Short Report

Screening of Urease Production by *Aspergillus niger* Strains

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ABSTRACT

In this study, urease production was investigated among thirteen strains of *Aspergillus niger;* seven strains isolated from soils of Semnan province in Iran and six strains obtained from Persian Type Culture Collection (PTCC). The enzyme production was screened in two submerged media quantitatively. The registered PTCC 5011 and the native S31 strains showed more urease production than the other eleven strains. The maximum enzyme productions by PTCC 5011 and S31 strains were 106 and 109 U.g^{-1}dry mass in submerged culture, respectively. Also, we used two solid media for screening all of the strains for urease production semi quantitatively. Due to the acceptable correlations between the two methods, the latter can be used as an ancillary method to mass screening of urease production by filamentous fungi. *Iran. Biomed. J. 8 (1): 47-50 2004*

Keywords: Urease, Screening, *Aspergillus niger*, Production

INTRODUCTION

Urease (urea amidohydrolase, EC 3.5.1.5) is a nickel-containing enzyme that catalyzes the hydrolysis of urea to yield ammonia and carbamat, the latter compound decomposes spontaneously to generate a second molecule of ammonia and carbon dioxide [1-5]. Urease has many industrial applications, e.g. in diagnostic kits for measuring urea, in alcoholic beverages as a urea reducing agent [6, 7], and in biosensors of haemodialysis systems for determining blood urea [8]. Urease is found in plants, algae, yeasts and filamentous fungi [5,9]. The urease from jack bean (*Canavalia ensiformis*) was the first enzyme to be crystallized and it remains the best-characterized urease [5]. There are many microbial sources for this enzyme including bacteria such as *Lactobacillus ruminis, Corynebacterium lillium*, *Lactobacillus fermentum*, and *Lactobacillus reuteri* [10, 11] and fungi such as *Aspergillus niger* [8], *Aspergillus nidulans* [12-14], and *Rhizopus oryzae* [15]. However bacterial strains yield low quantities of urease [8]. Filamentous fungi are the sources of about 40% of all available enzymes such as amylases, lactase, raffinase, dextranase, pectinase, and cellulase [16]. It is also the industry preference to use filamentous fungi, especially *Aspergillus* spp. as enzyme producers, because the fermentation industries are very familiar with the conditions required to maximize production of the homologous proteins by them [17]. Amongst the filamentous fungi, *A. niger* has been used for commercial production of many enzymes, e.g. pectinase, glucose oxidase, glucoamylase,

hemicellulase, glucanases, acid proteinase, and catalase [18-20]. Although urease production and purification have been studied in *A. niger* by Smith *et al.* [8], there is not enough information overall in this regard. In this study, we have screened urease production among thirteen registered and native *A. niger* strains. The purpose of the study was to assess the potentiality of urease production by these strains and to find an efficient urease producing strain for possible use in diagnostic kits of urea measurement in medical laboratories.

MATERIALS AND METHODS

Microorganisms and media. Six registered *A. niger* strains (PTCC 5010, 5011, 5012, 5057, 5223, 5154) obtained from the Persian Type Culture Collection (PTCC, Tehran, Iran) and seven native strains of *A. niger* (S17a, S19a, S20, S 25, S23, S30, S31) isolated from the soils of Semnan province in Iran, were studied for urease production.

The conidia were cultured on the slants of PDA (Potato Dextrose Agar). M1 and M2 media were utilized to assess urease production semi- quantitatively. The M1 medium contained: urea, 2.26 g; glucose, 20 g; $MgSO_4.7H_2O$, 0.5 g; potato infusion, 4 g; agar, 15 g; bacto phenol-red, 0.12 g; and distilled water up to 1 liter. The M2 medium contained: urea, 1.3 g; glucose, 20 g; $MgSO_4.7H_2O$, 0.5 g; $CaCl_2$, 0.3 g; agar, 15 g; bactophenol-red, 0.12 g and distilled water up to 1 liter. The M3 and M4 were utilized to assess urease production quantitatively. The M3 was a chemically-defined glucose-urea medium consisting: urea, 1.3 g; glucose, 20 g; $MgSO_4.7H_2O$, 0.5 g; KH_2PO_4, 13.3 g; K_2HPO_4, 0.348 g; $CaCl_2$, 0.3 g; $NiSO_4.7H_2O$, 0.032 g; and distilled water up to 1 liter (pH 4.8). The M4 was the production medium used by Smith *et al.* [8], consisting: glucose, 20 g; urea, 0.2 g; yeast extract, 10 g; yeast nitrogen base without amino acids, 10 g; $NiSO_4.7H_2O$, 0.032 g; Na_2HPO_4, 15 g; and distilled water up to 1 liter.

Culture conditions. The strains were cultivated on PDA at 30°C for 5 days in order to obtain conidia. The conidia were washed and suspended in normal saline containing 0.1% Tween 80 to give 10^5 conidia/ml. Then, 100 µl of the suspended conidia inoculated into M1 and M2 slant media for each strain. The growth was carried out at 30°C for 14 days. For submerged fermentation, the conidia suspension of each strain was inoculated in amounts to give 10^5 conidia/ml in 250 ml-Erlenmeyer flasks containing 50 ml of M3 or M4 medium. The fermentation was carried out on a rotary shaker incubator (200 rpm) at 30°C for 72 hours.

Preparation of homogenized mycelia. The fungal mycelia were filtered by filter paper (Whatman # 42). Mycelia recovered by filtration were washed three times with 0.1 M potassium phosphate buffer (pH 7.0). Preparation of homogenized mycelia was achieved by disrupting the washed mycelia in 0.1 M potassium phosphate buffer (pH 7.0) with a blender and resulting suspension was used for urease activity.

Dry mass measurement. A certain amount of washed mycelia dried in oven at 55°C and the dried mass was measured by an analytical scale several times to reach to a constant weight.

Enzyme assay. In the semi-quantitative screening method, the enzyme production was evaluated by visual inspection of the phenol red color change caused by ammonia liberation in the culture tubes. The amount of color change intensity was correlated to the enzyme production.

In the quantitative urease assay, the enzyme activity in media supernatants and homogenized mycelia was measured by Weatherburn method [21] with some modifications, i.e. Na_2HPO_4 was used instead of NaOH in alkaline hypochlorite solution and the 20-minute time for color development was elongated to 30 minutes. The reactions were done in micro tubes containing 100 µl of sample, 500 µl of 50 mM urea, and 500 µl of 100 mM potassium phosphate buffer (pH 8.0) in a total volume of 1.1 ml. The reaction mixture was incubated in a shaking water bath at 37°C for 30 min. The reaction stopped by transferring 50 µl of reaction mixture to the tubes containing 500 µl of phenol-sodium nitroprusside solution (0.05 g sodium nitroprosside + 1 g phenol/100 ml distilled water). Five hundred micro liters of alkaline hypochlorite (3.56 g Na_2HPO_4 + 1 ml sodium hypochlorite + 100 ml distilled water) was added to the tubes, and incubated at room temperature for 30 min . Finally, the optical density of the color complex was measured at 630 nm against the blank (500 µl phenol nitroprusside sodium + 500 µl sodium hypochlorite + 50 µl distilled water) with a spectronic 20D +

spectrophotometer and compared to a standard curve prepared with $(NH_4)_2SO_4$. Controls used for the enzyme reactions were reaction mixture without substrate and reaction mixture without incubation. One unit of urease activity was defined as the amount of enzyme liberating 1 µmol NH_3 from urea per minute, under the above assay conditions.

RESULTS AND DISCUSSION

The purpose of this study was to evaluate the potentiality of urease production by some *A. niger*

Table 1. Comparison of urease production among A. niger strains, in M3 and M4 media. All experiments were performed in triplicate for each strain in 3 batches at 30°C, (200 rpm) for 72h . Each value is the mean of nine determinations.

strain	Total activity unit in (M3)	Total activity unit in (M4)	Activity ($U.g^{-1}$) in (M3)	Activity ($U.g^{-1}$) in (M4)	Dry mass ($g.L^{-1}$) in (M3)	Dry mass ($g.L^{-1}$) in (M4)
5011	888	1980	106	96	8.40	20.60
5154	245	1609	62	77	4.40	21.00
5012	296	1012	74	52	4.00	19.80
5010	455	915	41	39	11.00	23.20
5223	415	864	42	37	9.80	23.20
5057	233	262	39	14	6.00	18.60
S31	960	2630	91	109	10.60	24.20
S25	715	1309	67	55	10.60	23.60
S30	421	1287	35	63	12.00	20.40
S19a	448	905	60	37	7.40	24.20
S17a	316	881	48	50	6.60	17.60
S23	264	721	9	31	32.00	22.80
S20	333	616	40	37	8.40	16.60

strains to select the best urease producer among them. Also, we compared a simple and reliable screening method for urease production with the conventional methods. *A. niger* strains were assessed for urease production on two solid (M1 and M2) and two liquid (M3 and M4) media. Liquid media are usually used for the screening of enzyme production [17]. Table 1 shows comparison of urease activity in homogenized mycelia of *A. niger* strains in M3 and M4 media. No enzymatic activity was detected in supernatants of the cultures. Based on these results, S31 shows the highest urease total activity in M3 and M4 media, i.e. 960 and 2630 $U.L^{-1}$, respectively. Among the registered strains, PTCC 5011 showed the most enzyme activities, although lower than those of the native strain S31 Smith *et al.* [8] reported a total activity of 1015 $U.L^{-1}$ by *A. niger* NRRL 003 (equivalent to PTCC 5012). Urease activity by S31was about 2.6 times more than that of the *A. niger* reported by Smith *et al.* [8]. Also, Mirbod *et al.* [9] and Lubbers *et al.* [5] studied urease production by a pathogenic fungus *Coccidioides immitis* and an ascomycetous fission yeast *Schizosaccharomyces pombe* with the total activity of 3340 and 1199 $U.L^{-1}$, respectively. Although, *Coccidioides immitis* has been reported to produce more urease, it cannot be used in industry due to its pathogenicity.

Table 2, shows that S31 and PTCC 5011 are the best urease producers on M1 and M2 media. Two strains PTCC 5010 and PTCC 5012 did not change the indicator color in the M1 medium and strains S20 and S23 did not change the indicator color in M2 medium. Table 3 shows the correlation among the various media we used for screening urease production by *A. niger* strains. Although, some *A. niger* strains show different behaviors on M1 and M2, due to distinct compositions, generally it can be said there is an acceptable correlation between urease production on these two media (M1 and M2), considering the results in Table 3.

M3 and M4 are chemically defined and complex media, respectively. A chemically defined medium is indeed the medium of choice for studying fermentation processes due to the ease of interpretation of the results comparing to complex media. [22]. We recommend M3 for improving culture conditions and screening programs among filamentous fungi due to an acceptable correlation obtained between M3 and M4 media, (Table 3).

The solid media we designed for screening of urease production by filamentous fungi were able to discriminate the high from low urease producers as efficient as the liquid media. Also the best correlation (r =

0.8, p = 0.001) was found between M2 and M3 media. Regarding to these facts and also the simplicity of working with solid media, we recommend using M2 medium in primary mass screening programs for urease production by filamentous fungi.

Table 2. Urease production on M1 and M2 media by *A. niger* strains.

Strains	5011	5154	5012	5010	5223	5057	S31	S25	S30	S19a	S17a	S23	S20
M_1	3+	3+	No	No	No	1+	4+	2+	3+	2+	1+	1+	2+
M_2	4+	3+	1+	1+	No	1+	4+	1+	3+	2+	1+	No	No

(No): No enzyme production;(1 + to 4+): Semi quantitative amounts of enzyme production.

Table 3. Statistical analysis of comparison of urease production in solid and liquid media.

	M2	M3	M4
M1	0.769 (0.002)	0.752 (0.003)	0.479 (0.098)
M2		0.800 (0.001)	0.681 (0.010)
M3			0.771 (0.002)

First number is Pearson correlation coefficient and number in the parentheses is p value.

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