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Isolation, Preliminary Enzyme Characterization and Optimization of Culture Parameters for Production of Naringinase Isolated from *Aspergillus niger* BCC 25166

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ABSTRACT

From isolation of fungi capable of hydrolyzing naringin by growing the fungi at 28°C on selective synthetic minimal medium, pH 5.8, containing 0.1% naringin, 348 fungi were isolated from 128 various host samples, collected from 11 different sources in Thailand and China. Primary screening of all 348 fungi was done by cultivation of the fungi at 40°C for 7 days in synthetic minimal medium, pH 4.0, added with 0.1% naringin, and by investigation of the naringin hydrolysis of the fungal culture filtrate at pH 4.0, 40°C, for 23-48 hours. Forty fungal isolates were obtained. Secondary screening was performed by measurement of both glycosidase activities, ⁄ -L-rhamnosidase and ∈D-glucosidase at 40°C, pH 4.0, and naringinase activity at the temperatures of 50, 55 and 60°C and at both pH 3.0 and 4.0 of the culture filtrates from all 40 fungal isolates. *Aspergillus niger* BCC 25166 was selected and genetically identified. The optimum pH and temperature of the enzyme in crude extract were investigated. The result showed that all naringinase, ⁄ -L-rhamnosidase and ∈D-glucosidase activities had identical optimum pH of 4.0. However, the optimum temperature of naringinase and ⁄ -L-rhamnosidase was 60° C, whereas that of ∈D-glucosidase activity was in the range of 60° to 70°C. Optimization of the medium and conditions for enzymes production in submerged fermentation found that the suitable inoculum concentration and medium were 10^5 spores/ml and Czapek-Dox medium, pH 4.0, containing 0.1% naringin, respectively. The maximum naringinase production of this fungus (117.77 U/mg protein) could be obtained by supplement of the medium with 3.75 g/l rhamnose as another carbon source and using 2.5 g/l $NaNO_3$ as its nitrogen source. For high production of ⁄ -L-rhamnosidase (303.20 U/mg protein), 2.5 g/l soya peptone should be used instead.

Key words: naringinase, naringin, ⁄ -rhamnosidase, ∈glucosidase, debitterness

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INTRODUCTION

The presence of bitterness has been a major limitation in the commercial acceptance of citrus juices (Puri and Kalra, 2005). All the processed citrus fruit juices contain naringin (4,5,7-trihydroxyflavonone-7-rhamnoglucoside) which attributes bitterness to the juices (Puri *et al.*, 2005). Albedo, the fruit membrane, is one of the major parts containing naringin, when it is squeezed, the naringin is extracted into the juice. Naringin is abundant in immature fruit but its concentration decreases as fruit ripens (Yusof *et al.*, 1990; Puri and Banergee, 2000). Since naringin is the main bitter component of citrus juices, thus, its hydrolysis with a concomitant decrease in bitterness is of industrial importance. Hence, it is of commercial interest that the flavors should be more acceptable by the consumer (Puri *et al.*, 1996; Norouzian *et al.*, 1999). The naringin level can be reduced by technologies such as adsorptive debittering, chemical methods, treatment with polystyrene divinyl benzene styrene (DVB) resins and €cyclodextrin. Because of the various drawbacks, the capabilities of non-enzymatic debittering technologies are limited (Puri and Banergee, 2000). A suitable debitterness can be achieved by treating the juice with an enzyme known as naringinase, which directly hydrolyses naringin. This enzyme contains both ⁄ -L-rhamnosidase (EC 3.2.1.40) and €D-glucosidase (EC 3.2.1.21) activities (Norouzian *et al.*, 2000). Naringin can be hydrolyzed by the ⁄ -L-rhamnosidase activity into rhamnose and prunin (4,5,7-trihydroxyflavonone-7-glucopyranoside), in which prunin can be further hydrolyzed by the €D-glucosidase activity into glucose and naringenin (4,5,7-trihydroxyflavonone) (Chien *et al.*, 2001). Due to the lesser bitterness of prunin than that of naringin about one-third, only activity of the ⁄ -L-rhamnosidase is essential (Norouzian *et al.*, 1999).

Historically, naringinase had been isolated from plant sources such as celery seeds and grapefruit leaves. However, for reasons of availability, only processes based on microbial naringinases are practicable (Puri and Banergee, 2000). The objectives of this research were to select a fungus with good ability to produce naringinase with high ⁄ -L-rhamnosidase activity and good catalysis at low pH but high temperature, and to optimize the media composition for naringinase production**.**

MATERIALS AND METHODS

Materials

Naringin, *p*-nitrophenyl-⁄ -L-rhamnopyranoside (pNPR) and *p*-nitrophenyl-€D-glucopyranoside (pNPG) were purchased from Sigma (St. Louis, MO, USA) while rhamnose was purchased from Fluka (Switzerland). Different carbon sources, organic and inorganic nitrogen sources were purchased from various sources, e.g. Hi-Media (Mumbi, India), Merck (Germany) and BDH (England). All other reagents were of analytical grade and were purchased from Merck (Germany), Fluka (Switzerland), Sigma (St. Louis, MO, USA) and BDH (England).

Microorganism

Among 348 fungal isolates from 128 host samples, one organism isolated from soil collected from Boh-Klueng hot spring in Ratchaburi province, Thailand, could hydrolyze naringin well in screening tests. This isolate which was subsequently identified as *Aspergillus niger*, designated as BCC 25166, was selected to study the optimal condition for naringinase production in submerged fermentation. This fungus was maintained in Sabouraud's dextrose agar modified with 0.01% naringin.

Culture media and growth conditions

Wild fungi were isolated from 128 various host samples collected from different

sources in Thailand and China by spread plate technique. These fungi were grown at 28°C on selective synthetic minimal medium (Ruiz *et al*., 1997) containing (g/l) 0.2 $MgSO_4.7H_2O$, 0.4 KH_2PO_4, 0.2 KCl, 5.0 NH_4NO_3, 0.01 $FeSO_4.7H_2O$, 0.01 $ZnSO_4$, 0.01 $MnSO_4$, 15 agar and 1.0 naringin, and its pH was adjusted to 5.8. The isolated fungi were then cultivated in Sabouraud's dextrose agar modified with 0.01% naringin for maintenance as stock culture. The Sabouraud's dextrose agar medium contains (g/l) 1.0 $MgSO_4.7H_2O$, 1.0 KH_2PO_4, 10.0 neopeptone, 20.0 glucose and 20.0 agar.

Aspergillus niger BCC 25166, the selected fungus was cultivated in Czapek-Dox medium (Puri *et al*., 2005), the optimal media for naringinase production. The composition of the medium was (g/l) 2.0 $NaNO_3$, 1.0 KH_2PO_4, 0.5 KCl, 0.5 $MgSO_4.7H_2O$, 0.1 $FeCl_3$ and 1.0 naringin. The pH of the medium was prior adjusted to 4.0. The fungus was grown at 40°C for 7 days. Then the sample was harvested and the culture filtrate was analyzed for naringinase activity, ⁄ -L-rhamnosidase activity, ∈D-glucosidase activity and protein concentration while the fungal mycelium was analyzed for cell mass.

Preliminary enzyme characterization

To determine the optimum pHs of the enzymes, naringinase, ⁄ -L-rhamnosidase and ∈D-glucosidase activities were assayed at different pH values, ranging of 3.0 to 10.0 at 1.0 unit interval. Buffers used for pH optimum study were citrate-phosphate (McIlvaine buffer) for pH 3.0-7.0, Tris-HCl for pH 8.0-9.0 and glycine-NaOH for pH 10.0. Once the optimum pH of the enzyme had been obtained, this pH was subsequently used for determination of the optimum temperature for enzyme activity by varying temperature from 30-80°C.

Media optimization

To optimize the medium for enzyme production, *Aspergillus niger* BCC 25166 was cultivated in Czapek-Dox medium, an optimal medium for enzyme production, at the initial pH of 4.0 in 250 ml Erlenmeyer flask containing 50 ml medium. This medium was inoculated with an optimized spore suspension at the concentration of 10^5 spores/ml. The fungus was incubated at 40°C for 7 days. Different carbon sources were added to the medium at the concentration of 1% w/v for selection of the suitable carbon source. To obtain the maximum enzyme yield, different concentrations of suitable carbon source between 0.125-2.5% w/v were added. Study of the suitable nitrogen source was also done by addition of different organic and inorganic nitrogen sources at the concentration of 0.5% w/v in the medium containing optimal carbon source. Different concentrations of the suitable nitrogen source between 0.125-2.5% w/v were also applied in the medium for studying the optimum concentration of nitrogen source for enzyme production.

Enzyme assay

⁄ -L-Rhamnosidase and ∈D-glucosidase activities were measured by 2 synthetic substrates, i.e. *p*-nitrophenyl-⁄ -L-rhamnopyranoside (*p*NPR) and *p*-nitrophenyl-∈D-glucopyranoside (*p*NPG), respectively, as described by Montreuil *et al*. (1994), at the optimum pH and optimum temperature of each enzyme tested. The enzyme activities were determined by incubation of the culture filtrate with their corresponding synthetic substrates for 10 minutes. The reaction was then stopped by addition of Na_2CO_3 solution and the absorbance was measured at 400 nm. All enzymatic measurements were done in triplicate.

For both glycosidases, 1 unit (U) of activity was defined as the amount of enzyme that could hydrolyze 1 μmol of either *p*NPR or *p*NPG to 1 μmol of *p*-nitrophenol per ml and minute at the assay conditions.

Naringinase activity was determined duplicately by the modified method of Davis

(1947), but slightly adjusted. Fifty µl of culture filtrate was incubated with 325 µl of 0.1% naringin in 0.1 M sodium acetate buffer, pH 4.0, for 12 hours at 60°C. The reaction was stopped by boiling for 2 min. Then, 10 µl of each reaction mixture was added with 200 µl of 90% diethylene glycol and 10 µl of 4N NaOH. Finally, the new mixture was left for 15 min at an ambient temperature and measured the absorbance at 420 nm using a microplate reader (Labscan). 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 hydrolyse 1 µmol of naringin per ml and minute at the assay conditions.

Analytical methods

Protein was determined by the dye-binding method (Bradford, 1976) using Serva Blue G-250 as a reagent dye with bovine serum albumin (BSA) as a standard protein.

Dry weight of fungal mycelium was determined by filtering the filtrate through Whatman No.1 filter paper, washing thoroughly with distilled water, drying overnight at 50°C and weighing its constant weight.

RESULTS AND DISCUSSION

Fungi selection and identification

Isolation of fungi capable of hydrolyzing naringin

Samples were collected from 11 different sources in Thailand and China. 128 various samples, e.g. soil, bark, leaf, fruit peel, lichen, waste water, etc., were obtained for fungal isolation. In this research, natural sources were used for fungal isolation as their natural diversity would be an important key for obtaining appropriated fungi being capable of producing high naringinase and / -L-rhamnosidase with required properties. Three hundred forty-eight fungal isolates were obtained and grown on the medium with 0.1% naringin. The pure isolates were maintained in Sabouraud's dextrose agar with 0.01% naringin for further screening.

Primary screening

As speculated, the finding of the fungi with the desirable properties of enzymes was based on the screening methods and medium used. To investigate the suitable screening medium, 10 positive and 10 negative fungal isolates were cultivated at 40°C for 7 days in various 7 media at the initial pH of either 4.0 or 3.0, namely: (1) synthetic minimal medium (Ruiz *et al*., 1997) added with 0.1% naringin, pH 4.0, (A^1); (2) synthetic minimal medium with 0.1% naringin, pH 3.0, (A^2); (3) Czapek-Dox medium (Puri *et al.*, 2005) containing 0.1% naringin, pH 4.0, (B); (4) synthetic minimal medium added with 0.1% naringin but replacing NH_4NO_3 (N-source) with peptone, pH 4.0, (C); (5) synthetic minimal medium with 0.1% naringenin, pH 4.0, (D); (6) synthetic minimal medium containing 0.1% grinded orange peel, pH 4.0, (E); and (7) potato dextrose broth (F). The culture filtrate were collected and investigated the naringin hydrolysis by incubation of the culture filtrate with 0.1% naringin in McIlvaine buffer, pH 4.0, at 40°C for 23-48 hours and observed the disappearance of substrate. The results indicated that synthetic minimal medium with 0.1% naringin (medium A^1) was suitable for use as a screening medium (result not shown). So this medium A^1 was used for cultivation of all 348 fungi in the secondary screening.

The naringin hydrolyses of the culture filtrates of these 348 fungal isolates from the primary screening revealed that only the culture filtrates of 40 isolates could hydrolyse naringin within 23-48 hours.

Secondary screening

Secondary screening was performed to select a fungus that produced high level of enzyme active at low pH and showed high activity at the

increasing temperature. Culture filtrates from 40 positive fungi obtained from primary screening were determined for ⁄ -L-rhamnosidase and ∈-D-glucosidase activities at 40°C and pH 4.0 (Table 1) and naringinase activity at the temperatures of 50, 55 and 60°C and at both pH 3.0 and 4.0 (data not shown). Criteria were based on the specific activity value.

The data from Table 1 demonstrated that fungal isolate code no. PTK-BS1.4 was one of the best fungi producing naringinase with good ability to catalyse naringin at high temperature but low pH and high specific activity of⁄ -L-rhamnosidase. This isolate was genetically identified (data not shown) as *Aspergillus niger* designated as BCC 25166 and selected for further study of media and conditions optimization. Though this fungus was in the same genus and species reported in many articles concerning the production of naringinase or⁄ -L-rhamnosidase (Bram and Solomons, 1965; Manzanares *et al.*, 1997; Martino *et al.*, 2000; Puri and Kalra, 2005; Puri *et al.*, 2005;), *Aspergillus niger* BCC 25166 seemed to produce much higher naringinase than the others reported so far. The specific activity of naringinase obtained from *Aspergillus niger* BCC 25166 was 169.76 U/mg while that of *Aspergillus niger* 1344 was 22.7 U/mg (Puri and Kalra, 2005) under the same assay conditions of 50°C and pH 4.0. Although the other types of naringinase or ⁄ -L-rhamnosidase producing fungi such as *Penicillium decumbens* (Norouzian *et al.*, 1999; 2000), *Aspergillus nidulans* (Orejas *et al.*, 1999; Manzanares *et al.*, 2000), *Aspergillus terreus* (Elinbaum *et al.*, 2002), *Aspergillus aculeatus* (Manzanares *et al.*, 2003), *Rhizopus nigricans* (Shanmugam and Yadav, 1995), *Fusarium solani, Mucor recemosus, Trichoderma longibrachiatum, Penicillium aureantiograiseum and Fusarium sambucinum* (Scaroni *et al.*, 2002) had been reported, *Aspergillus niger* BCC 25166 reported in this study showed higher specific activity of ⁄ -L-rhamnosidase (25.89 U/mg) than that of *Aspergillus nidulans* (9.32 U/mg protein) previously described by Orejas *et al.* (1999).

Preliminary enzyme characterization

Determination of the optimum pHs of naringinase, ⁄ -L-rhamnosidase and ∈-D-glucosidase were carried out at different pH values between 3.0-10.0, whereas the optimum temperature of each enzyme was determined at its optimum pH but at different temperatures between 30-80°C. Figure 1A shows that the optimum pHs of *Aspergillus niger* BCC 25166 naringinase, ⁄ -L-rhamnosidase and ∈-D-glucosidase activities are at the same pH 4.0. This optimum pH of naringinase activity resembled those of purified naringinase from *Aspergillus niger* 1344 (pH 4.0) (Puri and Kalra, 2005) and partially purified naringinase from *Penicillium decumbens* PTCC 5248 (pH 4.5) (Norouzian *et al.*, 2000) and lower than that previously described (pH 4.5-7.5) by Bram and Solomons (1965). The

Table 1 Groups of fungal isolates separated according to the specific activities of⁄ -L-rhamnosidase and ∈-D-glucosidase at 40°C, pH 4.0.

Fungal isolates (Code no.)					
⁄ -L-rhamnosidase activity(U/mg protein)			∈-D-glucosidase activity (U/mg protein)		
>20 U/mg	10-20 U/mg	<10 U/mg	>20 U/mg	10-20 U/mg	<10 U/mg
PTK-BS1.4	PTK3.5	The rest 31	PTK-BS1.4	PTK11.4	The rest 32
PTK-BL9.1	PTK11.4	isolates	PTK-BS2.2	PTK-BS5.4	isolates
PTK-BL5.1	PTK12.1		PTK-BL5.1	PTK-BS29.1	
PTK7.8	PTK20.5		PTK-PS10		
	PTK-BS2.2		PTK12.1		

optimum pH of ⁄ -L-rhamnosidase from *Aspergillus niger* BCC 25166 was 4.0, which was lower than those reported from the other strains such as *Rhizopus nigricans* (pH 6.5) (Shamugam and Yadav, 1995; Puri and Banergee, 2000), *Aspergillus nidulans* (pH 4.5-8.0) (Orejas *et al.*, 1999) and *Aspergillus nidulans* (pH 4.5-6.0) (Manzanares *et al.*, 2000).

From Figure 1B, it clearly showed that the optimum temperatures of both naringinase and ⁄ -L-rhamnosidase, at pH 4.0, were similar at 60°C, whereas that of ∈D-glucosidase was in the range of 60° to 70°C. This optimum temperature was higher compared to those of partially purified naringinase from *Penicillium decumbens* PTCC 5248 (55°C) (Norouzian *et al.*, 2000) and purified naringinase from *Aspergillus niger* 1344 (50°C) (Puri and Kalra, 2005), while this value was equivalent to that of purified *A. nidulans* ⁄ -L-rhamnosidase (Manzanares *et al.*, 2000) but was higher than that of crude *A. nidulans* ⁄ -L-rhamnosidase (40-50°C) (Orejas *et al.*, 1999).

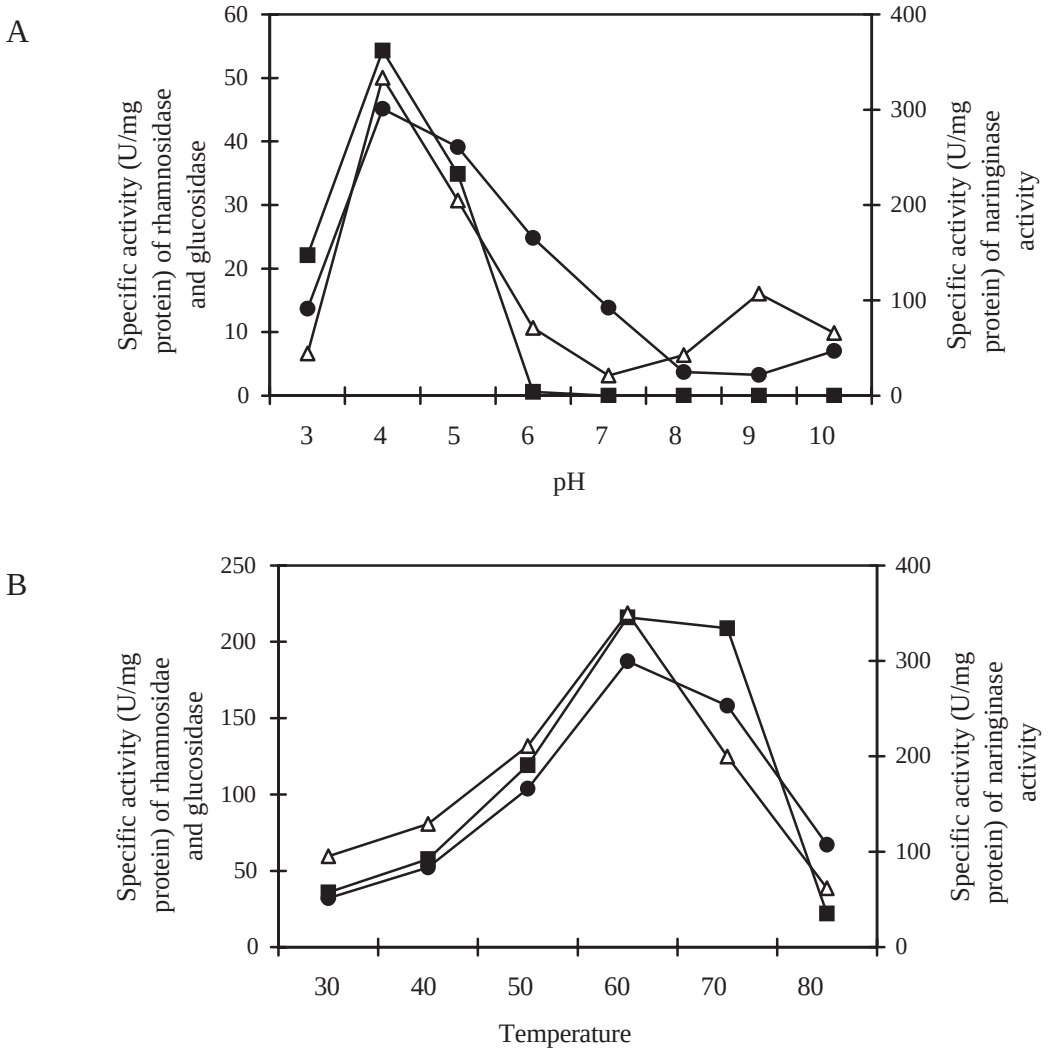

Figure 1 Optimum pH (A) and temperature (B) of naringinase, ⁄ -L-rhamnosidase and ∈D-glucosidase activities from *Aspergillus niger* BCC 25166.

However, this optimum temperature of ⁄ -L-rhamnosidase from *Aspergillus niger* BCC 25166 was lower than those reported such as of *Rhizopus nigricans* ⁄ -L-rhamnosidase (60-80°C) (Shamugam and Yadav, 1995) and of purified *A. niger* ⁄ -L-rhamnosidase (65°C) (Manzanares *et al.*, 1997).

Media optimization

Suitable media

Aspergillus niger BCC 25166 was cultivated in two media, synthetic minimal medium and Czapek-Dox medium of pH 4.0 at 40°C for 7 days for selection of a suitable medium for naringinase production. Due to the different composition of these two media, the criteria for consideration was based on enzyme activity compared to the total mass (Unit/g dry cell weight). When the growth of *A. niger* BCC 25166 on synthetic minimal medium and Czapek-Dox medium in the presence of 0.1% naringin were compared, Czapek-Dox medium appeared to encourage both higher cell growth and enzyme production (Figure 2). So this medium was selected as the optimum medium for cultivation of the selected fungus in enzyme production.

Effect of carbon source and its optimal concentration

Czapek-Dox medium was used as basal medium for optimization study by inoculation of *Aspergillus niger* BCC 25166 spore suspension of 10^5 spores/ml, the optimum inoculum concentration. Eight various carbon sources were supplemented in the medium containing 0.1% naringin at a concentration of 1% w/v. The result in Figure 3 indicated that each supplemented carbon source supported excellent growth of *A. niger* BCC 25166, while different level of enzyme production was obtained. Due to lesser bitterness of prunin than that of naringin about one-third, therefore, only ⁄ -L-rhamnosidase activity is essential (Norouzian, 1999). Thus, the criteria for selection of suitable supplemented carbon source would base on only ⁄ -L-rhamnosidase activity. From Figure 3, it showed that most of the supplemented carbon sources lowered ⁄ -L-rhamnosidase activity but promoted the fungus growth. Only rhamnose exhibited promotion of both ⁄ -L-rhamnosidase activity and fungus growth. However, the specific activity obtained from fungus cultivated in the medium supplemented with rhamnose was much higher

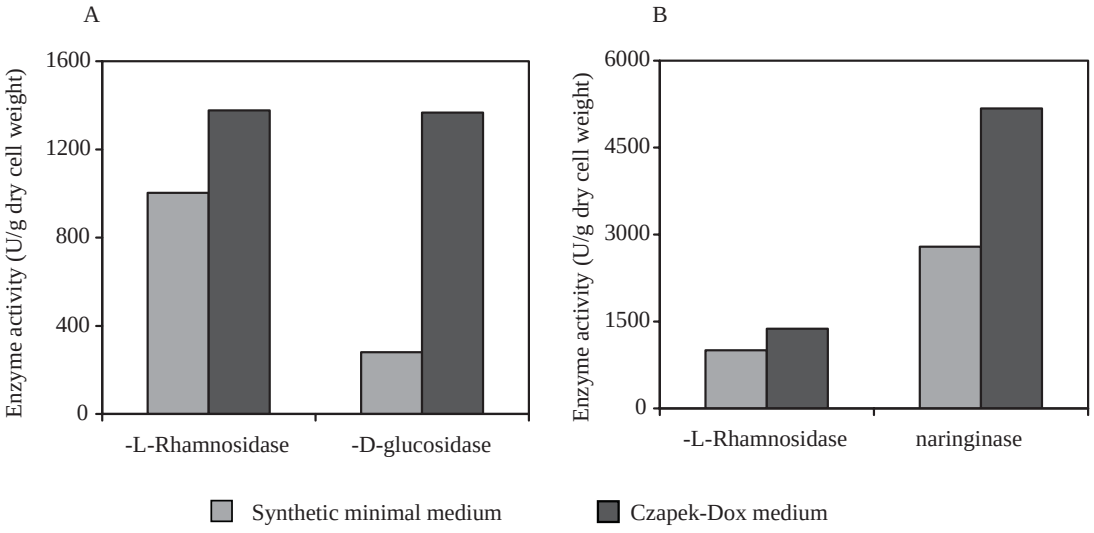

Figure 2 Effect of two different media on ⁄ -L-rhamnosidase, ∈D-glucosidase (A) and naringinase (B) production from *Aspergillus niger* BCC 25166 after cultivated for 7 days.

than that of the control (no supplemented carbon source). Thus, rhamnose was used as another carbon source for enzyme production in this medium. This rhamnose was reported by Elinbaum *et al.* (2002) that it 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. This work used both naringin and rhamnose, so the enzyme production would be sufficiently optimized. Puri *et al.* (2005) reported the use of molasses as carbon source in their medium. Cornsteep liquor was also reported to be used (Bram and Solomons, 1965). In these experiments, both sources were omitted.

Determination of optimum concentration of rhamnose was performed by cultivating the fungus in the Czapek-Dox medium containing different rhamnose concentration from 0.125-2.5% w/v and with $NaNO_3$ as nitrogen source. The results showed that the maximum specific activity of enzyme was 112.83 U/mg protein when added rhamnose at the concentration of 3.75 g/l (or 0.375% w/v) into the medium (Figure 4).

Effect of nitrogen source and its optimal concentration

Twelve different nitrogen sources were added in Czapek-Dox medium containing 0.1% naringin supplemented with 3.75 g/l rhamnose at a concentration of 0.5% w/v for studying of the suitable nitrogen source for enzyme production. Since the nitrogen sources used in this experiment interfered with protein assay, so the criteria for consideration was changed from specific activity

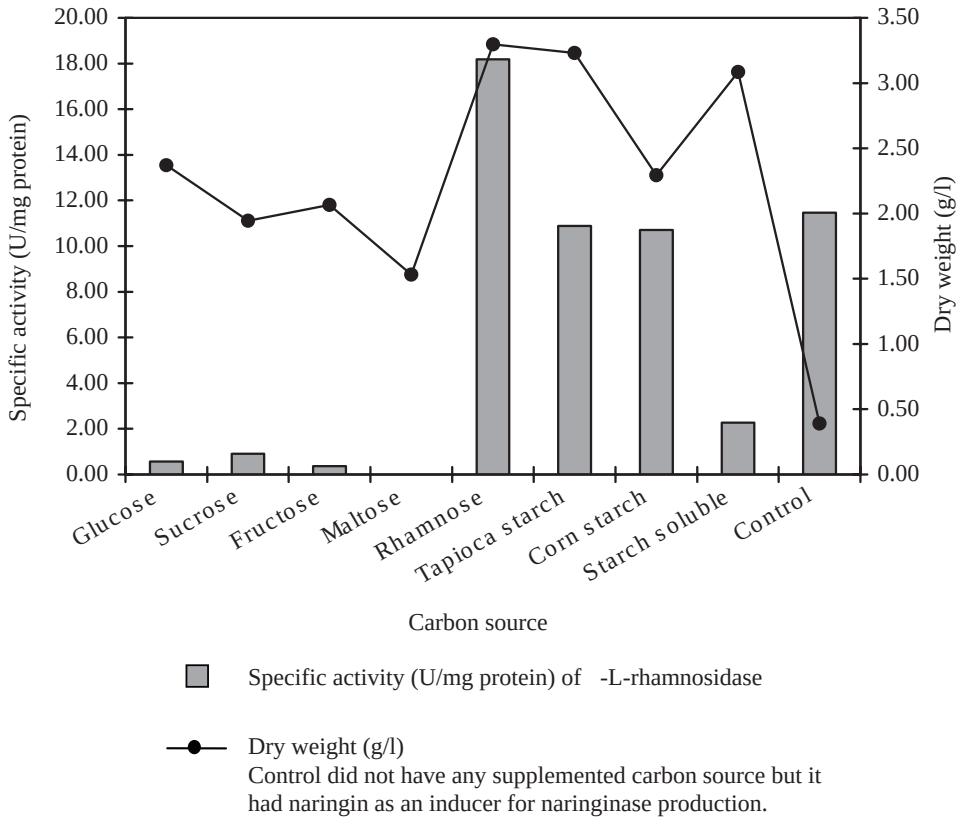

Figure 3 Effect of supplemented carbon sources on ⁄ -L-rhamnosidase production and growth of *Aspergillus niger* BCC 25166.

of enzyme to the enzyme activity compared to its total mass (U/g dry cell weight). The results in Figure 5 indicated that soya peptone gave the highest⁄ -L-rhamnosidase activity compared to all tested nitrogen sources and the control (containing $NaNO_3$ as nitrogen source), though all promoted fungus growth. These results were similar to those obtained in the previous study of the effect of carbon source on enzyme production in that all nitrogen sources supported the fungus growth, but the production of⁄ -L-rhamnosidase in all nitrogen sources, except soya peptone, was lower than that of the control. Therefore, soya peptone was the best nitrogen source in cultivation medium for increasing of ⁄ -L-rhamnosidase activity. Nevertheless, the fungus grew in the control (containing $NaNO_3$ as a nitrogen source) gave the highest naringinase activity (data not shown) compared to the others, so this inorganic nitrogen source could also be used as a nitrogen source in the cultivation medium but for high naringinase production. In contrast, yeast extract (Bram and Solomons, 1965) and peptone (Puri *et al*, 2005) were reported to increase naringinase production.

Finally, selected nitrogen sources, i.e. soya peptone and $NaNO_3$, were determined for their optimum concentrations. Different concentrations in the range of 0.125-25 g/l were tested. The results showed that the optimal concentration of soya peptone for increasing ⁄ -L-rhamnosidase activity was 2.5 g/l (Figure 6) which was similar to that of $NaNO_3$ which gave the highest naringinase activity at the concentration of 2.5 g/l (Figure 7). The maximum activities of naringinase and ⁄ -L-rhamnosidase obtained were 117.77 and 303.20 U/mg protein, respectively.

CONCLUSION

Three hundred forty-eight fungal isolates primarily screened showed that 40 isolates could hydrolyze naringin within 23-48 hours at pH 4.0, 40°C. From secondary screening, *Aspergillus niger* BCC 25166 was selected and identified. The crude naringinase, ⁄ -L-rhamnosidase and ⁄-D-glucosidase enzymes had the same optimum pH of 4.0. The optimum temperature of both

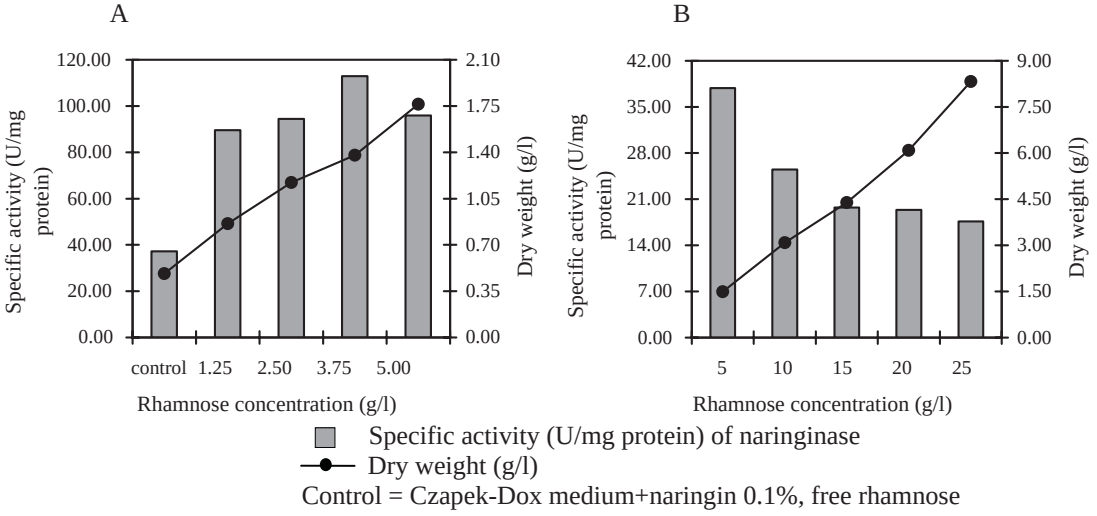

Figure 4 Effect of rhamnose concentration on naringinase production and growth of *A. niger* BCC 25166.

(A) Rhamnose concentration = 1.25-5 g/l,

(B) Rhamnose concentration = 5-25 g/l

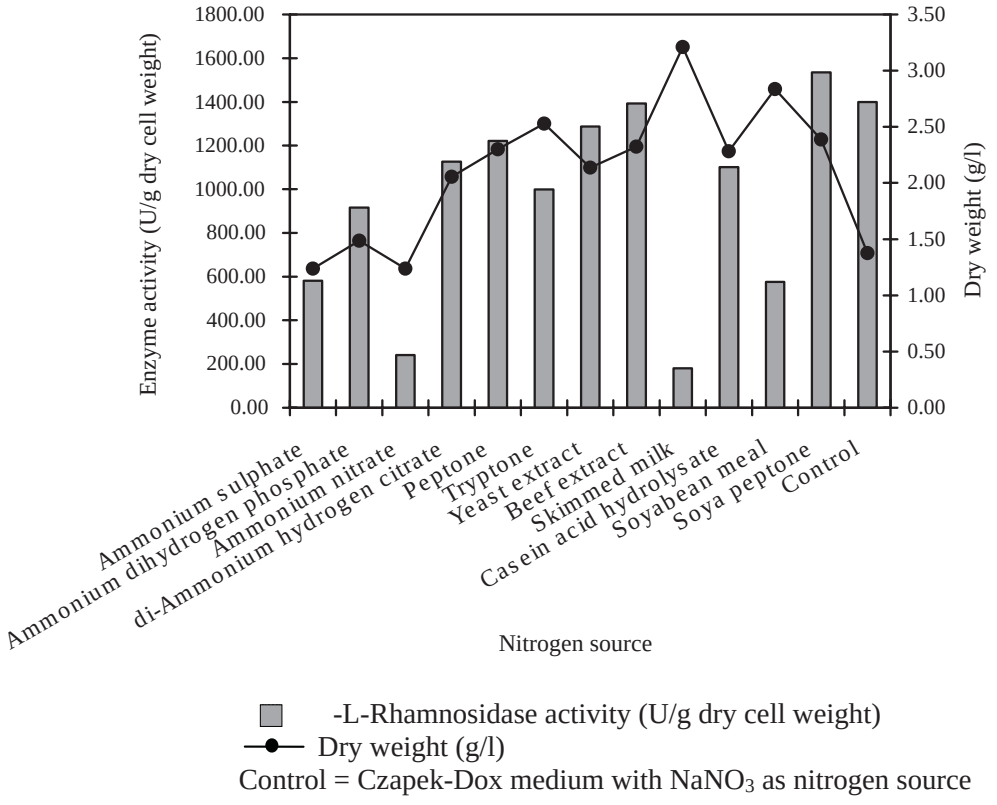

Figure 5 Effect of nitrogen sources on ⁄ -L-rhamnosidase production and growth of *A. niger* BCC 25166.

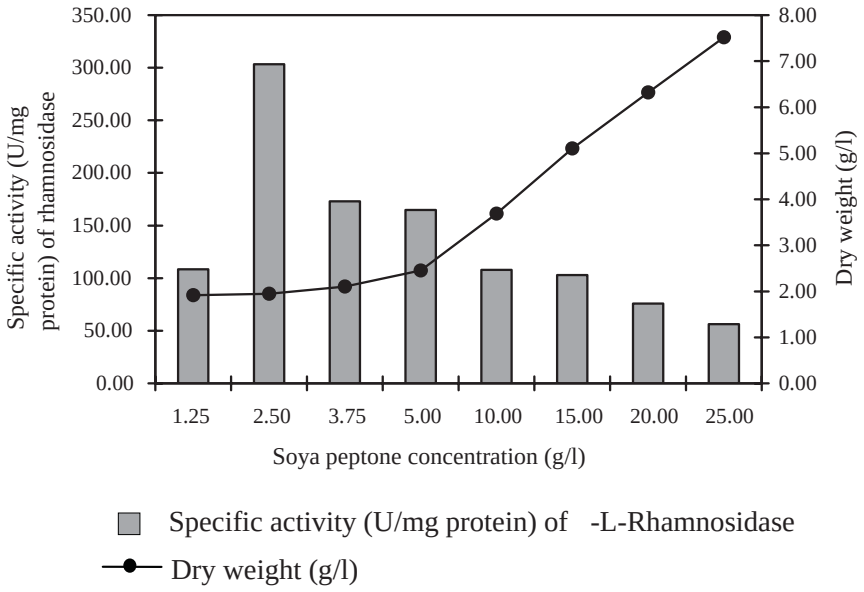

Figure 6 Effect of soya peptone concentration on ⁄ -L-rhamnosidase production from *A. niger* BCC 25166.

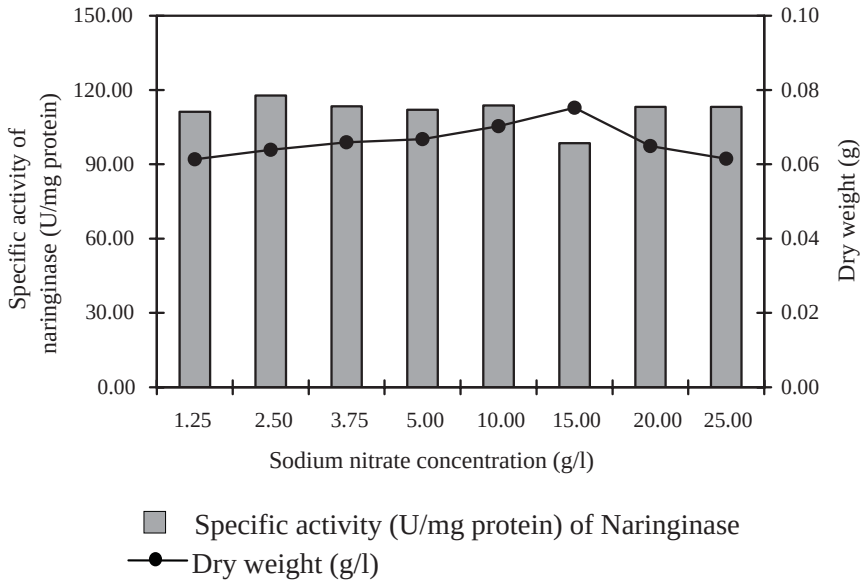

Figure 7 Effect of sodium nitrate concentration on naringinase production from *A. niger* BCC 25166.

naringinase and ⁄ -L-rhamnosidase was 60°C, whereas that of ∈-D-glucosidase activity was in the range of 60° to 70°C. The suitable inoculum concentration and medium were 10^5 spores/ml and Czapek-Dox medium at the initial pH of 4.0, respectively. The maximum naringinase (117.77 U/mg protein) and⁄ -L-rhamnosidase production (303.20 U/mg protein) could be obtained by supplement of the medium with 3.75 g/l rhamnose as another carbon source and using either 2.5 g/l $NaNO_3$ or 2.5 g/l soya peptone as a nitrogen source.

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