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Full Length Research Paper

Comparative studies on inducers in the production of naringinase from *Aspergillus niger* MTCC 1344

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This research provides detailed systematic study of the effect of different inducers (hesperidin, naringenin, naringin, rhamnose and rutin) in naringinase production by *Aspergillus niger* MTCC 1344. Cultures were carried out in shake flasks and they produce extracellular naringinase in a complex (molasses, peptone and salts) medium. The optimized concentration (%) of naringin, rhamnose, naringenin, rutin and hesperidin for maximized naringinase production are 0.1, 0.375, 0.01, 0.2 and 0.2, respectively. Compared with control, inducers increased the naringinase production by many folds in the order of naringin (6.63) > rhamnose (4.87) > naringenin (3.26) > rutin (2.84) > hesperidin (2.35). Under optimum conditions, about 9.68 units of enzyme per ml complex medium containing naringin were obtained on the 7th day. The activity to inducer (A/I) ratio was 968 Ug^{-1} naringin, and the cultivation time was shorter in submerged production. The results indicate that naringinase activity used naringin as an inducer which was significantly higher than the other four inducers. Therefore naringin is recommended for naringinase production.

Key words: Naringin, naringenin, rutin, hesperidin, rhamnose, naringinase, *Aspergillus*, inducer, molasses.

INTRODUCTION

Flavonoids like naringin and naringenin, rutin, quercetin, and hesperidin are functional chemicals with important properties in the fields of health care, cosmetics, food, and agriculture (Sekeroglu et al., 2006). Naringin, composed of aglycon naringenin and the disaccharide neohesperidose, is the principle bitter flavanone glycoside and the primary bitter component in grapefruit juice (Ferraria et al., 2008). Enzymatic hydrolysis is a possibility to overcome the bitterness and obtain compounds with improved biological activities. Naringinase is an enzyme complex composed of *α*-L-rhamnosidase (*α*R, EC 3.2.1.4) and *β*-D-glucosidase (*β*G, EC 3.2.1.21). Naringin can be hydrolyzed by α-L-rhamnosidase to rhamnose and prunin (one third of the bitterness of naringin), which can be further hydrolyzed by the β-D-glucosidase into glucose and tasteless naringenin. The potential application of naringinase is used in the debittering of grapefruit juice (Puri and Banerjee, 2000), deglycosylation of novel glycopeptide antibiotics, chloropolysporin (Sankyo, 1988); hydrolysis of hesperidin to release hesperetin glucoside, an important precursor in sweetener production (Chase, 1974); preparation of a rhamnose and prunin from naringin (Daniel et al., 1990), release of flavour compounds from terpenyl glycosides for the enhancement of aroma in grape juices and the derived beverages (Gunata et al., 1988) help in transformation of steroids like sapogenins and diosgenin (Elujoba and Hardman, 1987) and also in structural analysis of the plant's oligosccahrides, polysaccharides, glucosides and glycolipids produced successfully with rhamnosidase activity of naringinase (Young et al., 1989; Schols and Voragen, 1994).

For variety of applications, it is necessary to increase naringinase production. This can be achieved by optimizing culture conditions, especially by adding enzyme inducers. These inducers are usually natural substrates or substrate analogues for the enzyme. Medium which consists of rhamnose, molasses and corn steep liquor produced higher enzyme titers, as they have low carbohydrate contents. Naringinase is one of inducible enzymes (Puri and Banerjee, 2000), where the continuous or stepwise addition of an inducer increases its production (Mateles et al., 1965).

No reports were found regarding the effects of different substrates on naringinase production. This work gave a study of the effect of different elicitors (flavanoids and

sugars), on naringinase production from *Aspergillus niger* MTCC 1344. These results can provide a reference for the inducer selection for similar studies in the future.

MATERIALS AND METHODS

Medium and culture conditions

Aspergillus niger MTCC 1344 was obtained from Microbial type culture collection (MTCC), Punjab, India and maintained on czapeck dox agar. The medium components employed for naringinase production were (g/l) molasses 15; peptone 5; $NaNO_3$ 2.0; KH_2PO_4 1.0; KCl 0.5; $MgSO_4 \cdot 7H_2O$ 0.5; and $FeCl_3$ 0.1 (pH 4.5). 0.5 g/l of naringin, naringenin, rutin and hesperidin were added to different media. Instead of molasses, 5 g/l rhamnose was used in the production medium. A 72 h age and 15% size was used for inoculation. Flasks were incubated (27°C, 200 rpm) in a rotary shaker for 14 days. Samples were withdrawn aseptically at regular time intervals and analyzed for naringinase activity.

Assay

Assay for naringinase activity was carried out by slight modification in Thammawat method (2009) using naringin as substrate. 0.65 of 0.1% naringin in 0.01 M acetate buffer (pH 4.0) react with 0.1 ml of naringinase enzyme for 15 min at 60°C. From that reaction mixture, 0.2 ml was taken, mix with 4 ml of 90% diethylene glycol and 0.2 ml of 4N NaOH. The new mixture was left for 15 min at an ambient temperature with resulted yellow colour and the reading was measured at 420 nm. Determination of the enzyme activity was calculated using pure naringin as standard. One unit (U) of naringinase activity was defined as the amount of enzyme that could hydrolyze 1 µmol of naringin per ml and minute at the assay conditions.

Standards

Rhamnose, rutin (quercetin-3-O-β-D-glucose-[1,6]-O-a-L-rhamnose), hesperidin (hesperetin 7-*O*-β-D-glucose-[1,6]-O-α-L-rhamnose), naringenin (4,5,7-trihydroxyflavonone) and naringin (4',5,7-trihydroxyflavanone-7-rhamnoglucoside) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used were of analytical grade and supplied by Merck (Darmstadt, Germany).

RESULTS

A. niger was grown on a modified czapeck dox medium with rhamnose, naringin, naringenin, rutin and hesperidin as inducers. This organism produces significant amount of extra cellular naringinase in the medium.

Comparison of different substrates on enzyme production

The naringinase activity was observed in all five medium, maximum naringinase was produced in naringin (7.48 IU/ml) followed by rhamnose (6.72 IU/ml), rutin (3.71 IU/ml), naringenin (3.36 IU/ml) and hesperidin (2.11 IU/ml). Among the substrates, naringin act as a selective inducer, because it induces faster and maximized naringinase production (7^{th} day). Rhamnose induced production of naringinase on the 7^{th} day but the enzyme activity was lower than when naringin was used as the inducer. Whereas hesperidin induced naringinase production on the 5^{th} day but the enzyme activity was not detected on the 8^{th} day. With rutin and naringenin, naringinase was produced on the 8^{th} day but the enzyme activities were not distinguished on the 11^{th} day. Figure 1 describes the effect of different inducers on enzyme production.

Optimization of inducer concentration on enzyme production

The addition of flavanoids (hesperidin, rutin, naringin and naringenin) with various concentrations; 0.01, 0.05, 0.1, 0.15, 0.2, 0.25, 0.5% and a rhamnose sugar with various concentrations; 0.1, 0.25, 0.375, 0.5, 0.75 and 1.0% to the fermentation medium and their effect were studied. The optimized concentration (%) of naringin, rhamnose, naringenin, rutin and hesperidin used are 0.1, 0.375, 0.01, 0.2 and 0.2, respectively, and their corresponding enzyme productions (IU/ml) are 9.68, 7.12, 4.76, 4.19 and 3.44 (Figure 2).

DISCUSSION

Naringinase is an inducible enzyme; stepwise addition of small amounts of naringin was more effective than a higher concentration of the substrate added at the beginning of the fermentation. Reported inducers for naringinase production are rhamnose (Thammawat et al., 2008), hesperidin (Fukumoto et al., 1973), naringin (Bram and Solomons, 1965; Puri et al., 2009), naringenin (Puri et al., 2005). Different flavanoids and sugars tested for the stimulation of naringinase production showed an increase in enzyme activity. The optimized results concerning naringinase production are shown in Figure 2. The highest level of naringinase was obtained in the presence of naringin (seven fold increases referred to complex medium). Rhamnose and naringenin increased naringinase levels five and three fold respectively, and the rest of the compounds tested have less significant effect. In all cases, the maximum naringinase activity was found 3 days after addition of the flavanoids and sugar. The maximum naringinase production was observed on the 7^{th} day in both naringin and rhamnose, 8^{th} day in rutin and naringenin and being three days earlier with hesperidin.

It is noted that, both hesperidin and rutin can induce both α-L-rhamnosidase and β-D- glucosidase activity. Fukumoto and Okada (1973) reported the use of hesperidin as inducer for naringinase production by *Penicillium* sp. Rutin can be hydrolyzed by α-L-rhamnosidase to rhamnose and 3-glucosylquercitin, which can be

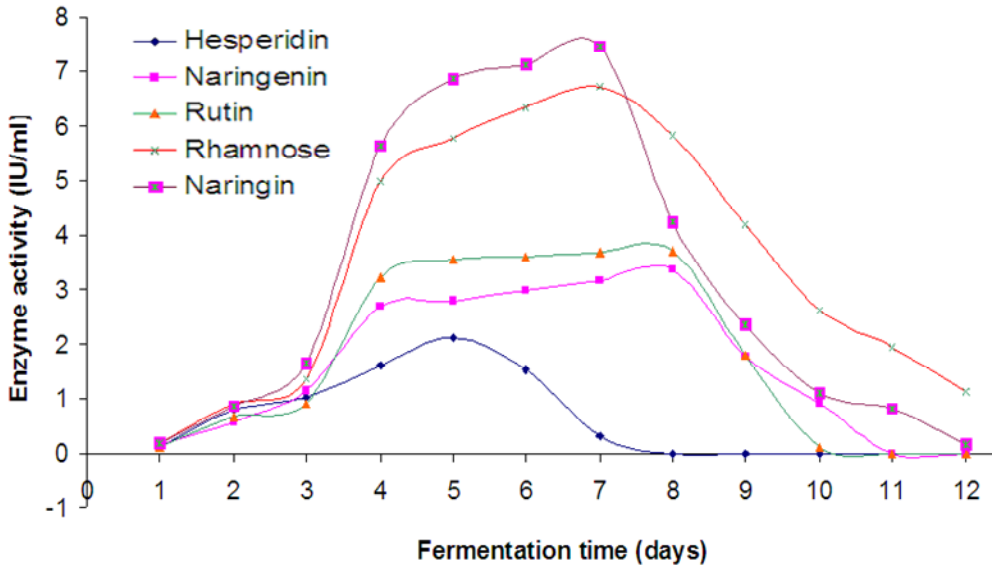

Figure 1. Effect of different inducers on enzyme production. Reaction conditions: Inducer concentration 0.05%; pH 4.5, inoculum age 72 h, inoculum size 15%, temperature 27°C and agitation 200 rpm.

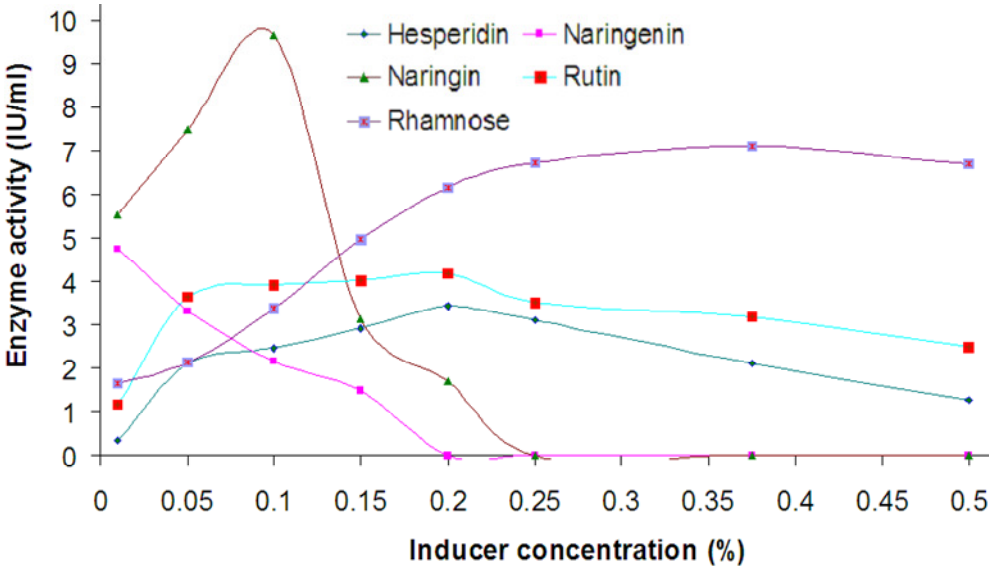

Figure 2. Effect of different concentration of inducers on enzyme production. Reaction conditions: pH 4.5, inoculum age 72 h, inoculum size 15%, temperature 27°C and agitation 200 rpm. Hesperidin, Naringenin, Naringin, Rutin and Rhamnose and their respective fermentation days are 5, 8, 7, 8 and 7.

further hydrolyzed by the β-D-glucosidase component of naringinase into glucose and quercetin (3, 3', 4', 5, 7-penta hydroxyl flavone) (Bokkenheuser et al., 1987). No reports were found for rutin as an inducer in the production of naringinase. Mateles et al. (1965) reported that rhamnose or plant meal containing rhamnose glucoside increased naringinase production. Elinbaum et al. (2002) mentioned that rhamnose could be used as an inducer in the production of *Aspergillus terreus* □-L rhamnosidase by solid state fermentation; however they reported that naringin was a better inducer than rhamnose. Same results were obtained in this study. Naringenin as an end product of naringin hydrolysis also induces naringinase. Production of naringinase by the same strain has been detected in the medium containing 0.05% naringenin (Puri et al., 2005). In *A. niger,* highest

enzyme titer of 385 IU/ml was achieved on 0.1% of naringin concentration (Bram and Solomons, 1965). Recently, Puri et al. (2009) reported that 0.5% of naringin concentration was used in the production of naringinase by *Staphylococcus xylosus* MAK2.

As illustrated, slight deviations from the optimum value gave great decrease in the production, which required a careful check of these inducers. The results indicated that naringinase activity using naringin as an inducer was significantly higher than the other four inducers. The activity to inducer (A/I) ratio is a major impact in any enzyme production. Elinbaum et al. (2002) mentioned that (A/I) ratio was 192 Ug^{-1} naringin by submerged fermentation and 400 Ug^{-1} naringin by solid state fermentation were obtained. Results in this study differ in the submerged fermentation, because the A/I ratio obtained was 968 Ug^{-1} naringin, 190 Ug^{-1} rhamnose, 4760 Ug^{-1} naringenin, 210 Ug^{-1} rutin and 177 Ug^{-1} hesperidin. A/I ratio results indicated that naringenin was the best inducer in naringinase production than naringin. Application of this result can be advantageous for an industrial production of the enzyme considering that the cost of naringin as a raw material is lower than that of naringenin. Therefore, it is recommended based on this study to use naringin as an inducer for the production of naringinase.

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