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Research Paper

DEBITTERING OF CITRUS FRUIT JUICE BY NARINGINASE OF PENICILLIUM PURPUROGENUM

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The bitterness in citrus fruit juice is mainly due to the presence of naringenin which makes the product unacceptable by some consumers. Removal of this bitterness, to certain extent, has been achieved by addition of partially purified naringinase to the citrus fruit juice. Naringinase produced by P. Purpurogenum is partially purified, and lyophilized before use. Maximum removal of naringenin content (74%) was achieved at naringinase concentration of 1.0g/L, with incubation at 40°C for 4 h.

Keywords: Debittering, Citrus, Naringinase, Naringenin, Penicillium

INTRODUCTION

India holds third rank in production of citrus fruits in the world. The citrus group of fruits includes sweet orange, mandarin, lemon, acid lime, pumello, grapefruit, etc. India produces nearly four million metric tons of citrus fruits per annum, but due to the poor post-harvest infrastructure facilities, the wastage of citrus fruits amounts to nearly one fourth of the total production. There is a need to reduce this wastage and make the fruits and fruit products available in market for consumers, so also to provide remunerative prices to the farmers during seasonal glut.

Commercial cultivation of citrus is mainly restricted in the northern, eastern and western parts of India. Punjab is known for its kinnow, mandarin and malta. Maharashtra is very famous for its mausami and Nagpuri santra (Mandarin oranges). Citrus fruits are considered to be the nutritious fruits as they are the rich source of β-carotene (source of vitamin A), ascorbic acid (vitamin C) and folic acid. Vitamins provide protection against many fatal diseases like cancer, heart ailments, etc. Citrus juice is also recommended for infants and senescent people as a supplementary source of vitamin C.

In recent years awareness of consumers about the health promoting ability of bitter flavonoids such as naringin has generated keen interest in processing of citrus fruit juice, such as grapefruit juice, not only for local consumption but also for export market. Some bitterness in the grapefruit products is acceptable, but excessive of it is one of the major objections to such products (Bell, 1955; Birdsall, 1955). Following are some of the several recommendations made to obtain citrus fruit juice free from bitterness:

- To use root stocks like trifoliate orange, tangello, cleopatra mandarins which are low in limonin
- Pre-harvest sprays of 250 ppm each of 2(4-ethyl phenoxy) triethyl amine and 2(3,4-dimethyl phenoxy) triethyl amine

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- Exposure of harvested fruit to 20 ppm of ethylene gas for four hour. The ethylene gas is easily available now a day in the form of ethylene ampoules
- Juice extraction without damaging citrus fruit tissues helps to lower limonin content in the juice
- Masking bitterness by addition of sweeteners like sucrose to juice
- Carbonation of juice immediately after extraction under chilling temperatures
- Enzymatic debittering by using immobilized enzymes or micro-organisms in bioreactors
- Debittering by membrane technology using cellulose polymers
- Debittering of juice in columns packed with polymer adsorbents

Tremendous information is available on naringin, the main bitter substance in grapefruit. Its intense bitterness, which is said to exceed that of quinine, is detectable in water at the concentration of as little as one part in 50,000 (Olsen and Hill, 1964). A possibility of removal of naringenin and debittering of fruit juices of by the enzyme naringinase has been reported by (Puri *et al*., 1996), (Chandler and Nicol, 1975), (Roe and Bruemmer, 1976), (Olson *et al*., 1979), Tsen and Yu, 1991. Naringinase enzyme has been reported to have two inherent activities such as -rhamnosidase and -glucosidase activity. Naringin is first hydrolyzed to prunin and rhamnose by -rhamnosidase. In the second step, prunin is broken down into naringenin and glucose by -glucosidase activity of the same enzyme (Olson *et al*., 1979). A recent application suggests that both naringin and naringenin have similar medical applications, indicating that the biological activity resides with the aglycone moiety and is not associated with the sugar residues. Both have been shown to be useful in inhibiting the accumulation of macrophage-lipid complex on the arterial endothelium and preventing or treating hepatic disorders in mammalian systems (Bok *et al*., 1999). Naringenin has also been shown to have a partial suppressive effect on adhesive glucan formation by Streptococcus mutans on human dental plaque (Masayoshi *et al*., 1984). Further, taking into its health promoting qualities naringin or naringenin can be included in a pharmaceutical composition, in a food preparation or even in a beverage in acceptable quantity. Thus, debittering of grape fruit juice by naringinase would not reduce the health promoting effects, as naringenin still remains in the treated citrus juice as an end product of nariginase action on the bitter principle, the narigenin.

MATERIALS AND METHODS

Fruits

Mature grapefruits (Citrus paradise) were identified by the taxonomists of University Department of Botany, RTM Nagpur University, Nagpur, purchased from the local market before use.

Microorganism, Culture Conditions & Production of Naringinase

P. Purpurogenum, indigenously isolated in our laboratory and identified by IMT, MTCC, Chandigarh, was maintained on PDA slants with periodic transfer at every 2 weeks.

The fungus was grown in Czapek Dox medium as suggested by Bran and Solomons (1965) containing 4% corn steep liquor, 4% yeast extract, 0.2% KH_2PO_4, 0.1% naringin and 0.5% $CaCO_3$. pH of the medium was maintained at 5.5 before sterilization. Enzyme production was carried out in 250 ml conical flasks. Each flask containing sterilized medium under standard condition was inoculated separately with 1 ml spore suspension of P. purpurogenum (10^8 spores/ml in sterile distilled water). Inoculated flasks were incubated at 30°C for 14 days under static condition.

Enzymes (Partially Purified Naringinase)

The culture filtrate, obtained after removal of mycelium from the culture flask, was clarified by centrifugation at 5000g for 20 min at 4°C. The supernatant obtained was used as crude source of enzyme. The crude enzyme was treated with ammonium sulphate to 80% saturation; the precipitate was collected by centrifugation at

12,000g for 15 min. The precipitate was dissolved in 10ml buffer (0.1M sodium acetate, pH 5.5) and the traces of ammonium sulphate present in the suspension were removed by dialysis against same buffer (2 L) for 18 h. All the procedures were carried out at 4°C. This partially purified naringinase was lyophilized under standard conditions and preserved in air tight containers until use. The lyophilized enzyme was found to have activity of approximately 100U/0.1g.

CHEMICALS

Ammonium Sulphate, Diethylene Glycol, Sodium Hydroxide and Naringin were purchased from Himedia laboratories, Mumbai. Other chemicals were of analytical reagent grade.

Estimation of Naringin Content in Fruit Juice

The mature fruits were washed under running tap water. The fruits were hand peeled and the segments were separated. The segment membrane was hand peeled and the juice sacs were used for the analysis. The juice sacs were mashed in a mortar and pestle, and the juice so obtained was passed through muslin cloth for removal of any debris, before it was used for analysis of naringin.

Naringin content in the fruit was estimated by the method of Davis (1947). To 10 ml of 90% diethylene glycol in a tube 0.2 ml of fruit juice was added. Thereafter, 0.2 ml of 4M NaOH was added to the tube, and the yellow color developed after 5 min of incubation at room temperature (30±2°C) was read in a spectrophotometer at 420nm. A standard graph of naringin was prepared in the range of 5-30 μg/ml.

DEATERMINATION OF NARINGINASE ACTIVITY

The partially purified naringinase used in this study contains β-glucosidase activity also. It was assayed for its activity by measuring the rate of glucose formation from the two step hydrolysis of naringin to prunin and rhamnose (by α-rhamnosidase) and of prunin to naringinin and glucose (by β-glucosidase). The reaction mixture consisting of 4.5 ml of a 0.1g/L naringin solution in 0.1 M Sodium acetate buffer at pH 5.5 and 0.5 ml of the enzyme was incubated at room temperature (30±2°C) for 30 min, after which 0.2 ml of reaction mixture was withdrawn for glucose estimation by DNS method (Miller, 1959). One unit of naringinase activity is defined as the amount of naringinase that liberates 1μmol of reducing sugars, expressed as glucose per minute, under the given assay conditions.

ENZYMIC DEBITTERING OF FRUIT JUICE

This study was carried out by following the procedure of Olsen and Hill (1964). The fresh fruit juice was extracted as described earlier. Partially purified naringinase enzyme was added at appropriate quantity to separate batches of 100ml juice so as to obtain desired final enzyme concentrations in the range of 25 to 100U/100ml of juice. Samples were then incubated for 1-4 h, at temperature from 25 to 40°C, after which the residual naringin content was estimated as described above. Reaction time of 1 to 4 h was selected and standardized based on a previous report (Roe and Bruemmer, 1977). The decrease in the naringin content was directly correlated with reduction in bitterness. From the amount of residual naringin present, the percentage reduction in bitterness was calculated.

RESULTS AND DISCUSSION

The enzyme naringinase was commercially isolated from a wide range of fungi grown on media containing naringin or rhamnose. They are known hydrolyze naringin within a few h at room temperature and at the natural pH of most citrus fruit juices (3.0-3.8) (Chandler and Nicol, 1975). Rombouts and Palnik (1978) have reviewed the major studies attempting to effect bitterness reduction in citrus juices by enzymatic treatments.

In the present investigation, enzymatic de-bittering of grapefruit juice using lyophilized naringinase produced by P. Purpurogenum with respect to enzyme concentration 0.25, 0.5 and 1g/100ml juice and temperature (25, 30 and

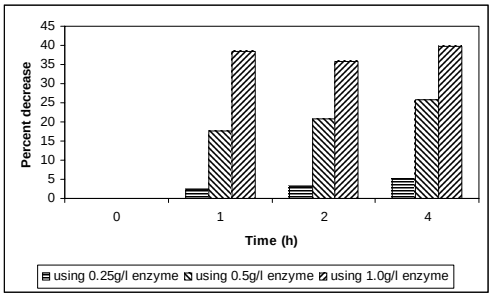
40°C) and time of incubation (1, 2 and 4 h) was optimized. In all three independent variables, enzyme concentration, temperature and time of incubation, were varied at three levels. In addition to that, controls were maintained for each set. From the results obtained (Table 1, Figure 1-3) it can be seen that when all the parameters i.e. the enzyme concentration, temperature and time of incubation were varied, there is a decrease in the naringin content. Maximum decrease of naringin (about 74%) was obtained at naringinase concentration of 100 U/100ml juice, with incubation at 40°C for 4 h.

Table 1: Effects of Varying Naringinase Concentration, Temperature and Time of Incubation on Grapefruit Juice Debittering

Trials	Naringinase g/L	Temp (°C)	Time (h)	Naringin content (µg/ml)
Control	**-**	**-**	**-**	**814**
1	0.25	25	1	795
2	0.5	25	1	671
3	1	25	1	502
4	0.25	30	1	756
5	0.5	30	1	634
6	1	30	1	355
7	0.25	40	1	630
8	0.5	40	1	478
9	1	40	1	292
10	0.25	25	2	789
11	0.5	25	2	644
12	1	25	2	522
13	0.25	30	2	690
14	0.5	30	2	612
15	1	30	2	345
16	0.25	40	2	625
17	0.5	40	2	467
18	1	40	2	310
19	0.25	25	4	773
20	0.5	25	4	603
21	1	25	4	491
22	0.25	30	4	704
23	0.5	30	4	524
24	1	30	4	375
25	0.25	40	4	533
26	0.5	40	4	354
27	1	40	4	210

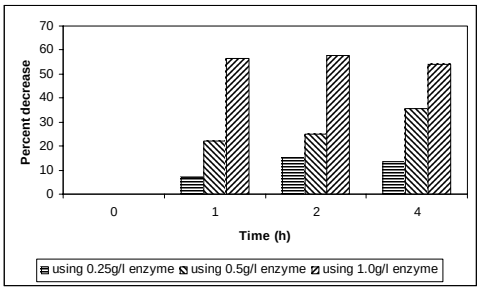
Results are mean of three replicates. (P value was set at > 0.05)

Figure 1: Percent Decrease in Naringin Levels Using Different Concentrations of Naringinase at 25°C.



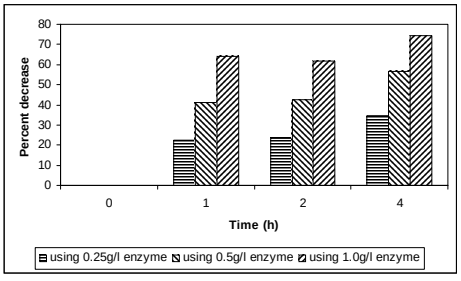
Results are mean of three replicates. (P value was set at > 0.05)

Figure 2: Percent Decrease in Naringin Levels Using Different Concentrations of Naringinase at 30°C.



Results are mean of three replicates. (P value was set at > 0.05)

Figure 3: Percent Decrease in Naringin Levels Using Different Concentrations of Naringinase at 40°C.



Results are mean of three replicates. (P value was set at > 0.05)

A critical review on debittering of citrus juice by using the enzyme naringinase has been reported by (Puri *et al*., 1996). Roe and Bruemmer (1976) suggested for the development of new varieties of citrus fruits where the naringin content would be less in quantity. (Olson *et al*., 1979), immobilized the naringinase enzyme on hollow fibers and tested it for the complete removal of bitterness from the citrus fruit juice and (Tsen and Yu, 1991) tried to remove bitterness of citrus juices by naringinase entrapped in cellulose triacetate fibers. The present work of removal of naringin content with lyophilized naringinase of P. Purpurogenum avoids the immobilization cost and removes approximately 74% naringin content from the grape fruit juice under controlled conditions as suggested above.

All results are mean of 3 replicates. The data obtained was analyzed using ANOVA technique as given by Ronald Walpole (1982). Significance was set at $P<0.05$.

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