Indian J Microbiol (October 2010) 50(Suppl 1):S46–S51
DOI: 10.1007/s12088-010-0029-6

ORIGINAL ARTICLE

Application of immobilized tannase from *Aspergillus niger* for the removal of tannin from myrobalan juice

Anita Srivastava · Rita Kar

Received: 23 October 2008 / Accepted: 4 February 2009

Abstract Tannase produced optimally on an agroresidue by an *Aspergillus niger* isolate under submerged fermentation immobilized on sodium alginate beads with 93.6% efficiency was applied for tannin removal from myrobalan/aonla (*Phyllanthus emblica*) juice. The pH and temperature optima of the immobilized enzyme were found to be 5.4 and 40°C while the corresponding values of the soluble enzyme were 5.8 and 35°C. Maximum tannin removal of 73.6% was obtained at 40°C and 150 rpm in 180 min with 36.6 U/ml of immobilized enzyme while the same amount of the soluble enzyme removed 45.2% of tannin at 37°C and 150 rpm in the same time period. The immobilized beads could be used repeatedly till 7th cycle with 77% efficiency. When preserved at 6°C the beads retained 71.7% of enzyme activity after 60 days. Reduction in vitamin C content, which is responsible for antioxidant property of the fruit, was minimum at only 2% during the treatment.

Keywords *Aspergillus niger* · Immobilized tannase · Myrobalan · Submerged fermentation · Sodium alginate

A. Srivastava · R. Kar (✉)
Department of Biochemical Engineering and Food Technology,
Harcourt Butler Technological Institute,
Kanpur – 208002, India

E-mail: rkarhbti@rediffmail.com

Introduction

Myrobalan (*Phyllanthus emblica*) popularly known as aonla in India is a tannin (17% w/w) and ascorbic acid (600 mg/100 g) rich tropical fruit. Studies have suggested that aonla increases hemoglobin percentages and stimulates anabolic processes such as protein synthesis. The dried fruit reduced cholesterol levels, indicating that aonla is safe to consume on a long-term basis. In spite of all these well established nutritional benefits aonla juice is not used as a popular beverage due to its high tannin content resulting in bitterness. Tannins are high molecular weight compounds composed of glucose and gallic acid molecules found in a variety of plant tissues.

Tannase (tannin acyl hydrolase EC 3.1.1.20) is the enzyme responsible for degradation of hydrolysable tannins, especially gallotannins to glucose and gallic acid [1]. Hydrolysis of tannin reduces the tannin content while preserving the gallic acid, a polyphenol having natural antioxidant properties and is responsible for stability of very high vitamin C content of aonla juice. Application of tannase in food industries such as preparation of instant tea, clarification and prevention of sediment formation in wines, beers and fruit juices, gallic acid and propyl gallate production have been reported in literature [2, 3]. Application of tannase for tannin removal, in myrobalan juice which has a bitter astringent taste may make it more acceptable as a health drink as the treatment reduces the tannin/bitterness level without much loss of its vitamin C and antioxidant properties.

In spite of its many potentials [4] tannase has rarely been used for tannin removal from fruit juices [5]. This is being reported for the first time and it can be of great advantage as the delicate composition of aonla which is essential for its nutritional benefits is not disturbed by enzymatic treatment.

Materials and methods

Chemicals

Sodium alginate from Himedia was used as a carrier for enzyme immobilization and all other chemicals used were of analytical grade.

Juice preparation

Fifty gram of aonla fruit was taken, cut into small pieces and ground in a mortar pestle. Juice was then extracted by adding 100 ml of distilled water and homogenizing in a blender followed by filtration through muslin cloth. The extracted juice was stored at 4°C for further use. The juice had an initial pH of 2.5.

Estimation of tannin content

The tannin content in the aonla juice was measured following the protein precipitation method of tannins [6]. To 1.0 ml of aonla juice, 2 ml of bovine serum albumin (1 mg/ml) was added and centrifuged at 5,000 rpm for 15 min. The supernatant was discarded and the precipitate was dissolved in 4 ml of sodium dodecyl sulphate (SDS)-triethanolamine solution. 1.0 ml of ferric chloride reagent was added, and the solutions were mixed immediately and absorbance was observed after 15–30 minutes at 550 nm. The tannin content was determined from a standard graph of pure tannic acid (100–500 μg).

Growth and maintenance of culture

Aspergillus niger isolate was grown on Czapekdox medium containing 1% (w/v) tannic acid and maintained on tannic acid agar slants stored at 4°C and subcultured routinely after every 3–4 weeks.

Medium composition and tannase production

Fifty milliliters of Czapekdox medium containing (g/l): $NaNO_3$ 6.0; KCl 0.52; $MgSO_4 \cdot 7H_2O$ 0.52; KH_2PO_4 1.52; $Cu(NO_3)_2 \cdot 3H_2O$ traces; $ZnSO_4 \cdot 7H_2O$ traces, $FeSO_4$ traces supplemented with 4% (w/v) pomegranate rind powder and 0.25% (w/v) tannic acid, pH 5.0 in 250 ml of Erlenmeyer flask was inoculated with 5.0×10^7 and incubated at 37°C in an orbital shaker for 96 h. Maximum tannase was produced after 72 h.

Measurement of ascorbic acid content

Ten milliliters of 0.1% standard ascorbic acid solution with 3% metaphosphoric acid was prepared and titrated against dichloro indophenol dye.

$$\text{Dye factor} = \frac{\text{mg ascorbic acid in sample titrated}}{\text{Titer value}}$$

The ascorbic acid content was measured by titrating 10 ml of aonla juice using the dye factor as following:

$$\text{Mg of ascorbic acid/100 g} = \text{titer} \times \text{dye factor} \times \text{dilution factor} \times 100/\text{aliquot analyzed}$$

Measurement of acidity

Twenty-five milliliters of juice was taken in a 100 ml of conical flask and three drops of phenolphthalein was added as an indicator. This was titrated with 0.1N NaOH and total acidity was determined.

Total acidity as citric acid %

$$= \frac{\text{Titer value} \times \text{normality of alkali} \times \text{volume made up} \times \text{Eq. wt of acid} \times 100}{\text{ml of aliquot taken for titration} \times \text{wt. of sample} \times 1000}$$

Acidity reduction (%) was determined from initial acidity and residual acidity after treatment with beads of immobilized enzyme.

Characterization of partially purified enzyme

The crude enzyme was partially purified by acetone precipitation followed by diethylaminoethyl (DEAE) cellulose column chromatography [7].

Optimum pH

pH optima of the soluble as well as immobilized enzyme was determined by varying the pH of the assay mixture in the range of 3.0–7.0.

Optimum temperature

For determination of optimum temperature of the enzyme, assays were carried out at different temperatures (30–45°C).

Immobilization of tannase

A 10 ml suspension containing 488 units of tannase and 4% sodium alginate was extruded drop wise through a 2 ml syringe into a 0.2 M $CaCl_2$ solution at 4°C to form

beads of 0.4 mm diameter. After 2 h the beads were washed with water and either preserved at a temperature of 6°C or used [8].

Assay of free and immobilized tannase

Free and immobilized tannase were estimated by the method based on chromogen formation [9]. For free enzyme reaction mixture containing 0.25 ml 0.01 M methyl gallate in 0.05 M citrate buffer pH 5.0 and 0.25 ml of broth whereas in case of immobilized tannase, the reaction mixture containing 0.5 ml of 0.01 M methyl gallate and 5 beads of immobilized enzyme (12.2 units) were incubated for 10 min at 35°C and 0.3 ml of methanolic rhodanine (0.667% w/v) was then added. After 5 min 0.2 ml of 0.5 M KOH was added. A control was run where enzyme was added after the addition of KOH in case of free enzyme. Finally the reaction mixture was diluted by 4.0 ml distilled water and incubated at 30°C for 10 min and absorbance was recorded at 520 nm. One unit of tannase is the amount of enzyme, which liberated 1 μm of gallic acid in 1 min.

Optimization of conditions for tannin removal from aonla juice by the immobilized enzyme

Effect of incubation period and enzyme concentration

Fifteen milliliters of aonla juice was treated with 5, 10, 15 and 20 beads of sodium alginate entrapped enzyme containing 12.2, 24.4, 36.6 and 48.8 units, respectively at 37°C, 150 rpm for 180 min and tannin content was analyzed at an interval of 60 min.

Effect of temperature

Fifteen milliliters of the juice was treated with either 0.75 ml of soluble enzyme (36.6 units) or 15 immobilized enzyme beads at 5 different temperatures 30, 35, 37, 40 and 45°C at 150 rpm for 180 min with tannin analysis at intervals of 60 min. After completion of the process, the beads were taken out from the juice and washed with distilled water.

Repeated use and preservation of the immobilized beads

Fifteen milliliters of juice was treated with 15 immobilized beads for 30 min and activity was measured. The beads were then washed with distilled water and again used to treat fresh juice. The process was repeated for 7 cycles. Activity of the enzyme in preserved beads was also checked at an interval of every 10 days for about 60 days.

Statistical analysis

A completely randomized design was used throughout this study. Data were subjected to analysis of variance (ANOVA) and mean comparison was carried using Student '*t*' test. Statistical analysis was performed using the statistical package for social sciences (SPSS for windows; SPSS Inc., Chicago, IL).

Results and discussion

Efficiency of immobilized beads

The efficiency of the immobilized beads were found to be 93.6% as the activity of the soluble enzyme was 48.8 U/ml whereas the same amount of immobilized enzyme showed an activity of 45.7 U/ml.

Characterization of soluble and immobilized tannase

The partially purified soluble and immobilized enzymes were found to have optimum temperatures of 35°C and 40°C and pH optima of 5.8 and 5.4, respectively (Figs. 1 and 2).

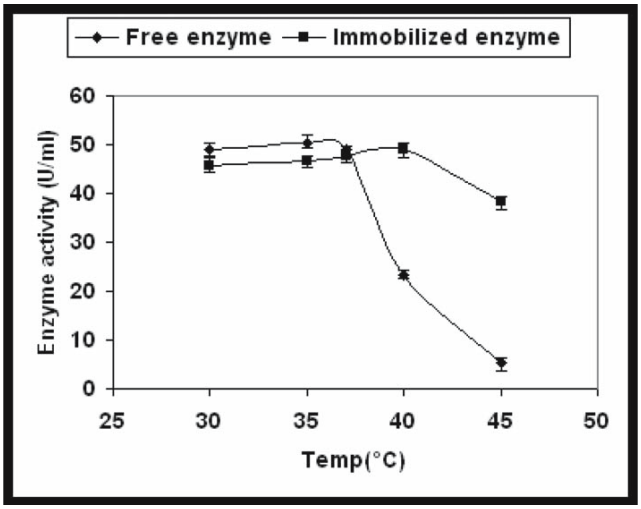

Fig. 1 Determination of the optimum temperature of soluble and immobilized tannase by the isolated A. niger strain. Reactions were carried out for 10 min with 0.01 M methyl gallate substrate in 0 .05 M citrate buffer, pH 5.0. Data are mean of three independent readings with significance of $P < 0.05$.

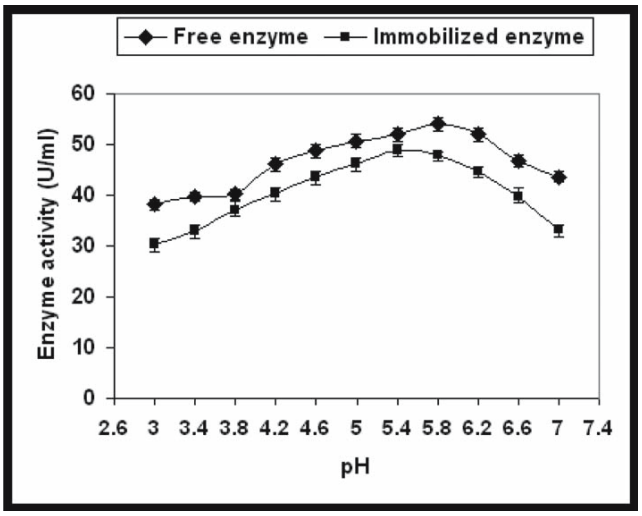

Fig. 2 Determination of the optimum pH of soluble and immobilized tannase by the isolated *A. niger* strain. Reactions were carried out for 10 min with 0.01 M methyl gallate substrate in 0.2 M citrate phosphate buffer at 35°C (with soluble enzyme) and 40°C (with immobilized enzyme), incubation period 72 h. Data are mean of three independent readings with significance of $P < 0.05$.

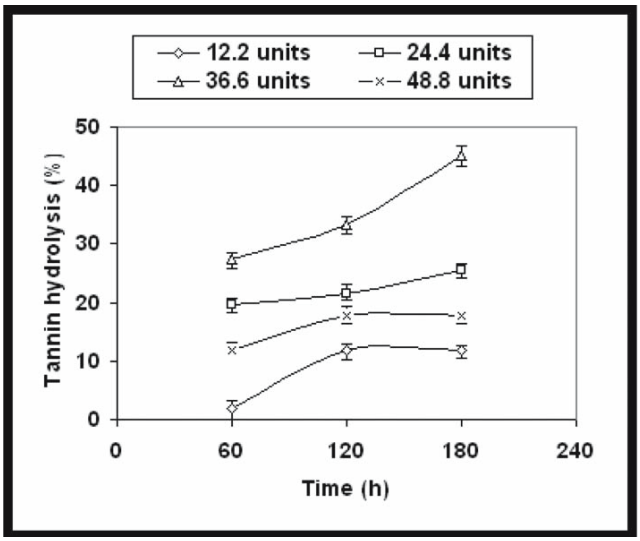

Fig. 3 Effect of soluble enzyme concentrations on tannin hydrolysis. The reaction was carried out with 15 ml of juice treated with different enzyme concentrations at 37°C, 150 rpm. 1.0 ml of aliquots were withdrawn and analyzed at 60 min intervals for tannin content. Data are mean of three independent readings with significance of $P < 0.05$.

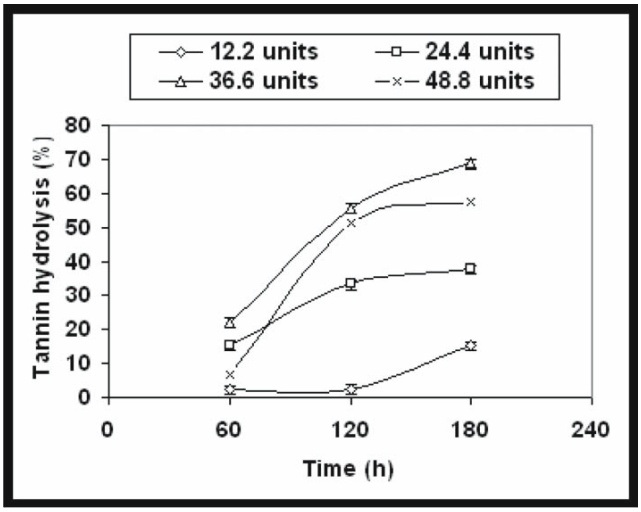

Fig. 4 Effect of immobilized enzyme concentrations on tannin hydrolysis. The reaction was carried out with 15 ml of juice treated with varying number of beads at 37°C and 150 rpm. 1.0 ml of aliquots were withdrawn and analyzed at 60 min intervals for tannin content. Data are mean of three independent readings with significance of $P < 0.05$.

Tannin removal in aonla juice with soluble and immobilized enzymes at different time intervals with varying enzyme concentrations

Maximum tannin of 45.2% (509 µg/ml) was hydrolyzed after 180 min on incubation with 36.6 U/ml of soluble enzyme whereas in case of alginate entrapped beads 68.7% (775 µg/ml) tannin was removed with 15 beads containing 36.6 U/ml at 37°C at 150 rpm (Figs. 3 and 4). In case of both soluble and immobilized enzyme tannin hydrolysis was reduced to 17.8% (202 µg/ml) and 57.7% (649 µg/ml) with higher enzyme concentration of 48.8 U/ml. The inhibition by higher enzyme concentration was much more significant with free enzyme.

Temperature as a function of tannin hydrolysis

The soluble enzyme showed a temperature optimum of 37°C (Fig. 5) with 45% tannin removal, but with immobilized enzyme maximum tannin removal of 73.6% was obtained at 40°C (Fig. 6). These results clearly indicating that the temperature optimum of the immobilized enzyme was elevated from 37°C (soluble enzyme) to 40°C. Moreover, the reaction was carried out for 3 hours and the enzyme remained thermally stable during this period. Entrapment within the gel protects the enzyme from adverse heat effects, while enhancing its activity. This is a clear advantage of immobilization. A shift in temperature optimum of the immobilized enzyme from 40°C to 55°C [10] and from 45°C to 55°C [11] have been reported.

Recycling of immobilized beads in aonla juice

The activity of the immobilized enzyme was tested after 30 minutes of juice treatment repeatedly for 7 cycles to test

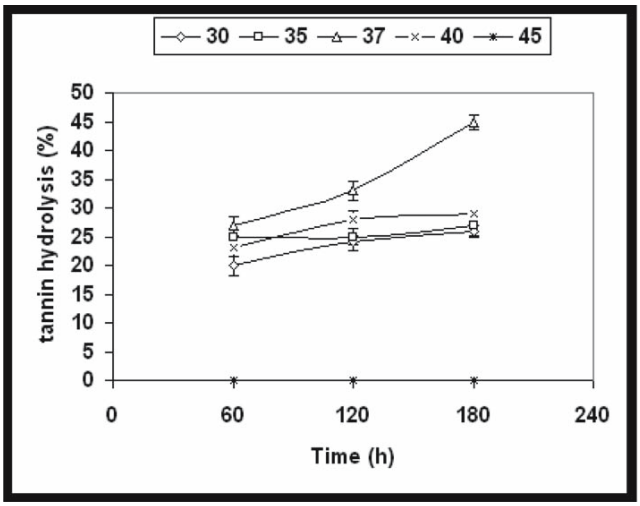

Fig. 5 Effect of temperature on tannin hydrolysis by free enzyme. The reaction was carried out with 15 ml of juice treated with 36.6 units of enzyme at 150 rpm. 1.0 ml of aliquots were withdrawn and analyzed at 60 min intervals for tannin content. Data are mean of three independent readings with significance of $P < 0.05$.

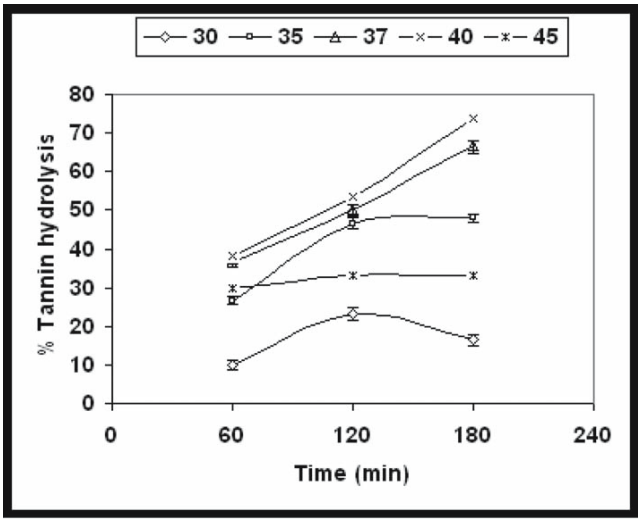

Fig. 6 Effect of temperature on tannin hydrolysis by immobilized enzyme. The reaction was carried out with 15 ml of juice treated with 15 beads of immobilized enzyme at 150 rpm. 1.0 ml of aliquots were withdrawn and analyzed at 60 min intervals for tannin content. Data are mean of three independent readings with significance of $P < 0.05$.

the activity retention. Fifteen milliliters of juice was treated with 15 beads of immobilized enzyme containing 36.6 U/ml of enzyme for 30 min under shaking conditions at 40°C, the beads were washed with distilled water and the activity of the immobilized beads was measured and found to be 45.7 U/ml as seen in Table 1. The process was repeated for 7 cycles with fresh juice. The activity of the beads was stable up to 3rd cycle and then decreased gradually with a residual efficiency of 77% after 7th cycle.

Table 1 Recycling of immobilized beads in aonla juice

Cycles	Immobilized tannase* (U/ml)	Efficiency (%)
1	45.7 ± 1.65	100
2	45.5 ± 0.86	99.5
3	45.5 ± 1.71	99.5
4	43.6 ± 0.92	95.4
5	40.4 ± 1.91	88.5
6	38.5 ± 1.21	84.2
7	35.2 ± 1.61	77

*Mean of the three independent readings ± SD.
15 ml of juice was treated with 15 beads for 30 min at 40°C and 150 rpm. Beads were washed with distilled water and then activity of the beads were measured. The process was repeated till 7th cycle.

Stability and activity of immobilized beads on preservation

The activity of the immobilized beads was found to be 45.7 U/ml which was fairly stable up to 30 days of preservation. The activity of the immobilized beads was checked till 60 days of preservation. The efficiency started decreasing after 30 days and was found to be 83.3% and 78.5% after 40 and 50 days, respectively and finally dropped to 71.7% after 60 days (Table 2).

Table 2 Stability and activity of immobilized beads on preservation

Days	Immobilized tannase* (U/ml)	Efficiency (%)
1	45.7 ± 1.39	100
10	45.7 ± 1.41	100
20	44.8 ± 0.99	98
30	42.4 ± 1.61	92.7
40	38.1 ± 1.72	83.3
50	35.9 ± 1.81	78.5
60	32.8 ± 1.91	71.7

*Mean of the three independent readings ± SD.
The reaction mixture containing 0.5 ml of 0.01 M methyl gallate as substrate and 5 beads containing 12.2 U/ml of enzyme. The activity of the immobilized tannase was tested at regular interval of 10 days for about 60 days.

Effect of immobilized tannase treatment on tannin, vitamin C and acidity levels of aonla juice

It can be seen from Table 3 that the treatment of juice resulted in significant removal of tannin (68.8%) with very little loss in vitamin C, which is responsible for high nutritional value and antioxidant property of aonla. Moderate reduction in acidity also contributes towards improvement in taste by reducing tartness.

Table 3 Tannin hydrolysis in aonla juice by immobilized enzyme as a function of time

Incubation period (min)	Tannin content (μg/ml)	Tannin hydrolysis (%)*	Vitamin C content (mg/ml)*	Reduction in vitamin C content (%)*	Acidity (%)*	Acidity reduction (%)*
0	1125	-	1.00 ± .01	-	1.20 ± .02	-
60	875	22.2 ± 1.1	0.995 ± 0.3	0.5 ± 0.3	1.17 ± 1.1	2.5 ± 0.3
120	500	55.5 ± 0.8	0.988 ± 1.1	1.2 ± 1.1	1.02 ± 0.7	15 ± 0.8
180	350	68.8 ± 0.7	0.980 ± 0.4	2.0 ± 0.3	0.90 ± 0.6	25 ± 0.8

*Mean of the three independent readings ± SD.
15 ml of juice was treated with 15 beads containing 36.6 units enzyme at 37°C, 150 rpm. 1.0 ml aliquot was withdrawn and analyzed at 60 min interval for tannin hydrolysis, vitamin C and acidity levels.

Conclusions

Tannin removal for bitterness reduction in aonla juice by immobilized tannase was found to have potential for further application. Hence the optimum conditions leading to maximum efficiency of the process and long-term utilization of the immobilized enzyme were evaluated. Since aonla, which is abundant and very popular in India, has very high nutritional value and antioxidant properties prone to damage by conventional processing methods, enzymatic treatment of the juice is of special significance. The concept of tannase application for fruit juice treatment and tannin removal of aonla, both are quite novel in nature.

References

1. Ibuchi S, Minoda Y and Yamada K (1972) Hydrolysing pathway substrate specificity and inhibition of tannin acyl hydrolase. Agric Biol Chem 36(9):1553–1562
2. Lekha PK, Ramakrishna M and Lonsane BK (1993) Strategies for isolation of potent cultures capable of producing tannin acyl hydrolase in higher titres. Chem Mikrobiol Technol Lebensm 15:5–10
3. Lekha PK and Lonsane BK (1994) Comparative titres, location and properties of tannin acyl hydrolase produced by *Aspergillus niger* PKL 104 in solid state, liquid surface and submerged fermentation. Proc Biochem 29:497–503
4. Cristobal N, Rodiguez R, Sanchez G, Augur C, Flavela Torus E, Lilia A, Prado B, Ramirez C and Equivel C (2007) Microbial tannases: advances and perspectives. Appl Microbiol Biotechnol 76:47–59
5. Rout S and Banerjee R (2006) Production of tannase under mSSF and its application in fruit juice debittering. Ind J Biotech 5:346–350
6. Haggerman AE and Butler LG (1978) Protein precipitation method for determination of tannins. J Agric Food Chem 26:809–812
7. Rajakumar GS and Nandy SC (1983) Isolation, purification and some properties of *Penicillium chrysogenum* tannase. Appl Environ Microbiol 46(2):525–527
8. Kierstan M and Bucke C (1977) The immobilization of microbial cells, subcellular organelles and enzymes in calcium alginate gels. Biotechnol Bioengg 19:387–397
9. Sharma S, Bhat TK and Dawra RK (2000) A spectrophotometric method for assay of tannase using rhodanine. Anal Biochem 279(1):85–89
10. Park NH and Chang HN (1979) Preparation and characterization of immobilized naringinase on porous glass beads. J Fermen Technol 57:310–316
11. Mishra P and Kar R (2003) Treatment of grapefruit juice for bitterness removal by amberlite IR 120 and amberlite IR 400 and alginate entrapped naringinase enzyme. J Food Sci 68(4):1229–1233