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Naringinase production by *Aspergillus niger* in the fermentation of different carbon and nitrogen sources

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ABSTRACT

Citrus fruit and frozen concentrated orange juice production is of great commercial importance for Brazil. Excessive bitterness is the main depreciator of juice value on the market. Naringinase, an enzymatic complex that degrades naringin, has potential application for debittering. This study investigated naringinase production by *Aspergillus niger* through sucrose, naringin, molasses and sugarcane juice, supplemented with peptone and sodium nitrate. Naringin, when used in the culture medium as inducer, was added in a single or fractioned doses. The base culture medium contained (g/L): 1.0 KH_2PO_4; 0.5 KCl; 0.5 $MgSO_4.7H_2O$ and 0.1 $FeCl_3$. The fermentations were carried out in 500mL Erlenmeyer flasks with 100mL culture medium, at initial pH 4.5, inoculum with 10^6spores/mL, 28°C and 180rpm. The molasses was more efficient when used at low concentrations for naringinase production, while at high concentrations it only increased invertase production. The greatest naringinase value of 178.6mUI/mL was attained with a combination of molasses (3g/L), peptone (10g/L) and naringin (0.5g/L). The fractioned addition of naringin as inducer did not improve production and therefore it is not recommended. *Aspergillus niger* showed promising potential as a biotechnological tool for naringinase production using a low-cost substrate. © 2010 Trade Science Inc. - INDIA

KEYWORDS

Naringinase;
Molasses;
Aspergillus niger;
Induction;
Bitterness;
Orange juice.

INTRODUCTION

Citrus fruit, mostly oranges, are one of the most produced fruit in the world. In 2008, about 12% of world fruit production was of oranges. Brazil is the largest world producer of oranges, with 18,389,752 ton in 2008[1] and the greatest producer and exporter of concentrated frozen orange juice, with 55% of world production in the same year[2].

The orange and its juice are appreciated throughout the world. However, the presence of a high concentration of naringin, a compound responsible for the characteristically bitter flavor of the juice, gives it an unpleasant flavor that reduces consumer acceptance and thus decreases its market value.

Several technologies have been suggested to

debitter citrus juices, but these alter the organoleptic characteristics of the juices and thus have limited application. However, enzymatic techniques are indicated for debittering, such as the addition of naringinase to the processes[3].

Naringinase, an enzymatic complex with α-L-rhamnosidase (EC 3.2.1.40) and β-D-glucosidase (EC 3.2.1.21) activity, has potential for application in rhamnosidase, prunin and antibiotic production, wine aroma enhancement and in the citric juice industry because it degrades naringin, forming less bitter compounds[3].

Naringinase production by fungal fermentation is interesting because of the possibility of using substrates such as sugarcane juice and molasses, a raw material and byproduct, respectively, from agroindustry that are cheap and widely available in Brazil.

Aspergillus niger is outstanding in biotechnological processes because it produces a variety of enzymes such as cellulose[4], lipase[5], amylase[6] and invertase[7], in addition to several compounds of interest[8-10]. Kishi et al.[11] reported *A. niger* as the best naringinase producer when they tested 96 mold strains and more recently it has been used by several other authors[12-14].

The present study used *A. niger* in sucrose, naringin, sugarcane juice and molasses fermentation for naringinase production.

EXPERIMENTAL

Microorganism

The filamentous fungus *A. niger* isolated from prunes was used, kept in BDA at 4°C and replicated every 30 days.

Fermentation culture medium and culture conditions

The cultures were carried out in 500mL Erlenmeyer flasks containing 100mL sterilized culture medium. The base culture medium contained (g/L): 1.0 KH_2PO_4; 0.5 KCl; 0.5 $MgSO_4.7H_2O$ and 0.1 $FeCl_3$. Naringin was added as inducer to the culture medium in a single dose (0.5g/L) or in fractioned addition, as reported later. The initial culture medium pH was corrected to 4.5, the inoculum was 10^6esporos/mL, the incubation temperature was 28°C and rotation was 180 rpm. The cultures were made in triplicate. The culture medium samplings and naringin addition (SIGMA, St. Louis, USA) were carried out under aseptic conditions. The molasses and sugarcane juice concentration referred to the total sugars.

To study the effects of naringin and sucrose as carbon sources for naringinase production, cultures were conducted with naringin (10g/L), sucrose (10g/L) and the combination of naringin (5g/L) and sucrose (5g/L) added to the base culture along with peptone (10g/L).

To determine the best carbon and nitrogen sources, cultures were carried out with molasses (10g/L) or sugarcane juice (10g/L) or a combination of molasses (5g/L) and sugarcane juice (5g/L) with peptone (10g/L) or sodium nitrate (10g/L) added to the base culture medium. The following concentrations were used to determine the best molasses concentration (g/L): 1.0; 3.0; 5.0; 10.0 or 15.0 with peptone (10g/L). Later peptone was used at concentrations (g/L): 6.0; 8.0; 10.0; 12.0 or 14.0 with molasses (3g/L).

The importance of adding the inducer during fermentation was investigated by adding naringin in a single dose (0.5g/L) at the start of fermentation or in five fractions (0.1g/L) at different fermentation times and at 24 hour intervals to the base culture medium with molasses (3g/L) and peptone (10g/L). Thus four experiments were carried out with fractioned naringin addition. In the first experiment, the naringin fractions were added at the times (0h, 24h, 48h, 72h, and 96h); in the second at times (24h, 48h, 72h, 96h and 120h); in the third at times (48h, 72h, 96h, 120h and144h) and in the fourth at times (72h, 96h, 120h, 144h and 168h).

Samples of 1.2mL of the cultures were removed every 24 hours and centrifuged for 15 minutes at 20.000g and 4°C. The supernatant was used for the analytical determinations of naringinase activity, invertase activity and total reducing sugars.

Assay methods

Naringinase activity was estimated by determining residual naringin using an adaptation of the Davis method[15]. A typical assay mixture comprised of 0.8mL 1g/L naringin dissolved in 0.5mL 0.1M sodium acetate buffer (pH 4) and 0.1mL culture centrifuged sample. The assay mixture was incubated at 50°C for 60min after which 0.1mL aliquot was added to 3mL 90% diethyleneglycol followed by the addition of 0.1mL 4N

NaOH. Samples were kept at room temperature (28°C) for 10min. The intensity of the resultant yellow color was determined at 420nm. One unit of naringinase activity was defined as 1µmol of naringin hydrolyzed under the above assay condition. The results were expressed in mUI/mL. Invertase activity was measured by estimating the release of reducing sugars due to invertase activity on sucrose by the reaction with dinitrosalicylic acid (DNS)[16] (Miller, 1959). The reaction mixture containing 0.5mL of culture centrifuged sample and 0.5mL of 0.5M sucrose in 1.5mL 0.2M acetate buffer (pH 5.0) was incubated at 30°C for 10min. One invertase unit was defined as the amount of enzyme needed to produce one µmol reducing sugar per min under the above assay condition. Total sugars was measured by the method of Dubois et al.[17]. The dry weight of the *A. niger* mycelium was determined after the last day of cultivation by gravimetry after filtering through filter paper and drying overnight at 80 °C.

RESULTS AND DISCUSSION

Effect of naringin and sucrose

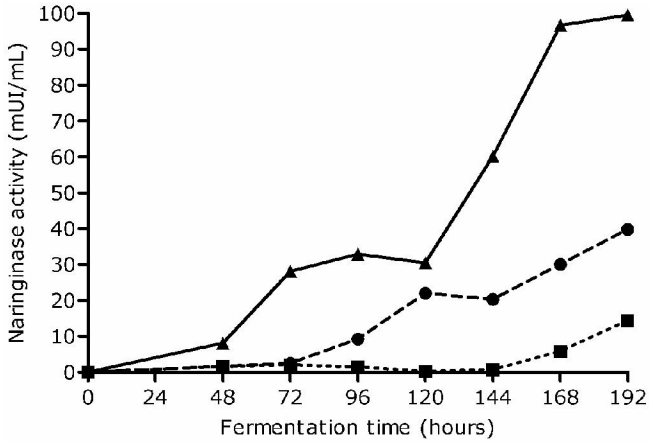

Figure 1 : Naringinase production by *Aspergillus niger* in sucrose and naringin fermentation. Additions were made to the fermentation base culture medium with the nitrogen source peptone (10g/L) and the carbon source naringin or sucrose or a mixture of naringin and sucrose. The values represent the mean of three replications. ▲ Sucrose (5g/L) and naringin (5g/L); ● Naringin (10g/L); ■ Sucrose (10g/L).

Sucrose as single carbon sources did not present significant naringinase activity until 144 hours culture, confirming its repressive role[14,18] (Figure 1). The naringinase value of 14.33mUI/mL (with standard deviation ± 2.3mUI/mL) was reached at 192 hour fermentation. The low naringinase activity, in the stationary phase of the culture, may have resulted from cell lysis and consequent extravasation of constitutional naringinase.

When naringin was used as a single carbon source the maximum activity was 39.80 ± 3.0mUI/mL with 192 hours culture. The results are line with those reported by Orejas et al.[19] who obtained 30.93mUI/mL in naringin fermentation (10g/L) as the only carbon source for *A. niger*. The results obtained showed that naringin acted as an inducer and can be used as a single carbon source for *A. niger*. However, due to the high cost of naringin and the values obtained of naringinase and biomass production, (values not shown), the use of naringin as a single carbon source is not recommended for large-scale naringinase production.

The best activities of 96.61 ± 8.0 mUI/mL and 99.51 ± 4.5mUI/mL were obtained when the carbon sources were combined, from 168 to 192 hours fermentation, respectively. The late naringinase production can be explained by catabolic repression by the sucrose. The microorganism used the sucrose initially, producing biomass, and then used naringin. The catabolite repression by glucose in naringinase production was also reported by Orejas et al.[19].

Therefore, in the subsequent experiments naringin was used only as inducer. Naringin was used as inducer at the concentration of 0.5g/L[12].

Effect of the carbon and nitrogen sources

The use of sodium nitrate or sugarcane juice in the culture medium did not favor naringinase production (Figure 2). Furthermore, the biomass values obtained were less than 4.6g/L. Low naringinase productions with sodium nitrate by *A. niger* were also reported by Bram and Solomons[12] and Puri et al.[14], while Gallego et al.[20] obtained good production with ammonia phosphate using *Aspergillus terreus*.

The greatest activity of 161.5 ± 6.0mUI/mL and 6.0g/L biomass was obtained with the combination of molasses and peptone at 145 hours culture, while with the combination of molasses and sugarcane juice with peptone the activity was 149.6 ± 1.9mUI/mL. Molasses, in addition to supporting the greatest naringinase production, is a byproduct of the sugar industry, cheap

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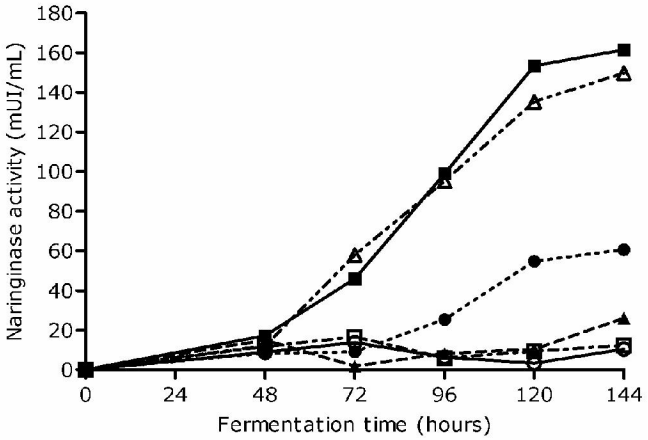

Figure 2 : Naringinase production by *Aspergillus niger* in the fermentation of different carbon and nitrogen sources. Additions were made to the fermentation base culture medium, containing naringin as inducer at the concentration of 0.5g/L, of molasses or sugarcane juice or a combination of molasses and sugarcane juice with peptone or sodium nitrate added. The values represent the mean of three replications. ■ Molasses (10g/L) and Peptone (10g/L); ▲ Molasses (10g/L) and $NaNO_3$ (10g/L); ● Sugarcane juice (10g/L) and Peptone (10g/L); □ Molasses (10g/L) and $NaNO_3$ (10g/L); △ Molasses (5g/L) and sugarcane juice (5g/L) and Peptone (10g/L); ○ Molasses (5g/L) and Sugarcane juice (5g/L) and $NaNO_3$ (10g/L).

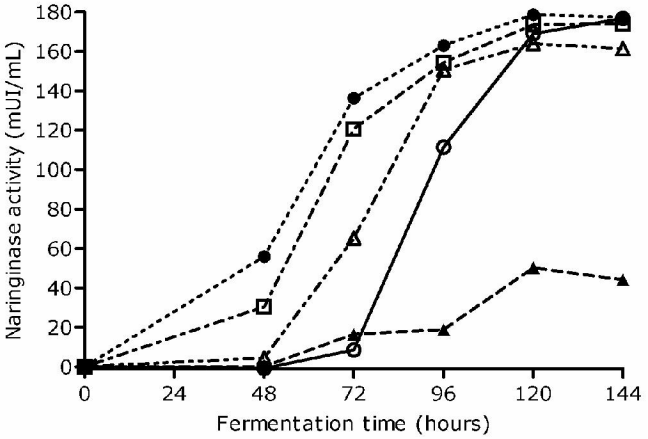

Figure 3 : Naringinase production by *Aspergillus niger* in the fermentation of peptone (10g/L) and different molasses concentrations. Additions were made to the fermentation base culture medium, containing naringin as inducer, with peptone (10g/L) and molasses at the concentrations (g/L): 1.0; 3.0; 5.0; 10.0 and 15.0. The values represent the mean of three replications. Molasses concentrations: ▲ 1g/L; ● 3g/L; □ 5g/L; △ 10g/L; ○ 15g/L).

and available throughout the year. Puri et al.[14] also reported the combination of molasses and peptone as the best for naringinase production by *A. niger* with 6.5UI/mL activity.

The invertase activity was measured because it is also important. The best condition for naringinase production (molasses and peptone) was also the most appropriate for invertase production, reaching 394 ± 15mUI/mL com 144h culture.

Although molasses and peptone have been used as carbon and nitrogen sources, in combination with naringin as inducer, it is important to establish an ideal concentration of these compounds that favors naringinase production. Thus peptone and molasses were used at different concentrations in the subsequent experiments.

Effect of molasses concentration

The best molasses concentration for naringinase production was 3g/L with 178.6 ± 13.1mUI/mL and 120 hours fermentation (Figure 3). Puri et al.[14] also obtained the best production with the lowest molasses concentration (4g/L) at the tested concentrations of 4.0; 6.0; 8.0 and 10.0g/L. The biomass values obtained were proportional to the molasses concentration used (results not shown) but the same did not occur with naringinase. The quantity of molasses influenced the start of enzyme production in the course of the culture, although the final naringinase values were similar. Thus at concentrations of 3g/L and 5g/L activity was detected at 48 hours, while with 10g/L and 15g/L the activity was detected at 72 hours and 96 hours, respectively. Yadav and Yadav[21] also reported influence of the concentration of the carbon sources on the start of naringinase activity during culture.

The comparison of naringinase with invertase, at different molasses concentrations during growth is shown in Figure 4.

The highest molasses concentration (15g/L) resulted in the greatest invertase activity (413 ± 20mUI/mL). Molasses quantities greater than 3g/L did not favor naringinase production, but did induce an increase in invertase production. The repressive effect of a sugar easily fermented on another was reported by Orejas et al. in *A. nidulans*[19] when studying α-L-rhamnosidase production (enzyme belonging to the naringinase complex) when glucose and rhamnose were present in the culture medium. In *A. niger*, the repressive effect of the glucose was also reported by Hanif et al.[22] in wheat bran fermentation and glucose for cellobiohydrolase production.

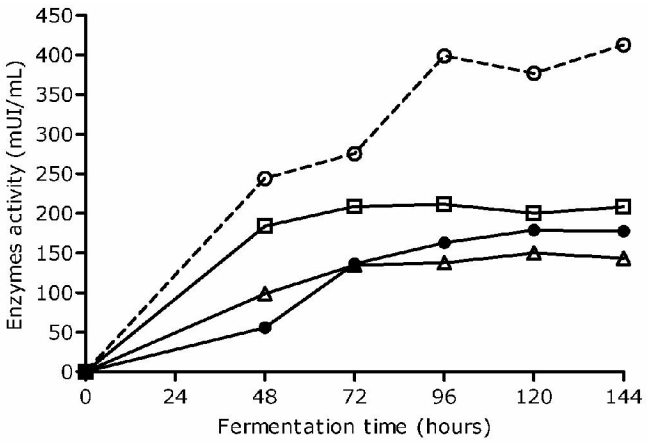

Figure 4 : Naringinase and invertase in peptone (10g/L) and different molasses concentrations fermentation by *Aspergillus niger*. The values represent the mean of three replications. Naringinase – molasses: ● 3g/L; Invertase - molasses: △ 1g/L; □ 5g/L; ○15g/L.

Therefore, the molasses concentration 3g/L was used in the following experiments to provide a greater activity and early naringinase production, and higher concentrations of molasses only favored invertase activity.

Effect of peptone concentration

Additions were made to the fermentation base culture medium, containing naringin as inducer, of molasses (3g/L) and peptone. The results are presented in Figure 5.

The best naringinase production (178.6 ± 13.1mUI/mL) was obtained with 10g/L peptone up to 120 hours fermentation. The increase in the peptone concentration up to 10g/L increased naringinase production but higher concentrations did not improve production. Thus

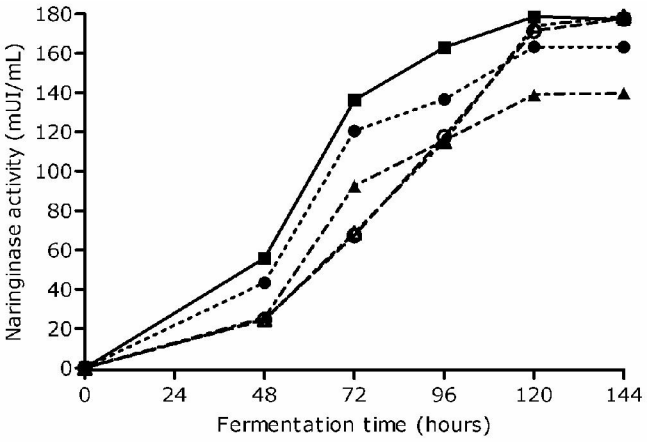

Figure 5 : Naringinase production by *Aspergillus niger* in the molasses fermentation (3g/L) and different peptone concentrations (▲ 6g/L; ● 8g/L; ■ 10g/L; △ 12g/L; ○ 14g/L). The values represent the mean of three replications.

the use of peptone (10g/L) and molasses (3g/L) at a nitrogen:carbon proportion (N:C) of approximately 3:1 seemed to be correct. Bram and Solomons[12] reported a higher proportion of nitrogen (N:C) 16:1 as best when they used two combined sources of nitrogen (yeast extract and corn steep liquor) for naringinase production by *A. niger*. However, Norouzian et al.[23] showed that the 1:1 ratio was the best condition with *Penicillium decumbens* using peptone, while Soria et al.[24] obtained greater production with peptone at the proportion of (N:C) 1:4 with *A. terreus*.

The final experiment was planned to investigate the effects of the method of adding naringin to the enzyme production, because different methods of using this inducer have been studied in naringinase production[12].

Induction method effect

Molasses, peptone and the inducer naringin were added to the fermentation base culture medium in a single or fractioned dose. The results are shown in Figure 6.

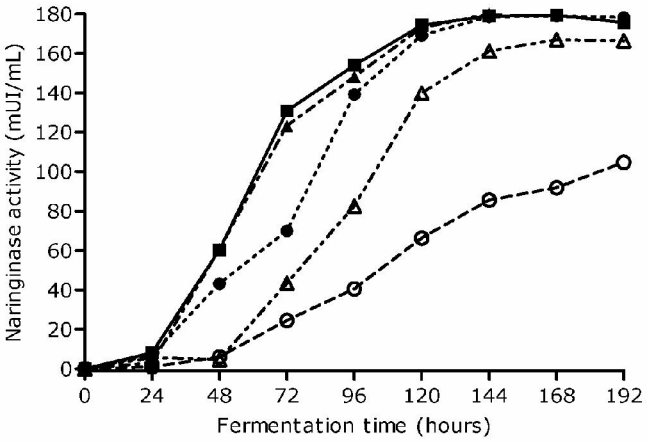

Figure 6 : Naringinase production by *Aspergillus niger* in the fermentation of molasses (3g/L), peptone (10g/L) and naringin in a single dose (▲ 0.5g/L) or fractioned, with the addition of the first of five doses of 0.1g/L at different times (● 0h; ■ 24h; △ 48h; ○ 72h). The values represent the mean of three replications.

The greatest naringinase production (179.9 ± 5,1mUI/mL) was obtained with 144 hours fermentation and the addition of the inducer in a single dose at the start of fermentation. However, the results for the fermentations whose first inducer doses were added at the times zero and 24 hours were similar to those of the best condition obtained. The start of inducer addition at later times (48 and 72 hours) decreased and delayed production. Bram and Solomons[12] obtained results that

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were not conclusive when they varied the naringin concentration added in a single dose. However, when they used a low naringin concentration, added in fractions to the culture medium, there was an increase from 328mUI/mL to 440mUI/mL in naringinase production by *A. niger*.

The results showed that for the line used in this study the fractioned addition of the inducer for naringinase production was not necessary.

A. niger showed promising potential in naringinase production using efficiently (consumption superior to 85% in all the experiments) molasses (3g/L) supplemented with peptone (10g/L) and in the presence of naringin. The use of this microorganism of biotechnological importance, fermenting an inexpensive and widely available culture medium, represented an advance for the biotechnological production of this important enzyme, particularly in a developing country such as Brazil.

CONCLUSIONS

A. niger showed promising potential in naringinase production using efficiently (consumption superior to 85% in all the experiments) molasses (3g/L) supplemented with peptone (10g/L) and in the presence of naringin. The use of this microorganism of biotechnological importance, fermenting an inexpensive and widely available culture medium, represented an advance for the biotechnological production of this important enzyme, particularly in a developing country such as Brazil.

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