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ISOLATION AND CHARACTERIZATION OF ENZYME NARINGINASE FROM *Aspergillus flavus*

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ABSTRACT

Naringin is the dominant flavanoid bitter principle in citrus fruit juices. The microorganism *Aspergillus flavus* were screened for the production of naringinase, in order to hydrolyze the naringin for removal of the bitter taste from juices. The Present study was conducted to isolate and characterize the naringinase enzyme from *Aspergillus flavus.* We elaborated the easy procedure for screening the naringinase producing microorganism by using 1% ferric chloride without assay methods. It is found that ferric chloride reacted with naringenin to give reddish brown color. The effect of pH and temperature were determined. This study revealed that the enzyme activity of the crude enzyme was 206 U/L. the pH profile of naringinase showed maximum enzyme activity at pH 3.8 to 5.0 with one peak at pH 4.5. The temperature vs. Enzyme activity showed one main peak and optimum at 45^{o}C. Naringenin, the end product of naringinase action on naringin was found to be best inducer at the concentration of 0.08 mg/L. Study on the effect of metal ions suggests that Mg^{2+} and Ca^{2+} ions are required for the better activity of the enzyme naringinase by *A. flavus* where as Fe^{2+} and Mn^{2+} show an inhibitory action on growth and enzyme production by *A. flavus.* The paper concludes that *Aspergillus flavus* can be source of naringinase enzyme for industrial purposes.

Keywords: Naringinase activity, Characterization, *Aspergillus flavus,* Naringenin, Ferric chloride.

[1] INTRODUCTION

Naringin, a bitter flavonone glycoside which is responsible for the bitterness in citrus fruits [1]. Naringin which consists of aglycone naringenin (4, 5, 7 - trihydroxy-flavonone) and sugar complex of α-L-rhamnoses and β-D-glucose [2]. Naringinase (E.C.3.2.1.40) is an enzyme which can hydrolyze the naringin into prunin and then into naringenin, which is non-bitter and tasteless. Hence this enzyme has two different enzyme activities on naringin. One is α-L-rhamonosidase (E.C.3.2.1.40) which can act on naringin and it releases prunin (aglycone and β-D-glucose) and α-L-rhamnose, second is β-D-glucosidase (E.C.3.2.1.21) which acts on prunin and releases aglycone naringenin and β-D-glucose in **[Figure-1]**[6].

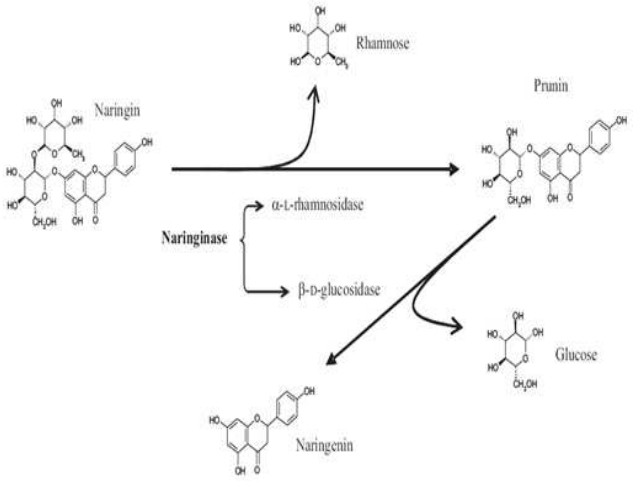


Fig. 1. Hydrolysis of naringin into prunin, rhamnose, glucose and naringenin by naringinase containing α-L-rhamnosidase activities and β-D-glucosidase activities

Naringinase has significant application in fruit juice industry to de-bitter the citrus fruit juices during its processing, and helps to improve the properties and stability of the juices [7]. For the industrial use the naringinase can be immobilized to reduce the cost of removing the bitterness from the juice. Many studies were carried out in the immobilization process [3]. The debittering process could be more cost effective and economically viable if naringinase production is achieved industrially using microorganisms. With this background, the study was conducted to isolate and characterize the extracellular naringinase from *Aspergillus flavus.*

[II] MATERIALS AND METHODS

2.1 Chemicals

Naringin and Naringenin were obtained from sigma, St. Louis, USA. The other culture media (Potato dextrose Broth and Agar) and different carbon and nitrogen sources were obtained from Hi-Media, Laboratories, Mumbai, India. All other reagents used were of analytical grade.

2.2 Isolation of Microorganism and its cultivation condition

Aspergillus flavus was obtained from the soil culture of Namakkal Poultry House, maintained on sterilized potato dextrose agar medium. The isolated species were grown on appropriate culture medium and culturing conditions. Based on morphology *Aspergillus flavus* were isolated and subcultured on PDA and PDB and it was incubated. The slants of *Aspergillus flavus* medium were stored at 5^{o}C in the refrigerator and sub cultured every month. All the culture media, unless otherwise stated, were sterilized at 15 lbs/inch2 pressure (121^{o}C) for 15 minutes. The vegetative spores, taken from slants and suspended in 0.85% sterile sodium chloride, were inoculated in the potato dextrose medium, pH 4.5. Flasks were incubated (28oC, 200rpm) in a rotary shaker for 8-10 days. Samples were withdrawn aseptically at regular time intervals and analyzed for naringinase activity. To study the effect on enzyme production, starch (carbon, 1%, w/v), peptone (nitrogen, 1%, w/v) were added to the culture medium from preliminary studies. The culture supernatant from the organisms was estimated for the protein concentration by Lowry method [5]. The maximum protein concentration was observed after 6 day old culture. Hence the sixth-day-old culture supernatant was used for all the activities.

2.4 Assay for naringinase activity

Naringin was estimated using Davis method. To the 0.3ml of pH 4 .0 sodium acetate buffer 1 ml of 0.1% naringin were added and 0.2ml of enzyme supernatant was added and incubated at 50^{o}C for 1 hr. From the incubated mixture 0.1 ml aliquot was taken and added in 5 ml of 90% diethylene glycol. The naringin present in the sample will give yellow color and the intensity is measured at 420nm [4].

2.5 Screening of Naringinase producing Organism

Naringinase producing organism was screened using 1% ferric chloride without using assay methods. It is found that ferric chloride reacted with naringenin to give reddish brown color [8]. Naringenin, the end product of naringinase action on naringin. Hence the potato dextrose agar medium containing naringin was used to grow the

naringinase producing organism. If the organism produces naringinase then the naringin present in the medium will be cleaved and the end product naringenin reacts with ferric chloride and produces reddish brown color. Hence this was tested with commercial naringenin.

2.3 Characterization of crude enzyme

2.5.1 Effect of pH

The optimal pH of the crude enzyme was found by dissolving the naringin at various sodium acetate buffer concentrations varies from pH 3.0 to 7.0.

2.5.2 Effect of Temperature

With the optimum pH of the crude enzyme the optimum temperature was found by incubating the enzyme with the substrate at varying temperature ranges from 30 to 70ºC.

2.5.3 Effect of metal ions

Different metal ions were used in the cultivation medium to determine the effect of metal ions on growth and naringinase production by *A.flavus*. Fe^{2+}, Mg^{2+}, Mn^{2+}, Ca^{2+}, Cu^{2+} were used at different concentrations of 5mM, 10mM and 30mM and the residual activity of the enzyme was found based on the assay condition.

2.5.4 Effect of inducers

To the PDB medium, the two studies were conducted to increase the activity of naringinase.

1. Effect of naringin
2. Effect of naringenin

To the culture medium of *A.flavus* different concentration of naringin and naringenin were used. Naringin concentration of 50 mg/L, 100 mg/L, 150 mg/L and 200 mg/L were taken to study the effect as inducer. Similarly, naringenin concentration of 0.002 mg/L, 0.004mg/L, 0.006 mg/L and 0.008 mg/L were taken to study the increase in activity of the naringinase enzyme.

[III] RESULTS AND DISCUSSION

The study investigated the screening method using 1% ferric chloride solution on naringinase producing organism directly over the petriplate in **[Figure 2]**, which shows the organism in the 3rd plate is producing Naringinase. Reddish brown color is due to the end product naringenin is confirmed in **[Figure 3]**. But again the test was done with the end product glucose to prove that the color was only due to naringenin in **[Figure 4]**.

Fig: 2. Screening using ferric chloride
1-Control plate with ferric chloride, 2-organism not producing naringinase, 3-organism producing naringinase, 4- organism producing naringinase in higher level, *Aspergillus flavus*

Fig: 3. Confirmatory test with pure naringenin 1-1% ferric chloride, 2-Naringin, 3-Naringenin, 4-Rutin

Fig: 4. Confirmatory tests with the another end product glucose

3.1 Characterization of crude naringinase enzyme

3.1.1 Effect of pH

The optimum pH of crude soluble naringinase was found to be pH 4.5 for *Aspergillus flavus* in **[Figure 5]**. The activity of the enzyme remains good at pH 5.0 also. Hence this enzyme can be used in wider range from low to high pH during the processing of citrus fruits. This pH obtained is comparatively same pH as reported in *Penicillium decumbens* pH 4.5 [9] and high in *Aspergillus niger* pH 4.0 [8]

3.1.2 Effect of temperature

The optimum temperature was found as 50°C for *Aspergillus flavus* in **[Figure 6].** This temperature is low as reported in *Penicillium decumbens* 55°C [9] and same as reported in *Aspergillus niger* 45°C [8].

Fig: 5. Optimum pH for the crude Naringinase enzyme in *Aspergillus flavus*

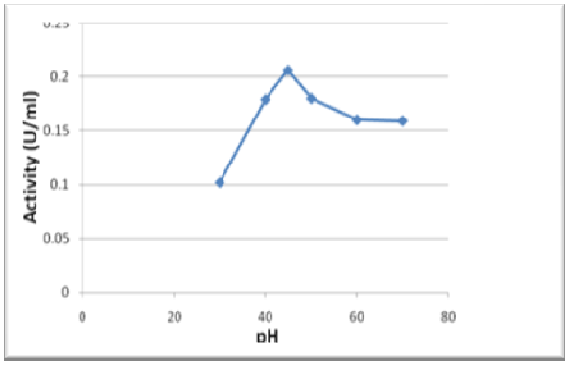
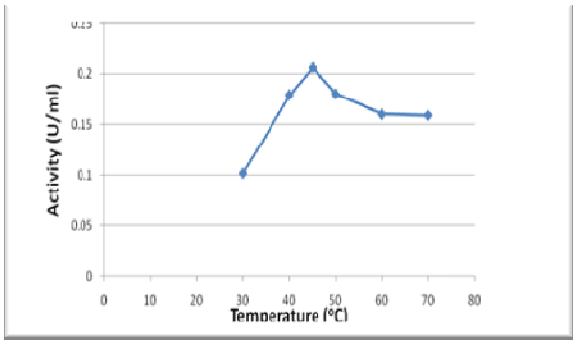


Fig: 6. Optimum temperature for the crude Naringinase enzyme in *Aspergillus flavus.*



3.1.3 Effect of inducers

Different concentration on naringin and naringenin were used to determine the best inducer of naringinase production from *A.flavus* (Table 1 & Table 2)

[Table-1]

Naringin (mg/L)	Activity (U/ ml)
50	1.007
100	1.002
150	0.957
200	0.988

Table: 1. Effect of naringin as inducer in naringinase activity from *A.flavus*

[Table -2]

Naringenin (mg/L)	Activity (U/ml)
0.02	0.690
0.04	1.152
0.06	1.158
0.08	1.160
0.1	1.146

Table: 2. Effect of naringenin as inducer in naringinase activity from *A.flavus*

The results revealed that the increasing concentration of naringenin increases the enzyme activity.

3.1.4 Effect of metal ions

Different metal ions were used in the cultivation medium to determine the effects of metal ions on growth and naringinase production by *A. flavus*. Fe^{2+}, Cu^{2+} and Mn^{2+} show an inhibitory action on growth and enzyme production by *A. flavus.* Along with Co^{2+}, Ni^{2+}, were found to be inhibitory both to growth and naringinase production by *A. niger* [8]. Cu^{2+} at a concentration of 5mM showed high inhibition of enzyme activity whereas Ca^{2+}, (5–10 mM), Mg^{2+}, (5–10 mM) stimulated naringinase synthesis. In the case of Mg^{2+}, the maximum activity 1.2 U ml−1 was observed at 10mM which accounted for a 24% increase in enzyme activity. Above 10mM enzyme activity decreased drastically. Ca^{2+} at 5–10mM also supported maximal production (1.1 U ml−1) of naringinase whereas at higher (30 mM) concentrations, a small decrease (0.9 U ml−1) in enzyme activity was observed. This suggests that Mg^{2+} and Ca^{2+}

ions are required for the production of naringinase by *A. flavus*. Fe^{2+} and Mn^{2+} show an inhibitory action on growth and enzyme production by *A. flavus* in **[Figure 7].**

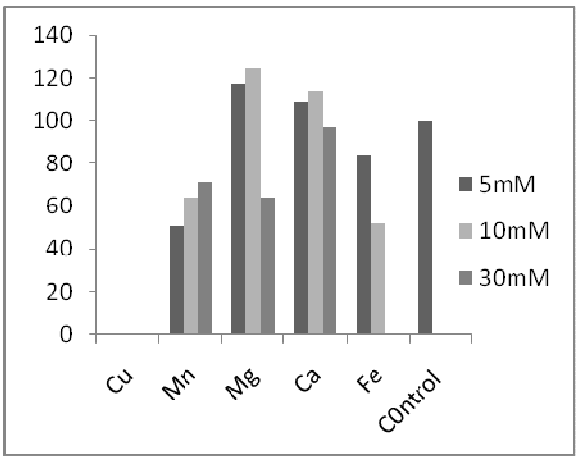


Fig : 7.
Residual activity profile of metal ions for naringinase

[IV] CONCLUSION

The present study investigated the naringinase activity obtained from *Aspergillus flavus* by establishing an easy screening method using ferric chloride. Using this easy procedure we studied the characterization of naringinase enzyme in different pH, temperature, inducer and metal ions. The study of naringenin on naringinase activity showed that it was a good inducer for the production of naringinase.

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