseudomonas putida* at pH 7

Time (hours)	$O.D._{660}$nm	Residual limonin(ppm)	% reduction in
0	.016 ± .005	25.5 ± 0.04	0
12	0.112 ± 0.027	20.83 ± 0. 61*	18.31
24	1.41 ± 0.022	19.4 ± 0.07***	23.92
48	2.1 ± 0.017	18.7 ± 0.04***	26.66
72	2.083 ± 0.029	15.8 ± 0.22***	38.03
96	2.089 ± 0.0073	13.5 ± 0.023***	47.05
120	2.02 ± 0.007	11.5 ± 0.13***	54.9

Values are Mean ± SEM of triplicates.
* P< 0.05, *** p< 0.001

Table 11:Growth and limonin biotransformation activity of *Pseudomonas putida* at pH9

Time (hours)	$O.D._{660}$nm	Residual limonin(ppm)	% reduction in limonin levels
0	.014 ± .0045	34.99 ± 0.76	0
12	0.51 ± 0.063	34.05± 0.84	1.96
24	1.11± 0.025	34.05 ± 0.25	2.01
48	1.8 ± 0.087	32.05 ± 0.44	6.42
72	1.85 ± 0.029	30.97 ± 0.63	10.9
96	1.823± 0.058	29.44 ± 0.34*	15.2
120	1.801 ± 0.021	25.25 ± 0.45**	27.2

Values are Mean ± SEM of triplicates.
* P< 0.05, *** p< 0.01

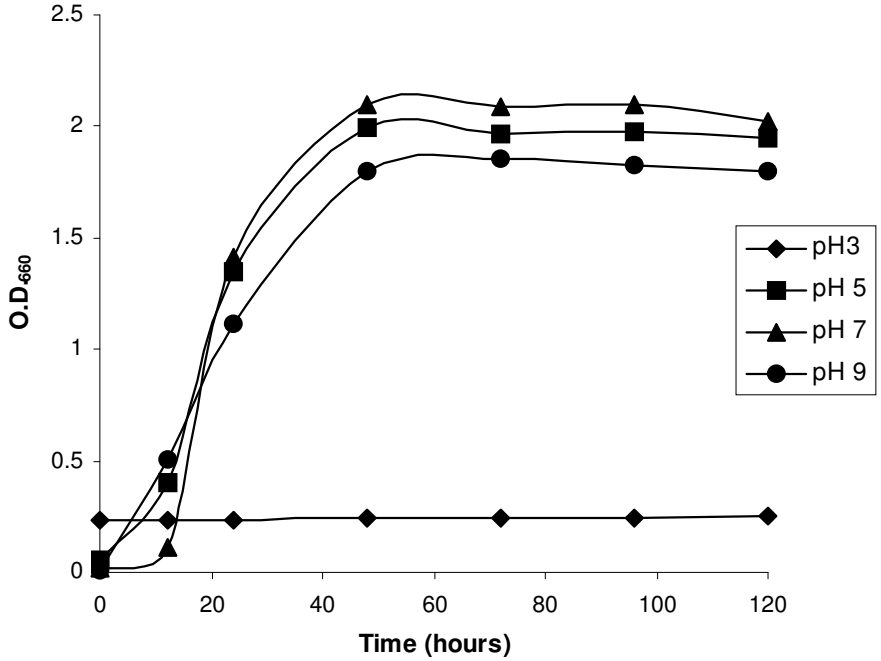


FIG.11 : Comparison of growth of *P.putida* at different pH

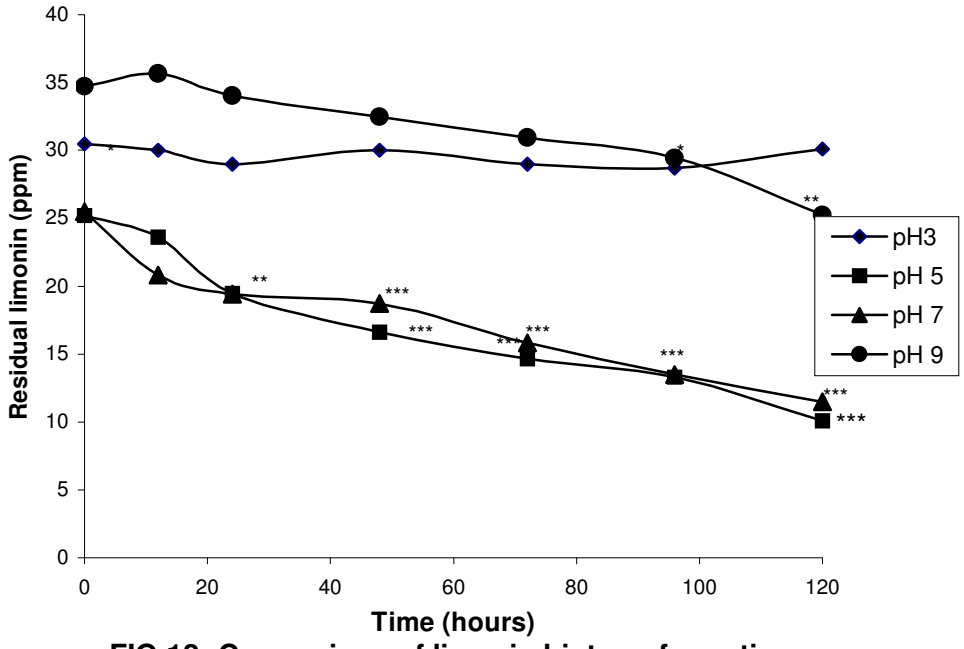


FIG.12: Comparison of limonin biotransformation activity of *P.putida* at different pH

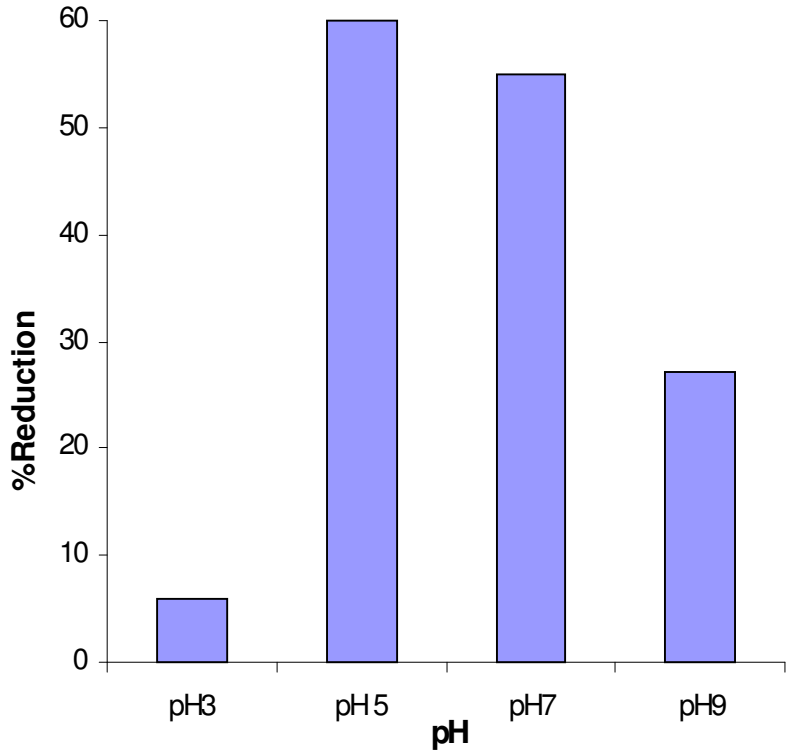


FIG.13 : Comparison of %reduction of limonin at different pH

Table 12:Growth and limonin biotransformation of *Pseudomonas putida* at 25°C

Time (hours)	O.D.$_{660}$nm	Residual limonin(ppm)	% reduction in limonin levels
0	0.059 ± 0.053	25.2 ± .043	0
12	0.098± 0.063	17.4 ± 0.055***	30.92
24	0.8 ± 0.0098	18.1 ± 0.263**	28.1
48	1.45± 0.007	17.8 ± 0.07***	29.36
72	1.4 ± 0.097	17.75 ± 0.21**	29.5
96	1.35 ± 0.097	16.2 ± 0.283***	35.7
120	1.32 ± 0.003	16 ± 0.25***	36.2

Values are Mean ± SEM of triplicates.

**P< 0.01, *** p< 0.001

pH range, that is at pH 5.0 ($p<0.001$), pH 7.0 ($p<0.001$) and pH 9.0 ($p<0.01$). Maximum reduction in limonin (60%) was observed at pH 5.0 (Fig. 13).

pH plays an important role in limonin biotransformation. Our results of better limonin conversion at this pH are corroborated by other reports also wherein reduction in limonin levels has been shown to increase when medium pH was changed from 4.5 to 5.0 (Manjón *et. al*., 1991; Marwaha *et al*., 1994). The results of this study also indicate towards the presence of an enzyme in the system that is capable of reducing limonin levels efficiently at lower pH. Since maximum reduction in limonin along with appreciable growth was achieved at pH 5 and since this pH is the nearest to that of Kinnow juice (3.5- 4.0), pH of the growth medium was kept at 5.0 in subsequent experiments.

4.5 Effect of Temperature

To determine the influence of temperature on the growth of *P.putida* and its limonin biotransformation activity, its growth as well as residual limonin levels in the growth medium were monitored over the temperature range 25°C- 40°C.

Effect on growth: Tables 12 to 15 and Fig. 14 show the growth of *P. putida* after different time intervals at different temperatures (25°C, 30°C, 35°C, 40°C). Growth of *P. putida* was seen to follow a typical growth curve involving lag phase, log phase and stationary phase at all of these temperatures. But absorbance at 660nm was maximum at temperature 35°C followed by that at 30°C.

Transformation of limonin as a function of temperature: Tables 12 to 15 and Fig. 15 summarize the data on residual limonin levels in the growth medium at different temperatures from 0 hours to 120 hours. Residual limonin levels did not decrease appreciably at temperatures 25°C, and 40°C, though significant reduction of limonin was observed at 30°C ($p<0.001$) and 35°C ($p<0.001$). A maximum reduction of 60% in limonin levels was observed at 35°C (Fig.16).

Table 13:Growth and limonin biotransformation of *Pseudomonas putida* at 30°C

Time (hours)	O.D.$_{660}$nm	Residual limonin(ppm)	% reduction in limonin levels
0	0.059 ± 0.0005	25.2 ± 0.098	0
12	0.25 ± 0.027	25.6 ± 0.69	-1.58
24	0.85 ± 0.022	24.2 ± 0.34	3.96
48	1.5 ± 0.017	24 ± 0.508	4.76
72	1.52 ± 0.029	21.13 ±0.45*	16.1
96	1.505 ± 0.097	18.7 ± 1.26*	25.6
120	1.51 ± 0.07	13.92 ± 0.32***	44.74

Values are Mean ± SEM of triplicates.
* P< 0.05, *** p< 0.001

Table 14: Growth and limonin biotransformation of *Pseudomonas putida* at 35°C

Time (hours)	O.D.$_{660}$	Residual limonin(ppm)	% reduction in limonin levels
0	0.059 ± .0005	25.2 ± .057	0
12	0.4± 0.038	23.6 ± 0.038	6.3
24	1.35 ± 0.025	19.46 ± 0.025*	23
48	1.99 ± 0.142	16.61 ± 0.07***	34.08
72	1.97 ± 0.27	14.64 ± 0.11***	41.9
96	1.974 ± 0.026	13.3 ± 0.037***	47.22
120	1.945 ± 0.26	10.09 ± 0.03***	59.96

Values are Mean ± SEM of triplicates.
* P< 0.05, *** p< 0.001

Table 15:Growth and limonin biotransformation of *Pseudomonas putida* at 40°C

Time (hours)	O.D.$_{660}$nm	Residual limonin(ppm)	% reduction in limonin levels
0	0.059 ± .0005	25.2 ±0.068	0
12	0.112 ± 0.009	24.8 ± 0.052	1.58
24	0.5 ± 0.008	21.13 ± 0.048***	16.15
48	1.01 ± 0.125	19 ± 0.036***	24.6
72	1.1 ± 0.008	18.01 ± 0.15***	30.9
96	1.01 ± 0.004	17.3 ± 0.023***	31.3
120	0.97 ± 0.0023	17.03 ± 0.011***	32.4

Values are Mean ± SEM of triplicates.
*** P< 0.001

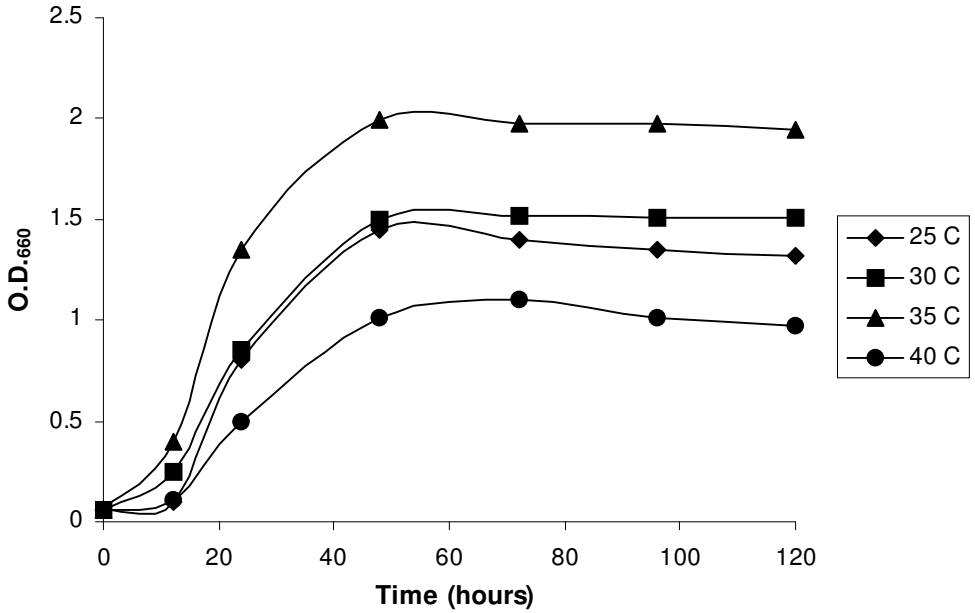


FIG.14: Comparison of growth of *P.putida* at different temperatures.

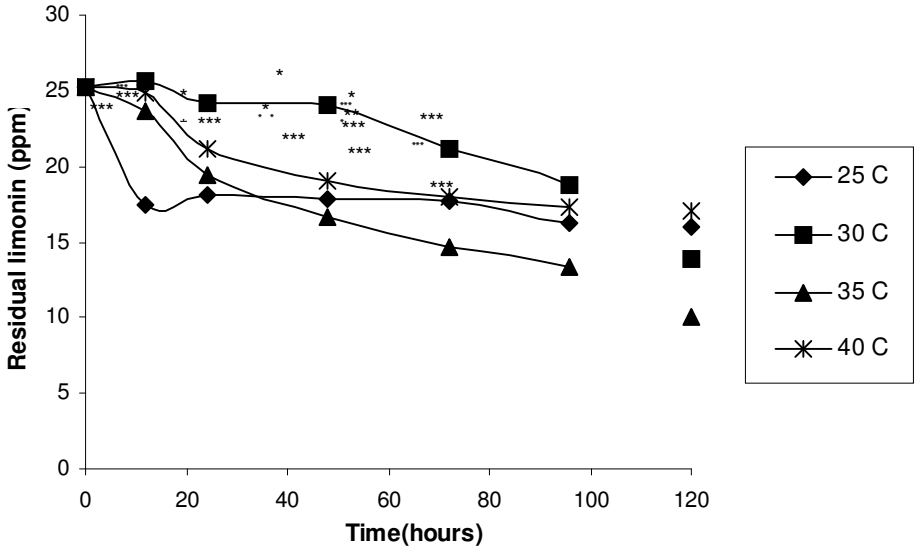


FIG. 15 : Comparison of limonin bitransformation activity of *P.putida* at different temperature

Values are Mean ± SEM of triplicates.
* P< 0.05, ** p < 0.01, *** p< 0.001

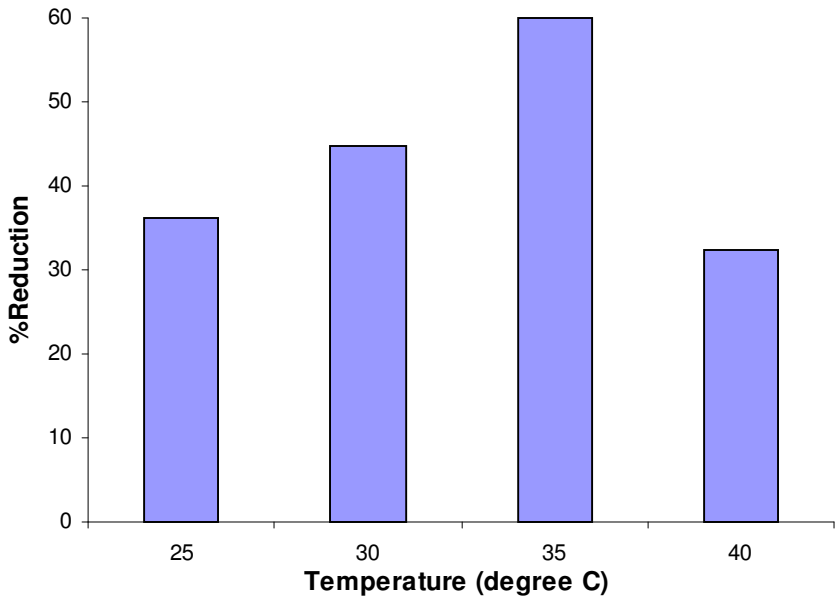


FIG.16:Comparison of % reduction of limonin at different temperature

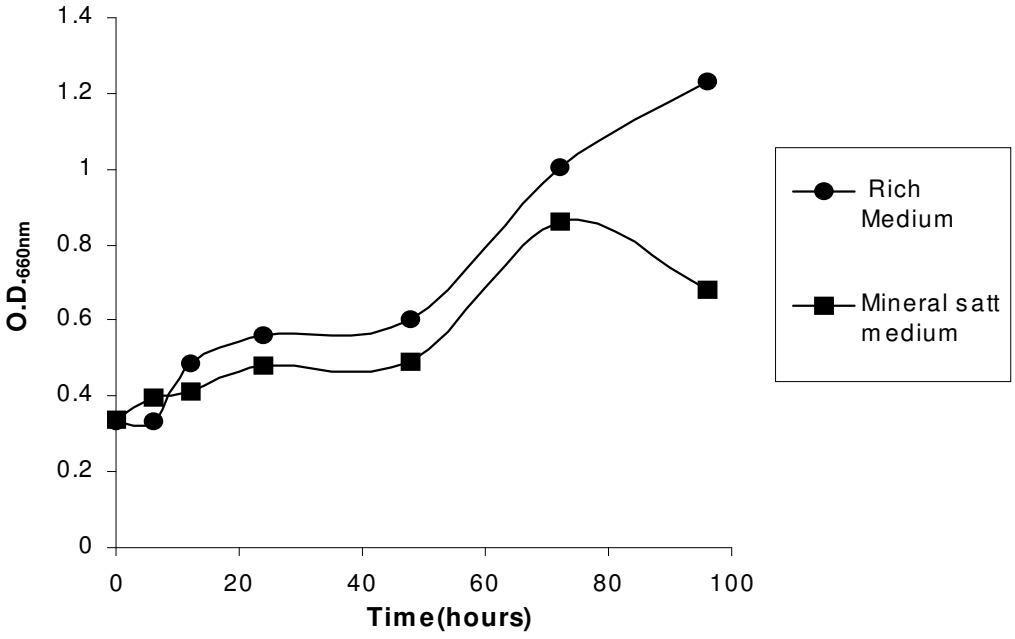


FIG.17 : Comparison of growth of *P.putida* in different media at pH 5

Since a typical growth curve was observed at 35^{o}C and maximum reduction of limonin was achieved at this temperature, therefore, in further experiments, incubation temperature was kept at 35^{o}C.

4.6 Effect of Time Interval

To determine the influence of duration on the growth and limonin biotransformation activity of *P. putida*, growth as well as residual limonin levels in the growth medium were monitored with different parameters (pH, temperature, medium, etc) at different time intervals.

Effect on growth: Tables 8 -15 showed the growth of *P.putida* in the growth medium after different time intervals under varying conditions of pH and temperature. Growth of *P. putida* was observed to reach maximum after a period 24 to 48 hours in most of the cases.

Transformation of limonin: The data on residual limonin levels in the growth medium from 0 hours to 120 hours shows that residual limonin levels did not decrease appreciably till 24 hours. A significant reduction was observed from 48 hours to 120 hours, maximum reduction in limonin being 75% in case of medium with pure limonin and 60% in case of medium with juice serum after 120 hours.

4.7 Effect of Media

To determine the influence of the type of medium used on the growth and limonin biotransformation activity of *P. putida*, it was grown in two different media- a rich medium containing a host of nutrients and a mineral salt medium containing only serum as the carbon source. Growth as well as residual limonin levels in the different growth medium were monitored.

Effect on growth: Fig. 17 showed the growth of *P. putida* at 35^{o}C and pH 5 in two different growth medium after different time intervals. Growth of *P. putida* was observed to follow a typical growth curve in both the media. Absorbance at

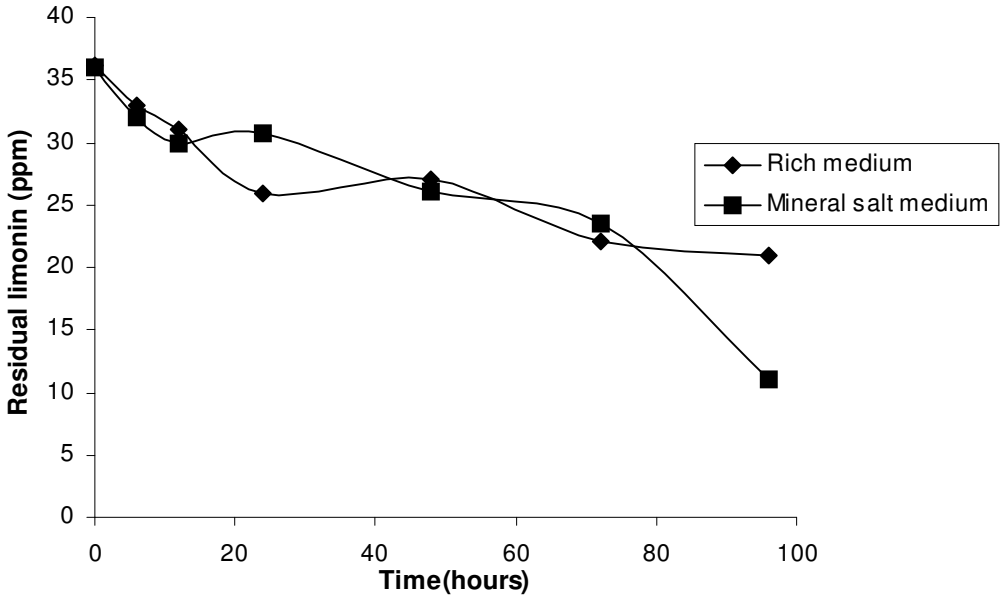


FIG.18:Comparison of limonin biotransformation activity of *P.putida* in different media

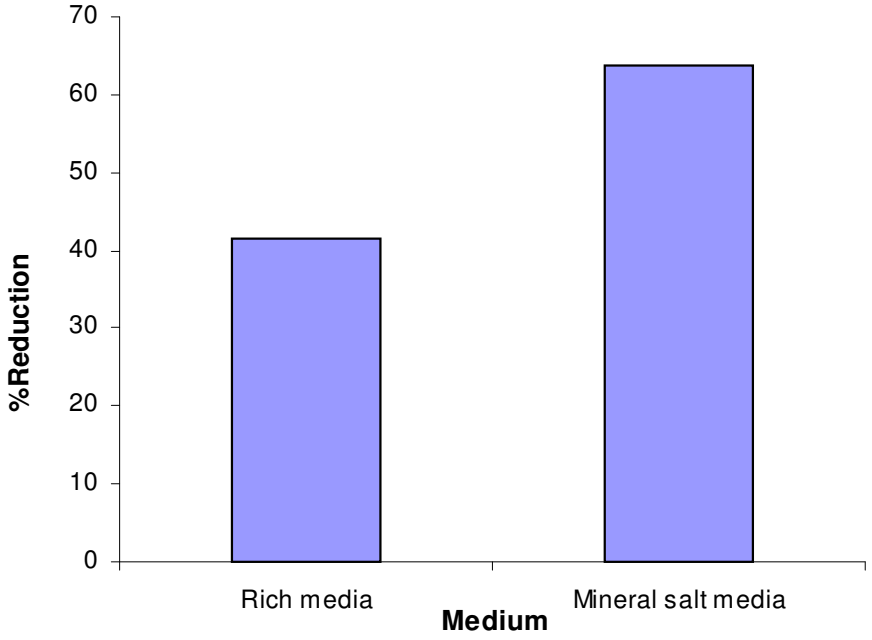


FIG. 19 :Comparison of% reduction of limonin in two medium

660nm was higher in case of rich medium (1.231) as compared to that in mineral salt medium (0.86).

Transformation of limonin: Fig. 18 summarizes the data on residual limonin levels in two different growth media from 0 hours to 120 hours. Residual limonin levels decrease appreciably in both media, but a higher reduction of limonin (63%) was observed in mineral salt medium as compared to that in the rich medium (41.9%) shown in Fig. 19. From above results it was found that although growth of *P. putida* was better in rich medium than in mineral salt medium, limonin biotransformation was more in mineral salt medium. This data shows that to some extent, limonin biotransformation occurs in rich medium also. This is an interesting observation since rich medium contains a host of other carbon and nitrogen sources apart of limonin and *P.putida* is utilizing limonin even in the presence of all these nutritional sources. This fact needs further detailed study.

Studies of optimization of parameters for limonin biotransformation by *P.putida* thus, show that maximum limonin biotransformation could be achieved by growing *P.putida* in mineral salt medium at pH 5 and a temperature of 35^{o}C for 120 hours. This was important keeping in view the low pH (3.5-4) encountered in Kinnow juice. It is necessary to get an enzyme that can work at or near the pH of Kinnow juice so that no or minimum upward adjustments of juice pH is done to carryout the debittering process. Otherwise, large amounts of enzyme would be required to efficiently reduce bitterness. The above mentioned results indicate the presence of an enzyme (s) in our experimental system that is able to reduce limonin levels at a pH of 5.0.

4.8 Identification of Reaction Products / Metabolites

After optimization of growth conditions showing maximum limonin biotransformaiton, identification of reaction products / metabolites of limonin was attempted. For this *P. putida* was grown either in medium containing limonin or in medium with serum. Cell supernatant was taken from 0 hour and 120-hour sample and sample preparation was done according to method described earlier.

Samples in the form of two fractions - dichloromethane (neutral) fraction and ethyl acetate (acidic) fraction, were analyzed using thin layer chromatography with solvent system cyclohexane – ethyl acetate (50:50). The compounds were visualized under UV light and also by spraying Ehrlich's reagent followed by exposure to warm HCl gas. Ehrlich's test constitutes a good evidence for the presence of a furan ring in the substrate, a characteristic of limonoids. Limonoids give characteristic off colored orange spots with this reagent. As a reference, commercially available limonin was also run simultaneously (Fig.20).

0 hours culture grown in medium with pure limonin did not show any spot with U.V illumination. But with Ehrlich's reagent, a single intense spot of R_f value 0.56 was observed in neutral fraction. R_f value of this spot was same as that of pure limonin. Acidic fraction from 0 hour sample did not show any spot either by UV illumination or with Ehrlich's reagent.

Analysis of 120 hours sample revealed four Ehrlich positive metabolites in growth medium- one in neutral fraction and three in acidic fraction. Mobility of neutral compound was found to be same as that of standard limonin (table 16). The intensity of this spot was lesser as compared to the spot of same R_f value in 0 hours sample indicating, thereby, that limonin was being transformed into some other metabolite/s. Mobility of the major compound observed in the acidic fraction was similar to that of deoxylimonoic acid (table 16) as reported in literature. UV spectrum of this compound showed a peak at 260nm (Fig.21).

Serum alone was extracted and two fractions analysed by TLC in cyclohexane/ ethyl acetate. The neutral fraction showed one major compound that had mobility similar to that of standard limonin as checked by two different solvent systems (table 16). One major compound was observed in the acidic fraction, which gave negative Ehrlich's reaction and showed a λ_{max} of 250nm in its UV spectrum (Fig.22). Similar, results were obtained from 0-hour culture medium containing serum. Neutral fraction of 120 hours sample containing serum showed one major compound with mobility as that of standard limonin. A minor compound was also

Table 16: Probable metabolites of limonin biotransformation

Sample	Fraction	Number of spots observed	R_f value in solvent system			probable metabolites
			1*	2*	3*	
Standard (Fig.20)	_	1	0.56		0.9	
Growth medium with limonin (0 hours)	Neutral	1	0.56		0.9	Limonin
	Acidic	3	_			-
Growth medium with limonin (120 hours)	Neutral	1	0.56		0.9	Limonin
	Acidic	3	0.1 0.4 0.57		0.2	Deoxylimonoic acid (Fig.21)
Juice serum	Neutral	3	0.53 0.37 0.16		0.9 0.2 0.2	Limonin
	Acidic	1	0.36	0.83	0.9	-ve to ehrilich's (Fig.22)
Growth medium with juice serum (120 hours)	Neutral	2	0.56 0.2	0.54	0.9	Limonin
	Acidic	2	0.12 0.35		0.23 0.9	Deoxylimonin (Fig.23) Deoxylimonoic acid (Fig.24)

1*Cyclohexane: Ethyl acetate (50: 50)

2* Ethanol

3* Benzene: Ethanol: Water: Acetic acid (200:47:15: 1)

WAVELENGTH SCAN/GB 04/29/03 15:08

nm	ABS
400.0	0.005
390.0	0.005
380.0	0.005
370.0	0.006
360.0	0.009
350.0	0.015
340.0	0.006
330.0	0.007
320.0	0.021
310.0	0.046
300.0	0.078
290.0	0.106
280.0	0.175
270.0	0.203
260.0	0.178
250.0	0.268
240.0	0.480
230.0	0.347
220.0	0.273
210.0	0.344
200.0	0.346
190.0	0.231

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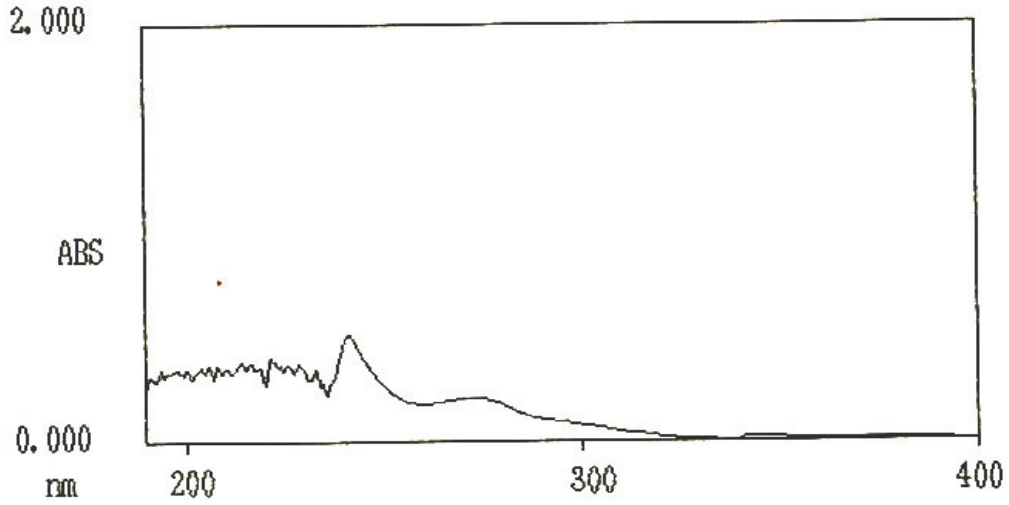

Fig. 20

UV Spectra of Pure limonin

WAVELENGTH SCAN/GB

04/29/03 16:32

nm	ABS
400.0	0.057
390.0	0.058
380.0	0.061
370.0	0.065
360.0	0.070
350.0	0.077
340.0	0.074
330.0	0.082
320.0	0.096
310.0	0.125
300.0	0.201
290.0	0.346
280.0	0.562
270.0	0.844
260.0	1.507
250.0	0.585
240.0	0.399
230.0	0.323
220.0	0.261
210.0	0.375
200.0	0.461
190.0	0.415

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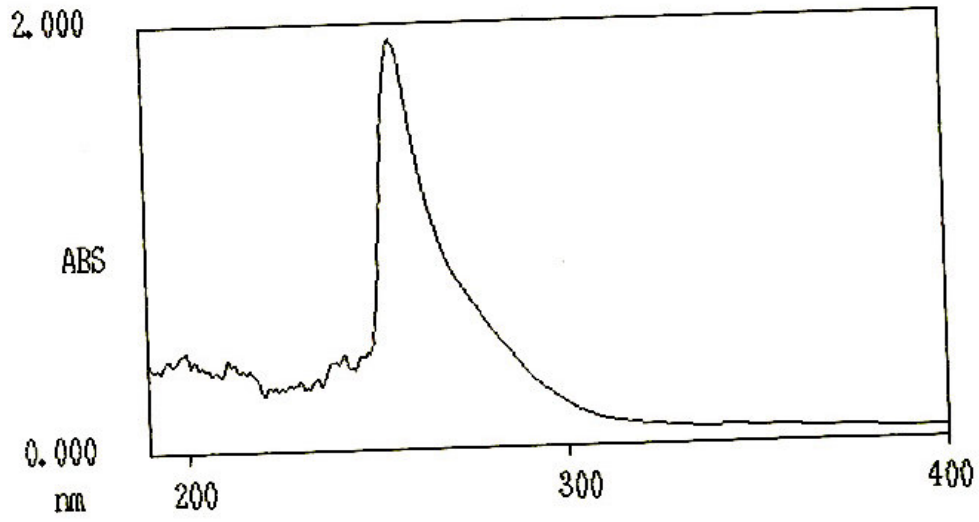

Fig. 21

WAVELENGTH SCAN/GB 05/05/03 10:21

nm	ABS
400.0	0.289
390.0	0.290
380.0	0.290
370.0	0.293
360.0	0.298
350.0	0.310
340.0	0.317
330.0	0.332
320.0	0.349
310.0	0.362
300.0	0.381
290.0	0.405
280.0	0.428
270.0	0.454
260.0	0.479
250.0	1.923
240.0	-1.218
230.0	-1.229
220.0	-0.239
210.0	-1.393
200.0	-1.136
190.0	-1.404

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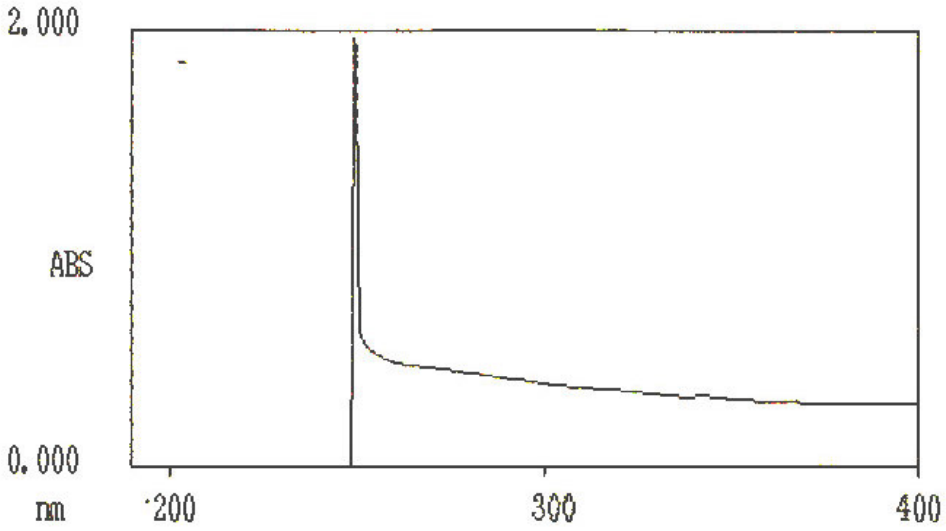

Fig. 22

revealed that had mobility as that of deoxylimonin as reported in literature (Hasegawa *et.al.,* 1972).

Acidic fraction of this sample (120 hours) revealed one major compound that had mobility as that of deoxylimonoic acid with the data in literature (Hasegawa *et.al.,* 1972). This was confirmed by TLC in two different solvent systems (table 16). UV spectra of this compound further showed a λ_{max} of 260nm again indicating it to be deoxylimonoic acid (Fig. 23). A second minor compound was also observed in this fraction.

These results indicate towards the following pathway in *P.puitda*:

Limonin ⟶ Deoxylimonin ⟶ Deoxylimonoic acid

These results are well supported by earlier reports. *Pseudomonas 321-18* has been shown to metabolize limonin, via two pathways- one through deoxylimonin and deoxylimonoic acid and other through 17-dehydrolimonoate (Fig.25) (Hasegawa *et.al.,* 1976). The same pathway was proposed for *Acinetobacter*, a bacterium isolated from soil (Vaks and Lifshitz, 1981) and for *Arthrobacter globiformis* II and its variants isolated from soil (Hasegawa *et. al*., 1983).

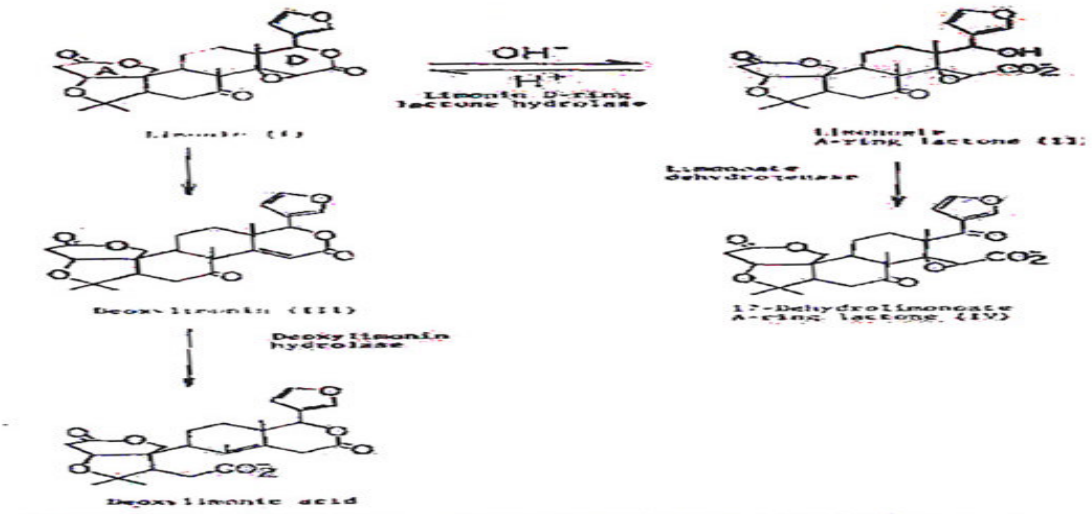

Figure 25 Metabolic pathways of limonoids in *Pseudomonas* sp. 321-18.

350.0	0.372
340.0	0.415
330.0	0.482
320.0	0.566
310.0	0.681
300.0	0.818
290.0	1.006
280.0	1.250
270.0	1.548
260.0	1.629
250.0	0.713
240.0	0.549
230.0	0.576
220.0	0.750
210.0	0.592
200.0	0.600
190.0	0.598

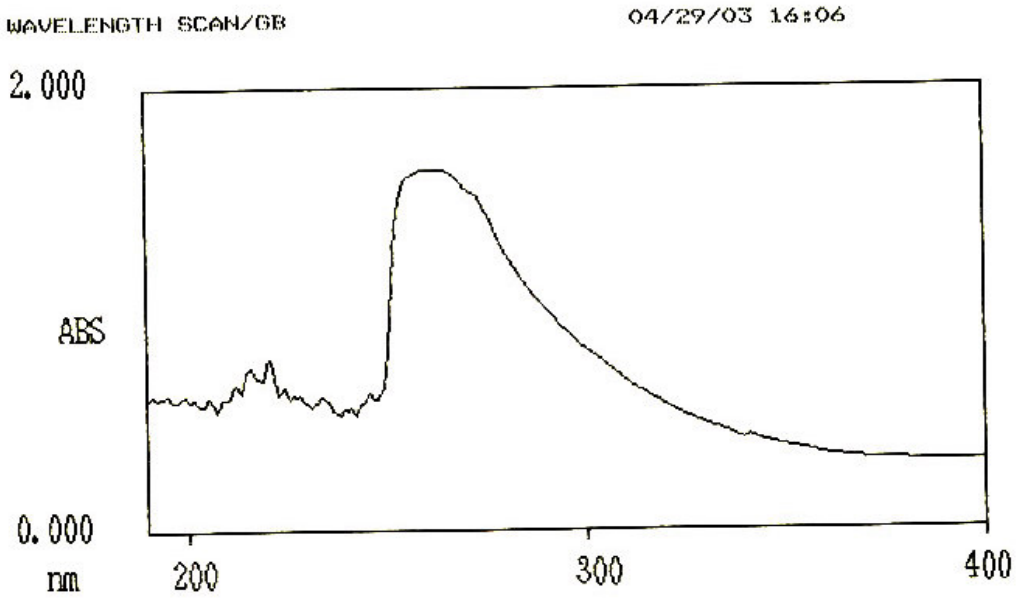

Fig. 23

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nm	ABS
400.0	0.107
390.0	0.110
380.0	0.117
370.0	0.128
360.0	0.155
350.0	0.201
340.0	0.267
330.0	0.348
320.0	0.414
310.0	0.516
300.0	0.642
290.0	0.810
280.0	1.042
270.0	1.339
260.0	1.494
250.0	0.620
240.0	0.446
230.0	0.412
220.0	0.347
210.0	0.370
200.0	0.380
190.0	0.373

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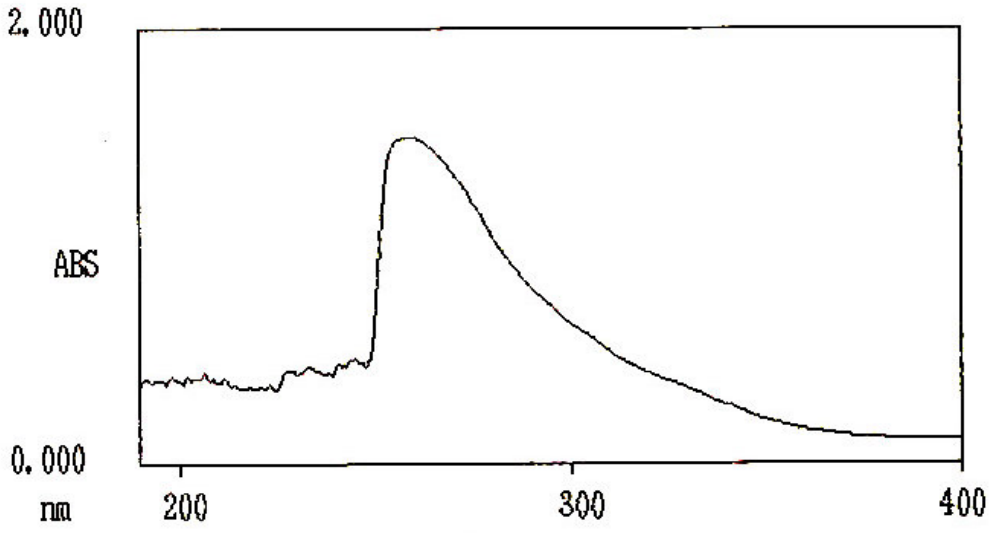

Fig. 24

The enzymes involved in these pathways, limonin-D-ring lactone hydrolase, limonoate dehydrogenase and deoxylimonin hydrolase have been isolated and characterized (Hasegawa *et.al.,* 1972, 1974). The enzyme that catalyzes the formation of deoxylimonin is yet to be identified. Conversion of limonin to LARL and vice-versa is a reversible reaction, favoring limonin formation at acidic pH. That is how LARL present in juice is converted to limonin after extraction. The conversion of limonin to non-bitter LARL, on the other hand is favored by alkaline pH. Since in the present study, juice serum has been used as source of limonin and pH of the growth medium was kept at 5.0, limonin is probably not being converted to LARL, the precursor of 17-dehydrolimonoate-A-ring lactone. It seems likely that *P.putida* (MTCC 1072) converts limonin to deoxylimonin, which is then converted to deoxylimonoic acid. It would to pertinent to add here that the results of the present study are preliminary and need confirmation regarding the identification of metabolites of limonin by Nuclear Magnetic Resonance and Mass Spectroscopy. Also, the enzymes involved in the metabolic pathway need to be isolated and characterized. Work in this regard is in progress.

SUMMARY AND CONCLUSIONS

Limonoids, a group of oxygenated tetranortriterpenoids, are of general occurrence in genus *Citrus.* Two of these compounds, limonin and nomilin, have a bitter taste at a level as low as 2-5 ppm. As they occur in economically important Kinnows, oranges and grapefruits, removal or transformation of limonin is of much interest to the citrus fruit industry.

Several physico-chemical and biotechnological methods have been tried to reduce bitterness in juices from citrus fruits. However there is no such technique on the suitability of debittering techniques in Kinnow juice. Development of such a technology could help accelerate the processing of Kinnow fruits.

Thus, the present study concentrated on the biotransformation of limonin by *Pseudomonas putida* (MTCC1072) in an attempt to control the development of bitterness due to formation of limonin in juice with time. Biotransformation of limonin was studies in two ways- first using pure limonin as the sole carbon source in the growth medium and secondly using Kinnow juice serum as source of carbon and limonin.

To start with, growth conditions for maximum limonin biotransformation were optimized. After the optimization of growth conditions, metabolites were analysed using preparative TLC and UV spectroscopy. Studies comparing glucose and limonin as the carbon source showed that *P. putida* is able to utilize limonin from the growth medium. A comparison of pure limonin and juice serum revealed significant reduction in limonin levels in both the cases.

Studies of optimization of parameters for limonin biotransformation by *P. putida* showed that maximum limonin biotransformation could be achieved by growing *P. putida* in mineral salt medium at pH 5, temperature 35^{o}C for 120 hours. This also indicates the presence of enzyme(s) in the system that can efficiently reduce limonin levels at a low pH that is close to that of Kinnow juice. The results of identification of metabolites formed indicate towards the conversion of limonin to deoxylimonin and then to deoxylimonoic acid. Still, to the best of our knowledge, no study has so far been reported in literature showing limonin biotransformation activity of *P. putida* for Kinnow juice.

It must be added here that the results of the present study are preliminary and need confirmation regarding the identification of metabolites of limonin by Nuclear Magnetic Resonance and Mass Spectroscopy. Also, the enzymes involved in the metabolic pathway need to be isolated and characterized. Work in this regard is in progress.

Composition of limonin(Ehrlich's) reagent:

4- dimethyl aminobenzaldehyde	: 0.1g
Glacial acetic acid	: 3ml
Perchloric acid (70%)	: 2.4ml

Composition of M63 medium :

Phosphate dihydrogen orthophosphate	: 13.6g
Ammonium Sulphate	: 1g
Iron Sulphate	: 0.005g
Magnesium Sulphate	:0.246g
Distilled water	: 1000ml

Composition of rich medium:

Yeast extract	: 0.1%
Beef extract	: 0.2%
Peptone	: 0.5%
Sodium chloride	: 0.5%

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