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Full Length Research Paper

Naringinase production from filamentous fungi using grapefruit rind in solid state fermentation

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This study involved the production of naringinase by twelve filamentous fungi in solid-state fermentation (SSF) using orange and grapefruit rind as substrates; these agro industrial residues contain naringin, an important inductor for this enzyme. The percentage of naringin hydrolyzed from grapefruit rind by *Aspergillus foetidus, Aspergillus niger* and *Aspergillus niger* HPD-2 was 81, 80 and 79% respectively. The volumetric and specific naringinase activity of each strain was influenced by temperature, pH and water activity (Aw). The culture conditions optimal for *A. foetidus*, *A. niger* and *A. niger* HPD2 were as follows: pH 5.4, 35°C, 0.5222 Aw; pH 5.4, 35°C, 0.7533 Aw; pH 5.4, 40°C, 0.7533 Aw; respectively. The highest volumetric activity was obtained using *A. foetidus* with 2.58 U ml^{-1}, when grew on grapefruit rind. Among the strains used, optimal naringinase production was with *A. foetidus*. The use of grapefruit rind as a substrate gave a higher yield of naringinase production than using orange rind.

Key words: *Aspergillus foetidus*, naringin, solid-state fermentation, grapefruit rind, naringinase.

INTRODUCTION

Naringin (4',5,7-trihydroxyflavanone-7-rhamnoglucoside) is the main bitter component of several citrus fruits (e.g. grapefruit). Its hydrolysis with a concomitant decrease in bitterness is important for industrial applications. Debittering can be achieved by treating the juice with an enzyme known as naringinase, which is an enzyme complex containing both α-L-rhamnosidase (EC 3.2.1.40) (Elinbaum et al., 2002; Puri and Kalra, 2005a) and β-D-glucosidase (EC 3.2.1.21) (Roitner et al., 1984), which are both known to produce prunin and rhamnosa, and naringenin and glucose respectively. Naringinase is produced by many microorganisms but there are only few reports on the commercial production of this enzyme (Puri and Banerjee, 2000).

Nonetheless, other industrial applications have been proposed for naringinase or its subunits such as: biotransformation of antibiotics and steroids (Thirkettle, 2000). Elimination of hesperidin crystals from orange juices (Terada et al., 1995), aroma enhancement of wines (Caldini et al., 1994) and enzymatic preparation of hydrolysis by products of natural glycosides (Roitner et al., 1984). Moreover prunin, rhamnosa, and naringenin, as previously reported, show biological activities (Amaro et al., 2009) and can be used as raw materials for synthesis of substances utilized in pharmaceutical, cosmetic and food product (Ellenrieder et al., 1998).

On the other hand, solid state fermentation (SSF), which has been used infrequently to produce naringinase, is an important tool for the production of industrial fungal enzymes, due to automation capabilities and operating experience with many other large-scale solid-substrate fermentation processes (Chisti, 1999; Pandey et al., 1999; Ooijkaas et al., 2000). In this work orange and grapefruit rinds were utilized, agro industrial wastes with high concentration of naringin, as substrate to produce naringinase by experimenting with several filamentous fungi in a SSF system.

MATERIALS AND METHODS

Twelve filamentous fungi, obtained from different culture collections (Table 1) were used in this study and maintained on PDA medium.

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Table 1. Microorganisms used in this study.

Microorganism	Origin
A. foetidus	NRRL-341
A. niger HPD-2	Veracruz Institute Technologic
A. niger	IIBM-UNAM collection
A. oryzae	IIBM-UNAM collection
Penicillium caseicolum	Chemistry faculty, UNAM
Penicillium chrysogenum NRRL	IIBM-UNAM collection
Penicillim glaucum	LACTO LAB
Penicillium roqueforti I	Isolated from Cheese
Penicillium roqueforti II	IIBM-UNAM collection
Penicillium roqueforti CNRZ	IIBM-UNAM collection
Penicillium roqueforti Milano	IIBM-UNAM collection
Rhyzopus delemar	IIBM-UNAM collection

The inoculum was produced by cultivating each microorganism in a PDA medium at 30°C for six days. Subsequently, the spores were recovered in a mineral salt solution without suspending the mycelium. The spore suspension was adjusted to 14 X 10^4 spores ml^{-1} for each fungus (Ortiz et al., 1993).

The cultures were carried out in a 500 ml Erlenmeyer flask with 10 g of orange or grapefruit rind (particle size: 0.86, 1.19, 1.69 and 2.38 mm) and 10 ml of mineral salt solution (KH_2PO_4, 13.77 g l^{-1}; K_2HPO_4, 17.629 g l^{-1}; NH_4Cl, 0.2 g l^{-1}; $MgSO_4.7H_2O$, 0.2 g l^{-1}; $FeCl_3$, 0.001 g l^{-1}). The orange or grapefruit rind and the salt solution were sterilized separately for 15 min at 121°C and 103 kP; the initial pH of the medium was adjusted to 4.5. The medium was inoculated with the spore suspension. The spores were then inoculated on orange or grapefruit rind and thoroughly mixed. Flasks were incubated at 30°C, without agitation. Samples were withdrawn (flask/sample) aseptically at regular time intervals and analyzed for extracellular protein content, naringinase activity and residual substrate.

Recovery of the enzymatic extract

The extract was recovered using 75 ml of sodium acetate buffer 0.1 M, pH 4.0, and stirring with a magnetic bar for 30 min at 4°C. Subsequently, the suspension was filtered and centrifuged (14000 rev min^{-1}, 30 min, 4°C).

Assay methods

Naringinase activity was estimated by determining the residual naringin by using the Davis method (Davis, 1947). Typical assay mixtures included 1 ml 0.05% naringin dissolved in 0.1 M sodium acetate buffer (pH 4) and 1 ml culture filtrate. The assay mixture was incubated at 40°C for 60 min, after which 100 µl aliquot was added to 5 ml 90% diethylene glycol followed by the addition of 100 µl 4 N NaOH; taking into account the initial naringin content in the crude extract as time zero for reference. Samples were maintained at room temperature (28°C) for 10 min. The intensity of the resultant yellow colour was determined at 420 nm. One unit of the activity of naringinase was defined as 1 µmol of naringin hydrolyzed under the above assay conditions. In order to determine the percentage of hydrolysis, naringin content of grapefruit and orange rind was quantified before and after fermentation. Extracellular protein was determined means the method developed by Lowry et al. (1951).

Table 2. Culture conditions used for naringinase production.

Condition	pH	Aw	Temperature (°C)
1	5.4	0.5222	40
2	5.4	0.5222	35
3	5.4	0.7533	40
4	5.4	0.7533	35
5	6.4	0.5222	40
6	6.4	0.5222	35
7	6.4	0.7533	40
8	6.4	0.7533	35

Evaluation of environmental factors on naringinase production

To investigate the effects of environmental factors on enzyme production diverse conditions such as pH, temperature and water activity were evaluated. The experiments (four repetitions each) were carried out by following an experimental design (factorial design 2^3). In order to measure water activity, 10 g of orange or grapefruit rind was homogenized with 10 or 15 ml of salt solution and measured utilizing Aqualab equipment CX2 (Pullman, WA). Due to the nature of this substrate, conditions of the medium were as follows: initial pH 5.40 or 6.40, temperature 35 or 40°C and water activity 0.5222 or 0.7533. Table 2 shows the culture conditions used for this experiment.

RESULTS

The fungi strains were evaluated using orange or grapefruit rind as a substrate with minimum liquid required in order to avoid drainage. Eleven fungi were capable of producing naringinase on either orange or grapefruit rind substrates (data not shown). *Penicillium roqueforti* Milano was the only strain unable to produce the enzyme. It was observed that all fungi with the ability

Table 3. Naringinase production by several filamentous fungi grown on grapefruit rind.

Microorganism	Volumetric activity (U ml-1) a	Specific activity b
Aspergillus foetidus	1.46	0.205
Aspergillus niger HPD-2	1.068	0.179
Aspergillus niger	1.46	0.187
Aspergillus oryzae SS	0.322	0.058
Penicillium caseicolum	0.016	0.015
Penicillium chrysogenum NRRL	0.012	0.012
Penicillium glaucum	0.101	0.008
Penicillium roqueforti I	0.160	0.017
Penicillium roqueforti II	0.012	0.008
Penicillium roqueforti CNRZ	0.008	0.005
Rhyzopus delemar	0.012	0.005

(a) Volumetric activity (U)= µmol of naringin hydrolyzed per hour at 40°C, pH 4.5. (b) Specific activity = Volumetric activity/ Extracellular Protein.

Table 4. Volumetric activity and specific activity of naringinase.

Microorganism	Volumetric activity (U ml-1) a	Specific activity b
Aspergillus foetidus	1.46	0.194
Aspergillus niger HPD-2	1.03	0.132
Aspergillus niger	1.43	0.172
Aspergillus oryzae SS	0.287	0.032
Penicillium roqueforti I	0.287	0.027

(a) Volumetric activity (U) = µmol of naringin hydrolyzed per hour at 40°C, pH 4.5. (b) Specific activity = Volumetric activity/ extracellular protein.

to produce naringinase had higher yields of the enzyme, with grapefruit rind yielding higher levels than orange rind. Accordingly, grapefruit rind was selected as the substrate. The eleven strains were grown in grapefruit rind by using 14 x 10^4 spores ml^{-1} as inoculum in all cases. Based on the results (Table 3), it can be seen that crude extracts of *Aspergillus foetidus*, *Aspergillus niger* HPD.2, *Aspergillus niger*, *Aspergillus oryzae*, and *Penicillium roqueforti* I, had the maximum titles of both volumetric and specific activity. This experiment was repeated using those five fungi strains; the results of the activities (Table 4) showed that *A. foetidus*, *A. niger*, and *A. niger* HPD-2, had the best volumetric and specific activity. The percentage of hydrolyzed naringin from the substrate was determined and it was also observed that *A. foetidus*, *A. niger* and *A. niger* HPD-2 hydrolyzed 81, 80 and 79% of the content of naringin in the substrate respectively. These results are similar to those reported by Puri et al. (1996a) who were able to register 82% of the hydrolysis of the naringin. However, these authors used immobilized enzymes.

In contrast, *A. oryzae* and *P. roqueforti* I, metabolized 74 and 71% of the naringin from the substrate, respectively; nevertheless, the levels of volumetric and specific naringinase activity were substantially lower than the other three fungi. The effect of initial pH, temperature and water activity on naringinase production by strains of *A. foetidus*, *A. niger* and *A. niger* HPD-2 was determined by measuring the volumetric and specific activity (Figures 1 and 2).

A. foetidus (Figure 1) produced the highest levels of volumetric activity with 2.58 U in condition 2; *A. niger* and *A. niger* HPD-2 produced 2.06 and 1.68 U in condition 4, respectively. The analysis of variance of the data from 2^3 factorial designs showed temperature and pH as factors with the highest influence on enzyme production. It was also observed that the interactions of the factors pH-temperature and Aw-temperature were the most important. Figure 2 illustrates the specific activity and demonstrates that *A. foetidus* had higher specific activity than the other strains of *Aspergillus*. The maximum levels of specific activity were observed in the conditions 1, 3 and 4 (optimal condition) with *A. foetidus*, *A. niger* HPD-2 and *A. niger*, respectively.

Finally, we conducted studies utilizing optimal condition for each fungus by following the naringinase production every two days. *A. foetidus* demonstrated constant production of naringinase during the fermentation, with maximum level obtained on the 8th day in all cases (Figure 3). The specific activity of *A. foetidus*, *A. niger* HPD2 and *A. niger* was determined obtaining 0.335, 0.195 and 0.17, respectively.

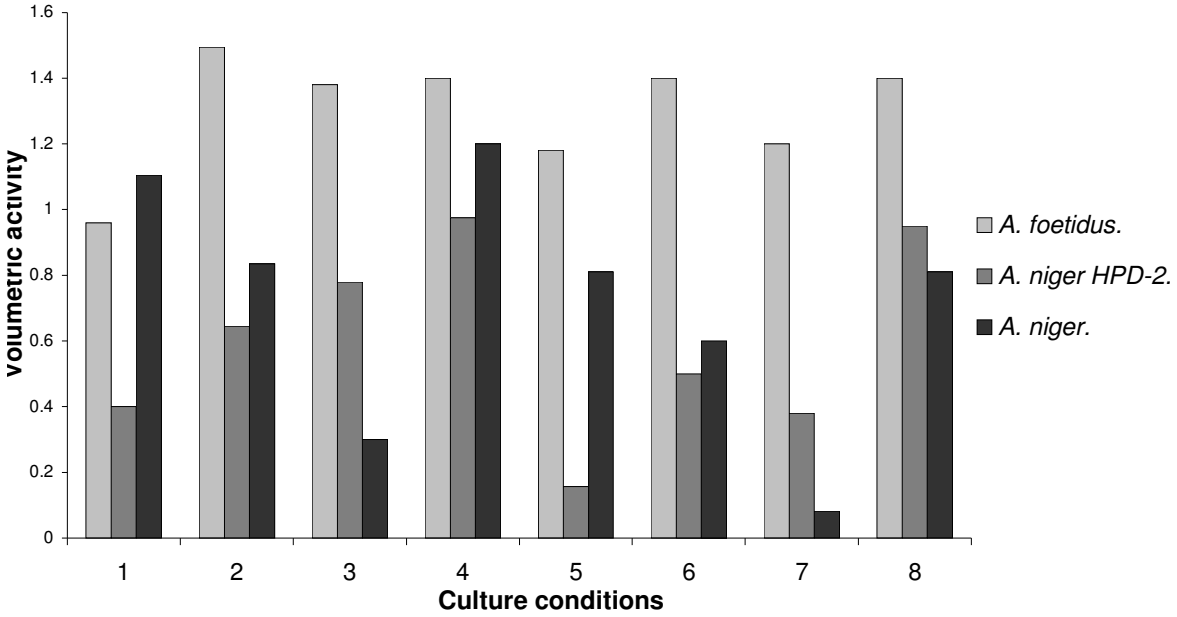

Figure 1. Volumetric activity of *A. foetidus*, *A, niger* HPD-2 and *A. niger* at different conditions (pH, Temperature, Aw).

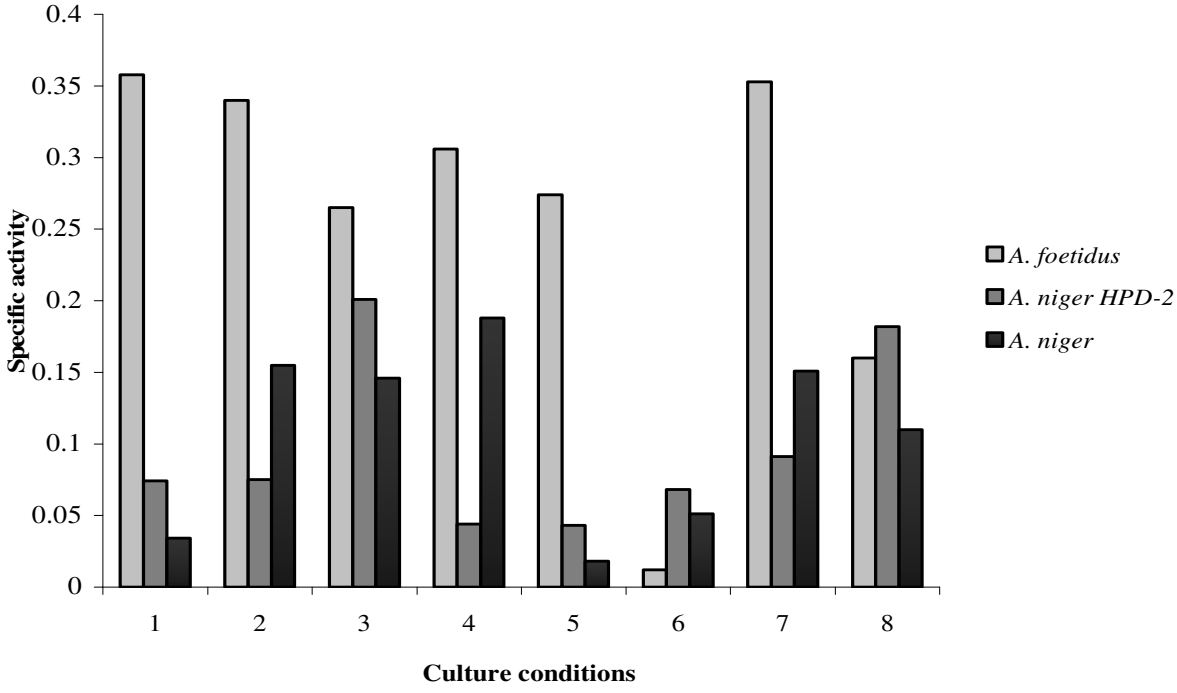

Figure 2. Effect of culture conditions on specific activity of naringinase produced by *A. foetidus, A. niger* HPD-2 and *A. niger*.

DISCUSSION

Naringinase production was possible using only citrus rind in a solid state fermentation. As the results indicate, all tested fungi were capable of growing in citrus rind, but only certain fungi were able to produce naringinase. Indeed, three of the four *Aspergillus* strains grown in grapefruit rind had the highest enzyme yield. It is important to mention that *A. foetidus*, a fungus uncommonly reported as a naringinase producer, was our optimal producer for naringinase enzyme in this study. Although, fungi of the genera *Penicillium* have been

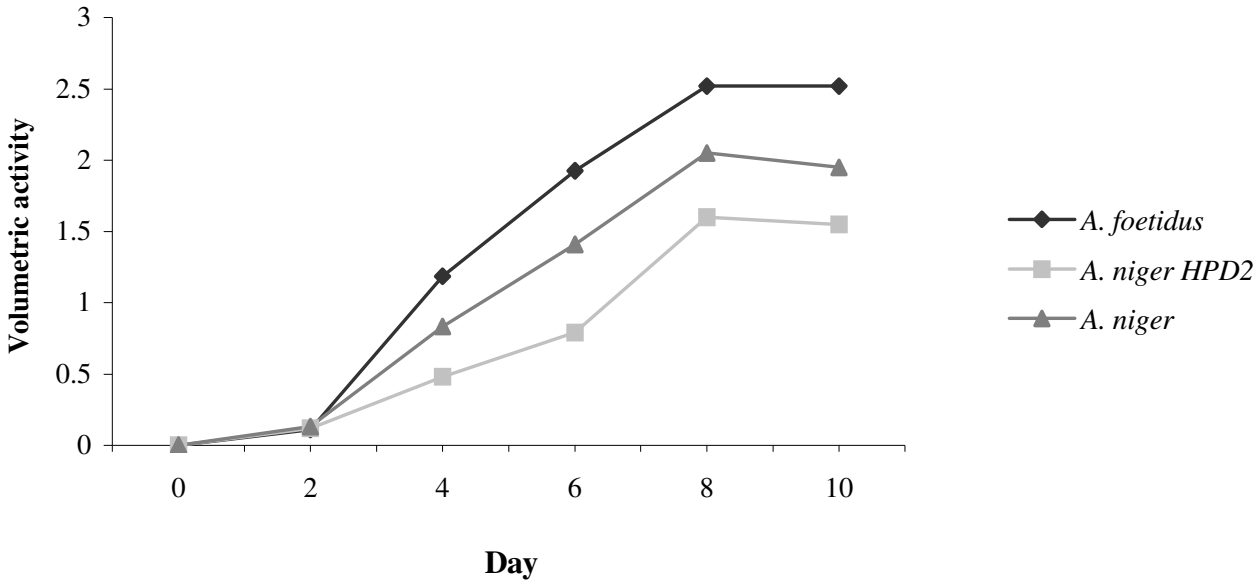

Figure 3. Naringinase production of *A. foetidus*, *A. niger* HPD-2 and *A. niger*.

reported as producers of this enzyme (Olson et al., 1979; Michon et al., 1989; Puri et al., 1996b; Cowan, 1991; Tsen and Yu, 1991; Elinbaum et al., 2002), *Aspergillus* genus had been used preferentially forenzyme production for a number of years. This bias is due to its high level of extracellular enzyme production seen in this genus. The other fungi strains produced naringinase; however, specific activity was low, which means they produce large amounts of protein but minimal amounts of naringinase; this could be a problem when enzyme purification is needed.

Although we can obtain naringinase with both orange and grapefruit rind, maximal enzyme production was observed on grapefruit rind as substrate. This is probably due to highest naringin content in grapefruit rind (572 µmol of naringin per gram of fresh weight) (Berhow and Vandercook, 1989) and lowest content of naringinase inhibitor compounds, such as monosaccharides (fructose and glucose) (Puri et al., 2005b; Bram and Solomons, 1965). Thus, this substrate, due to high naringin content, induces by itself enzyme production without the need of naringinase-inductor addition, as described by Puri et al. (2005b) and Elinbaum et al. (2002). All three *Aspergillus* strains produced significant amounts of enzyme in optimal temperature of 40°C, optimal pH of 5.4 to 6.4 and in a relatively low water environment; this is an advantage for SSF due to its minimal water requirement. Modifying different environmental conditions produced almost twice the levels of volumetric activity and specific activity was increased. Taking into account the levels of volumetric and specific activity, we found that *A. foetidus* produced optimal levels of naringinase.

In recent years the enzyme production industry (biotech) has encountered problems with undesirable levels carbohydrate content in the synthetic medium utilized in reduced-bitter enzyme production. The presence of those substances posses an inhibitory effect on naringinase production. Some researchers have used media incorporating naringin in order to increase enzyme production in a SSF system. In this work we elucidate the suitability and advantages of using grapefruit rind, an abundant and economical agricultural waste product, as a substrate for naringinase production.

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