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Production of Naringinase by a new soil isolate of *Serratia* Sp.: Effect of different carbon and nitrogen sources

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Abstract

Four strains of Naringin degrading bacteria were isolated and tested for naringinase activity. All the four isolates showed extracellular naringinase activity. The one which showed consistently good activity in three different media was selected (2 U/L) and identified by phenotypic characterization as Serratia Sp. *In shake-flask trials, effect of various carbon and nitrogen sources was studied. Among all the carbon sources, glucose enhanced the naringinase production. Peptone supplemented with ammonium nitrate was found to be favourable. Maximum of 9.2 U/L naringinase activity was achieved in the medium comprising naringin, glucose, peptone, ammonium nitrate and salts.*

Keywords: Debittering, Naringinase, *Serratia* Sp., Carbon and nitrogen sources.

Introduction

Bitterness in the commercial citrus juices especially grapefruit juice is posing a serious challenge to fruit juice processing industries. This bitterness is due to the presence of flavonone called naringin, which is said to be bitterer than quinine. Naringin concentration is found to be linked with the maturity of the fruit. As the fruit ripens, concentration of the Naringin tends to decrease [17]. Natural grape fruit contains 0.017-0.025% Naringin and the orange peel contains 0.036% Naringin which is responsible for making the juice bitter. The taste threshold of naringin to impart bitterness is about 50 mg L^{-1} [16]. In the past, reduction of Naringin content was carried out using chemical methods which had several drawbacks resulting in the inferior quality of fruit juice. Debittering of the juice using enzyme naringinase is a promising approach as it causes minimal damage to the nutritional quality and enhances organoleptic properties.

Naringinase is an enzyme complex, containing α-rhamnosidase (EC 3.2.1.40) and β-glucosidase (EC 3.2.1.21). Naringin hydrolysis is a two step reaction where α-rhamnosidase first splits the naringin to L-Rhamnose and Prunin and β-glucosidase hydrolyses Prunin into non-bitter Naringenin and D-Glucose. Apart from debittering of citrus juices, Naringinase also finds applications in the production of glycopeptide antibiotic. Chloropolysporin from *Faenia interjecta* is having strong activity against *Staphylococcus aureus* and *Enterobacteria*[12].

Naringenin is shown to have suppressive effect on adhesive glucan formation by *Streptococcus mutans* on human dental plaque[7].With wide range of applications, Naringinase has indeed become one of the biotechnologically important enzymes.

Naringinase has been reported in plants, yeasts, fungi and bacteria[13]. Microbial enzymes are gaining special importance in the recent days due to cost effective production and economically viable process. Microbial naringinases has completely replaced the chemical methods of Naringin reduction in industries. Production of Naringinase has been very well studied in fungal sources, however very limited reports are available on bacterial Naringinase. Naringinase is still a very expensive enzyme and is being sold by the Japanese producer with a price tag of 360 $/kg [8]. From this it appears that there is a great scope for fermentation process development using new isolates, which would result in the commercially viable processes.

Thus, isolation of new promising naringinase producers is the need of the hour. Once new strains are isolated, identification of nutritional factors such as carbon and nitrogen sources and their effect on the production needs attention. In the present study, an effort is made to isolate new bacterial strains. Further, screening of different carbon and nitrogen sources to enhance the production of naringinase is carried out.

Material and Methods

All the chemicals used were of analytical grade. Naringin was procured from Sigma Aldrich (St. Louis), USA.

Isolation and identification of naringinase producing bacteria: About 1g of soil sample was added to 100 ml of Nutrient broth, incubated in Incubator shaker for 48 hours at 30˚C, 150 rpm. 1 ml of turbid broth was serially diluted and then inoculated into a selective medium[14] having the composition (per L) 5g NH_4NO_3, 0.2g KCl, 0.4g KH_2PO_4, 0.01g $FeSO_4 \cdot 7H_2O$, 0.01g $ZnSO_4$, 0.01g $MnSO_4$,15g Agar, 0.2g $MgSO_4 \cdot 7H_2O$ and 1g Naringin and its initial pH was 6 at 30°C. The cultures showing substantial growth were transferred to fresh medium. After three more transfers, turbid broth was diluted appropriately and spread plated on nutrient agar and discrete colonies were picked up and were preserved at 4˚C on nutrient agar slants. Strains were identified based on their morphological, physiological and biochemical characteristics (Table 2) according to Bergey's Manual of Determinative Bacteriology[4].

Screening of the production medium: Three different media (Table 1) were screened for the production of naringinase. The pH of these media was adjusted to 6 after the preparation before steam sterilization.

Preparation of inoculum: Inoculum was prepared by transferring loop full of culture into 250ml Erlenmeyer flask containing 100ml nutrient broth and incubated in an Orbital shaker 150 rpm and 30°C. A 12h old culture[10] was used as the inoculum for the fermentation medium.

Fermentation: 5% (v/v) culture[10] was inoculated into 100ml of media in a 250 ml Erlenmeyer flask and incubated for 48h at 30°C (pH 6). Contents of the flasks were centrifuged at 8000 rpm for 15min and cell free supernatant was used for the determination of naringinase activity.

Estimation of naringinase activity: The fermented culture solution was centrifuged at 10000 rpm for 10min at 4°C and supernatant was collected for estimation of naringinase activity. 0.1 mL supernatant was mixed with 0.9 mL of 0.05% (w/v) naringin dissolved in 0.1 M sodium acetate buffer (pH 4.0). The mixture was stirred for 30 min at 50°C and the reaction was terminated with 0.5mL Trichloroacetic acid (10%, w/v). Then, 0.1mL of the reaction mixture was added to 5mL of 90% (v/v) diethylene glycol followed by the addition of 0.1mL of 4N NaOH. This mixture was kept for colour development at room temperature at 10min. The resulting yellow colour was measured at 420nm. One unit (U) of naringinase activity was defined as the amount of enzyme that could hydrolyze 1 μmol of naringin/min at the assay conditions[4].

Biomass estimation: The growth of the organism was estimated by measuring the biomass content of the fermentation broth every 12 h interval up to 48h of incubation. The broth was centrifuged at 8000 g for 5 min at 4°C. The sediment was dried at 80°C for 24h in a preweighed eppendorf tubes. The weight of the biomass obtained was expressed in g/L.

Effect of different carbon and nitrogen sources: Different carbon sources (0.5%, w/v) tested was Glucose, Sucrose and Rhamnose and the nitrogen sources (0.3%, w/v) were peptone with sodium nitrate, peptone with ammonium nitrate and yeast extract. The Naringinase production and biomass content was determined during the course of fermentation.

Results and Discussion

Isolation and Identification: Four pure cultures were obtained as an outcome of isolation activities and were named as 1A, 1B, 2DP and 3P. All the four cultures were grown in three media (Table 1) and the naringinase activities of the cell free broth was determined every 12 hours till 48 hours. One isolate 2DP showed consistently high activities in all the 3 media (Fig. 1). This isolate was chosen for further investigations. Various morphological, physiological and biochemical characteristics were studied according to Bergey's manual of Determinative Bacteriology[4]. Based on the results (Table 2), the chosen organism (2DP) was presumptively identified as *Serratia* Sp.

More than 30 reports have been published so far on new microbial sources of naringinase. Among them, only one paper has reported the production of naringinase from bacteria[13]. Puri et al[10] have reported the isolation and fermentation studies of *Staphylococcus xylosus* MAK2. A quick survey of literature pertaining to the hydrolysis of various biomolecules shows that a large variety of bacteria are found to produce hydrolytic enzymes. As naringin is a relatively simple, energy rich molecule produced by plants, it is highly likely that several varieties of bacteria possess the ability to metabolize these kinds of natural biomolecules. However, in case of Naringin hydrolysis, only one bacterium was reported so far. In this context, successful isolation of four strains of extracellular Naringin producers from soil is a significant development.

Screening of the production medium: Among three media tested for naringinase production, medium I was a synthetic medium having nitrate as the nitrogen source and Naringin as the sole carbon source. Medium II had peptone as the organic nitrogen source and Naringin as the carbon source. Medium III had yeast extract as the nitrogen source and naringin as the carbon source. Each media had different salt components. Among these three different media studied, Medium III gave Naringinase production of 2.2 U/L, while Medium II gave 2 U/L. Among them, though Medium III showed the highest productivity, due to the interference caused by its ingredients during assay, it was eliminated. Medium II was taken for all the further trials.

Effect of various carbon sources on naringinase production: Glucose, rhamnose and sucrose were the 3 different carbon sources studied. Inclusion of glucose in the medium resulted in the peak production of 5 U/L after 36 h of fermentation with 4 g/L biomass production (Fig.2). Sucrose showed a moderate naringinase yield (3.44 U/L) with high biomass production (5.6 g/L) after 36 h of fermentation. Rhamnose inclusion resulted in the reduction of enzyme productivity, which is in concurrence with earlier reports[1,11]. As glucose enhanced the production, glucose (5g/L) was used in further trials.

Effect of various nitrogen sources on naringinase production: Among all the nitrogen sources tested with glucose as carbon source, peptone supplemented with ammonium nitrate showed highest enzyme production (9.18 U/L) after 36h of fermentation (Fig. 3). Yeast extract also exhibited significant naringinase production (8.3 U/L) and also favored maximum biomass production. Peptone supplemented with $NaNO_3$ did not show significant effect on naringinase production (5.16 U/L). The pH

measurement towards the end of fermentation showed that the pH was 7.9. The reason for this could be that when organism utilizes $NaNO_3$, both Na^+ and nitrate ions cause drift in the pH of the medium towards alkalinity. As the bacterium was isolated from organic rich soil, perhaps the production of naringinase requires acidic conditions and the alkaline pH had inhibited naringinase production. Several authors[9,15] have reported that Peptone and Yeast extract were found to increase the naringinase production.

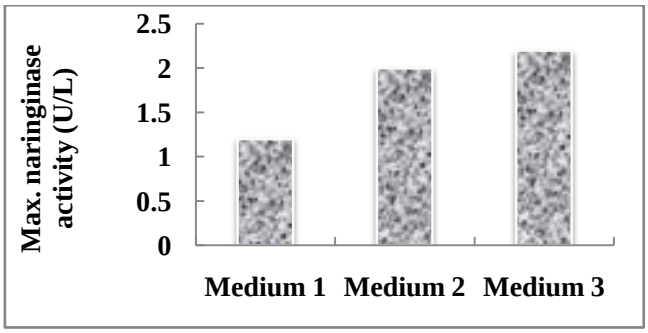

Fig. 1: Screening of media for the production of naringinase

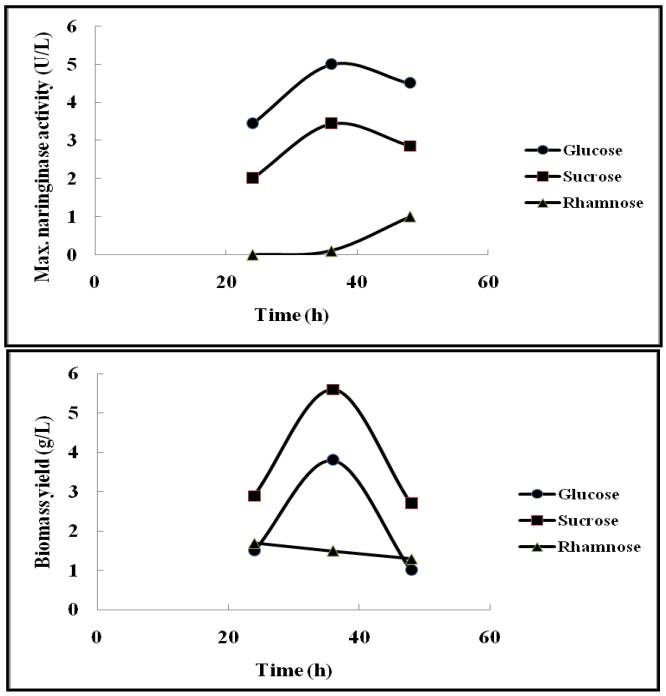

Fig.2: Effect of carbon sources on naringinase and biomass production

Conclusion

Glucose enhanced enzyme production which was contrary to the earlier reports on fungi and bacteria. Among different nitrogen sources studied, yeast extract gave maximum biomass yield while peptone with ammonium nitrate gave higher enzyme production, indicating organism needs both organic and inorganic nitrogen sources for the enzyme production and growth. The enzyme activity was enhanced to 9.18 U/L as an outcome of shake flask trials, which is quite encouraging. Further, optimization of the culture conditions and media using statistical approach is envisaged to increase the enzyme production.

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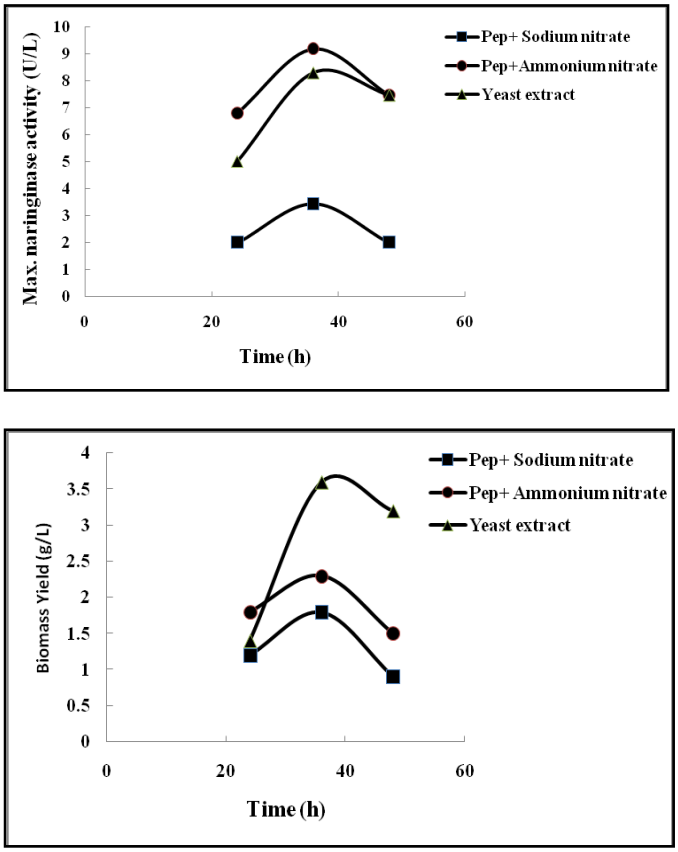

Fig. 3: Effect of nitrogen sources on naringinase and biomass production

Table 1
Different media used for screening

Medium 1 (g/L)	Medium 2 (g/L)	Medium 3 (g/L)
Naringin- 1	Naringin-1	Naringin-1
$NaNO_3$ - 3	Peptone-3	Yeast extract-2
$MgSO_4$- 0.5	$MgSO_4$-0.5	$MgSO_4$-0.5
$CaCl_2$ - 0.5	KCl- 0.5	$FeSO_4$- 0.5
K_2HPO_4- 1	KH_2PO_4- 1	NaCl-0.5
		K_2HPO_4- 0.5 KH_2PO_4- 0.5

Table 2
The morphological, physiological and biochemical characteristics used to identify 2DP isolate.

Colony configuration	**circular**
Colony margin	entire
Colony elevation	convex
Colony surface	smooth
Gram staining	Negative
Pigmentation	Pink
Cell shape	rod
Motility	motile
Spore	absent
Indole	Negative
Simmon citrate	Positive
Voges- Proskaer	Positive
Lysine Decarboxylase	Positive
H_2S production	Negative
Urea hydrolysis	Negative
Catalase	Positive

References

1. Bram B. and Solomons G. L., Production of the enzyme naringinase by *Aspergillus niger*, *App. Environ. Microbiol.,* **13**, 842–845 **(1965)**

2. Caldini C., Bonomi F., Pifferi P.G., Lanzarini G. and Galente Y. M., Kinetic and immobilization studies on fungal glycosidase for aroma enhancement in wine, *Enzyme Microb. Technol.,* **16**, 286-291 **(1994)**

3. Daniels L., Linhardt R.J., Bryan B.A., Mayerl F. and Pickenhagen M., Methods for producing rhamnose, US Patent, **4**, **933,** 281 **(1990)**

4. Davis D.W., Determination of flavonones in citrus juice, *Anal. Chem.,* **19**, 46–48 **(1947)**

5. Elujoba A. A. and Hardman R., Diosgenin production by acid and enzymatic hydrolysis of fenugreek, *Fitoterapia,* **58**, 299–303 **(1987)**

6. Manzanares P., DeGraff L. H. and Visser J., Purification and characterization of a α-L-rhamnosidase from *Aspergillus niger*, *FEMS Microbiol. Lett.,* **15**, 279-283 **(1997)**

7. Masayoshi I., Masaru U., Takefumi I. and Yoshikatsu N., Flavonoids as a possible preventive of dental caries, *Agric. Biol. Chem.,* **48**, 2143-2145 **(1984)**

8. Ni H., Li L., Xiao A., Cao Y., Chen Y. and Cai H., Identification and characterization of a new naringinase producing strain, *Williopsis californica* Jmudeb007, *World J Microbiol Biotechnol*., **27(12),** 2857-2862 **(2011)**

9. Norouzian D., Hosseinzadeh A., Nouri Inanlou D. and Moazami N., Production and partial purification of naringinase by *Penicillium decumbens* PTCC 5248, *World J Microb. Biot.,* **16**, 471-473 **(2000)**

10. Puri M., Kaur A., Barrow C. J. and Singh R. S., Citrus peel influences the production of an extracellular naringinase by *Staphylococcus xylosus* MAK2 in a stirred tank reactor, *Appl. Microbiol. Biotechnol.,* **89(3)**, 715-722 **(2010)**

11. Puri M., Banerjee A. and Banerjee U. C., Optimization of process parameters for the production of naringinase by *Aspergillus niger* MTCC 1344, *Process Biochem.,* **40**, 195–201 **(2005)**

12. Puri M. and Banerjee U. C., Production, purification and characterization of the dibittering enzyme naringinase, *Biotechnol Adv.,* **18**, 207–217 **(2000)**

13. Ribeiro M.H., Naringinases: occurrence, characteristics and applications, *Appl. Microbiol. Biotechnol.,* **90**, 1883-1895 **(2011)**

14. Thammawat K., Pongtanya P., Juntharasri V. and Wongvithoonyaporn P., Isolation, preliminary enzyme characterization and optimization of culture parameters for production of naringinase isolated from *Aspergillus niger* BCC 25166, *Kasetsart J Nat. Sci.,* **42**, 61–72 **(2008)**

15. Vinoth Kumar V. and Revathi Babu P.K.S., Optimization of Fermentation Parameters for Enhanced Production of Naringinase by Soil Isolate *Aspergillus niger* VB07, *Food Sci. Biotechnol.,* **19(3)**, 827-829 **(2010)**

16. Yalim S., Ozdemir Y. and Ibrahim Ekiz H., Naringin in Turkish Orange Juices and Its Reduction by Naringinase, *J Food Drug Anal.,* **12**, 273-276 **(2004)**

17. Yousof S., Ghazali H. M. and King G. S., Naringin content in local citrus fruit, *J. Food Chem.,* **37**, 113-117 **(1990).**

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