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Short Communication

L-Asparaginase Production by *Bipolaris* sp. BR438 Isolated from Brown Rice in Thailand

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ABSTRACT

Thirty-six filamentous fungal isolates obtained from brown rice in Thailand were screened for their ability to produce L-asparaginase. Using modified Czapek Dox (mCD) agar containing L-asparagine and phenol red as indicator, 24 L-asparaginase producing fungal isolates could be preliminary identified by observing pink colour formation. It was found that isolate BR438 exhibited highest activity (6.3 $\pm$ 0.65 U/ml) when cultured in the mCD medium containing 1% L-asparagine and 0.4% glucose at 30°C for 72 h. Its asparaginase was also proved to be non-cytotoxic when tested against Vero cell lines. The identification based on internal transcribed spacer (ITS) regions of ribosomal DNA and morphological study showed that isolate BR438 was *Bipolaris* sp. and was closely related to *Bipolaris australiensis* and *B. ovariicola.*

Keywords: *Bipolaris*, brown rice, L-asparaginase, cytotoxicity.

1. INTRODUCTION

Asparaginase (L-asparagine amido hydrolase, E.C.3.5.1.1) is an enzyme which converts L-asparagine to L-aspartic acid and ammonia. The therapeutic potential of this enzyme is well established, as it has remarkably induced remission in most patients suffering from acute lymphoblastic leukemia (ALL) [1]. It has also been used for treatment of lymphosarcoma and in many other clinical experiments relating to tumor therapy in combination with chemotherapy [2]. Many research groups have studied asparaginase production and purification in an attempt to minimize impurities that produce allergenic reactions [3-4]. Moreover, L-asparaginase degradation gives rise to free amino acid, which is a precursor of acrylamide before baking. It is also used in the food industry to reduce the acrylamide formation in food products [5]. Preventase (production from *Aspergillus niger* by DSM Company) and Acrylaway (production from *A. oryzae* by Novozyme Company) are commercially available asparaginase currently used in food industry. The Acrylaway has been used to reduce acrylamide formation in French fries. After blanched and unblanched potato strips were treated with 10000 U/l asparaginase solution before final frying, it was reported that acrylamide formation was reduced by 30% and 60%, respectively [6].

L-asparaginase production using microbial systems has attracted considerable attention, owing to the cost-effective and eco-friendly nature. A wide range of microorganisms such as filamentous fungi, yeasts, and bacteria have proved to be beneficial sources of this enzyme [7-11]. Although asparaginase from *Escherichia coli* and *Erwinia* sp. have been used as anti-tumor or anti-leukemia agent, minor side effects such as allergic reaction and vomiting have been reported [1, 12-13]. Many fungi were isolated from different habitats for metabolites production but less intention in rice, the main staple food for Thai people, especially brown

rice. Since only the hull is removed, brown rice retains most of its nutritional value and is probably a good habitat for fungi. Thus, fungi isolated from edible source may have less metabolic side effect for human use.

In this study, filamentous fungi isolated from brown rice in Thailand were investigated for their ability to produce L-asparaginase.

2. MATERIALS AND METHODS

2.1 Fungal Isolates

Rice seeds obtained from 5 rice research centers in Phatthalung, Phrae, Sakonnakorn, Suphanburi and Ubonratchathani provinces, Thailand. They were aseptically dehulled as brown rice at the Fungal Research Laboratory, Faculty of Science, Chiang Mai University, for fungal isolation. Thirty-six isolates were selected and identified based on their morphological and molecular characteristics. They were cultured on potato dextrose agar (PDA) and incubated at 30°C for 48 h before L-asparaginase screening.

2.2 Preliminary Screening for L-asparaginase Production

The methodology was modified based on Gulati *et al.* [14]. Modified Czapek Dox (mCD) medium containing 0.2% (w/v) glucose, 1% (w/v) L-asparagine, 0.152% (w/v) K_2PO_4, 0.052% (w/v) KCl, 0.052% (w/v) $MgSO_4.7H_2O$, 0.003% (w/v) $CuNO_3.3H_2O$, 0.005% (w/v) $ZnSO_4.7H_2O$, 0.003% (w/v) $FeSO_4.7H_2O$, 1.8% (w/v) agar, initial pH 6.2 was supplemented with 0.009% (v/v) phenol red as indicator. Control plates were mCD medium containing $NaNO_3$ as nitrogen source instead of asparagine. The plates were inoculated with the 36 selected fungal isolates and incubated at 30°C for 48 h. The isolates that showed pink zone around the colonies indicated L-asparaginase production and were selected for determination of enzyme activity.

2.3 Quantitative Assay for L-asparaginase Activity

The fungi were cultured on mCD agar plates and incubated at 30°C for 48 h. Plugs were taken from the edge of the colonies, using a 5 mm diameter cork borer. One disc was added to a test tube containing 5 ml of mCD broth and incubated at room temperature (27-30°C) for 96 h on a reciprocal shaker at 150 rpm. The cultures were then filtered through Whatman No.1 filter paper. Enzyme activity was determined by Nesslerization method [15] and expressed as U/ml. One international unit of L-asparaginase activity is defined as 1 μmol of ammonia per min under the conditions of the assay. The isolate which gave the highest enzyme activity was then studied for the effect of glucose and L-asparagine concentrations on L-asparaginase production.

2.4 Effect of Glucose and L-asparagine Concentrations on L-asparaginase Production

Three discs of the fungus were inoculated into Erlenmeyer flasks (250 ml) containing 30 ml mCD broth with different concentrations of glucose at 0.0, 0.2, 0.4, 0.6, 0.8 and 1.0 %, initial pH 6.2 and incubated at 30°C for 96 h on a reciprocal shaker at 150 rpm. The fungus was harvested by filtration through Whatman No.1 filter paper, dried at 150°C and the dry weight was determined. The filtrates were then measured for pH and enzyme activity. After determination of suitable glucose level, the optimal of L-asparagine as nitrogen source was studied at 0.0, 0.2, 0.4, 0.6, 0.8 and 1.0 % with the same conditions.

2.5 Effect of Incubation Time on L-asparaginase Production

The fungal culture was then inoculated in mCD broth containing 0.4% glucose and 1% L-asparagine. It was incubated at 30°C for 96 h on a reciprocal shaker at 150 rpm. During the fermentation, the fungal broth was sampled every 24 h and used for determination of L-asparaginase activity as previously described.

2.6 Cytotoxicity Test

Three discs of the fungal culture were inoculated in an Erlenmeyer flask (250 ml) containing 50 ml of mCD broth with 0.4% glucose and 1% L-asparagine. The culture was incubated at 30°C for 72 h on a reciprocal shaker at 150 rpm and filtered through Whatman No.1 filter paper. The extract was then freeze-dried and the obtained powder was packed into sterile microfuge tube and sent to the Bioassay Laboratory, BIOTEC, Bangkok for cytotoxicity analysis. The test was done against Vero cells (African green monkey kidney), using Green Fluorescent Protein (GFP) based assay.

2.7 Molecular Identification of L-asparaginase Producing Fungus

Total genomic DNA was extracted from fresh mycelia using a protocol outlined by Jeewon *et al.* [16]. Primer pair ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) [17] were used to amplify the ITS1, ITS2 regions and 5.8S gene. Amplification was

carried out in a 50 µl reaction volume. The thermal cycle consisted of 5 min initial denaturation at 95°C, followed by 30 cycles of 1 min denaturation at 95°C, 1 min primer annealing at 55°C, 1 min extension at 72°C and a final 10 min extension at 72°C. PCR products were purified using GFXTM PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Catalog no. 27-9602-01) following the manufacturer's protocol. The purified PCR products were directly sequenced at Macrogen Sequencing System, Korea with primer ITS4 and ITS5 as sequencing primers. Nucleotide sequences were aligned using ClustalW program [18]. Maximum Parsimony method (MP) was used in the construction of a phylogenetic tree using the program in MEGA4 [19]. The topological analysis was performed with 1000 bootstrap replicates.

3. RESULTS AND DISCUSSION

Thirty-six fungal isolates from brown rice in Thailand were identified to belong to the following genera *Acremonium, Aspergillus*, *Bipolaris, Cochliobolus, Curvularia, Fusarium, Hansfordia, Phaeotrichoconis* and mycelia sterilia fungi. They were examined for L-asparaginase production. Among these, 24 isolates showed pink zone around the colonies on mCD agar containing phenol red as indicator, indicating the increase in pH which originated from ammonia accumulation in the medium [14]. The dye indicator is yellow at acidic condition and turns to pink at alkaline condition. Since this method is very simple and rapid for the detection of L-asparaginase activity, it has been used for primary screening of L-asparaginase production from fungi such as *Aspergillus, Penicillium* and *Fusarium* spp. [7] and endophytic fungi isolated from Thai medicinal plants [20].

Bipolaris sp. isolate BR438 showed the highest L-asparaginase activity of 5.54 U/ml, it was therefore selected for further study. The enzyme activity was higher than other fungi that reported by Gulati *et al.* [14]. Nevertheless, the strain of fungi that have been reported to produce L-asparaginase is not diverse. There is no report about L-asparaginase production from *Bipolaris* spp. Most study was done with *Aspergillus* spp. [7-8, 21]. Enzyme activity of some isolates was not correlated to the size of pink zone diameter. This result was similar to Hölker *et al.* [22] and Lee *et al.* [23] that reported the ability of fungi to produce enzyme was different in solid and liquid state fermentations.

The optimum glucose at 0.4% showed highest enzyme activity of 5.8 ± 0.66 U/ml. The highest amount of cell dry weight (1.46 ± 0.04 mg/ml) was obtained in the medium with 0.4% glucose. However, there was no statistically differences in enzyme production at 0.2, 0.4 and 0.8 % glucose ($p < 0.05$). Thus, to economize fermentation, 0.2% glucose was optimum, and enzyme production did not depend on fungal biomass.

Maximum enzyme (6.2 ± 0.20 U/ml) was released when 1.0% L-asparagine was added to the medium One-way analysis of variance indicated significant ($p < 0.05$) effect of L-asparagine level on enzyme production. Maximum cell dry weight (1.56 ± 0.06 mg/ml) of fungi was observed with 1.0% L-asparagine as nitrogen source. Our results suggested that L-asparaginase production was regulated by glucose and nitrogen level which are congruent with those investigated by Sarquis *et al.* [7]. The optimal glucose level in present study was higher than those reported by Gulati *et al.* [14] (0.2%), while L-asparagine concentration was similar (1.0%).

Enzyme activity increased after incubation for 24 to 72 h and slightly decreased after 72 h when used 4% glucose and 1% L-asparagine. L-asparaginase production was highest after incubation for 72 h (6.3 ± 0.65 U/ml) at final pH 8.5 (Figure 1). At the same time, the biomass of fungi was 1.54 ± 0.14 mg/ml. Incubation times of 72 or 96 h had no significant effect on enzyme production and biomass of fungi at $p < 0.05$. This result was different from Sarquis *et al.* [7] who reported the highest L-asparaginase activity of

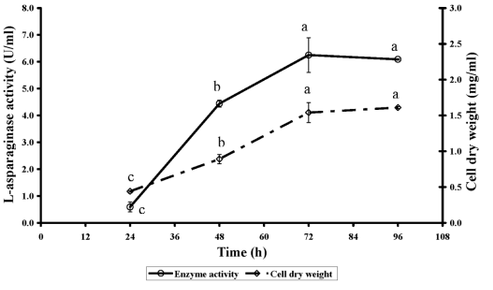

Figure 1. Effect of incubation time on enzyme and cell dry weight production by *Bipolaris* sp. BR438 in mCD broth, different significant: $p < 0.05$ (one-way ANOVA), values are mean of triplicates ± SD

A. terreus in liquid medium was found at 48 h while in solid medium the optimal period for enzyme production was 96 h [21]. This may reflect the different growth rate between *Aspergillus* and *Bipolaris*. Mishra recommended SSF as an advantage method to increase the yield of L-asparaginase [21]. Hence, investigation on production of L-asparaginase by solid state fermentation (SSF) using agricultural wastes should be carried out to verify such recommendation.

Preliminary screening for cytotoxicity of the crude extract indicated that the maximum final concentration of the crude extract, 50 mg/ml, more than 50% of the cells could grow, suggesting that the crude extract of BR 438 had no cytotoxic property. Previous studies by Rossi *et al.* [12] and Saviola *et al.* [13] indicated that the toxic side effects of some L-asparaginase currently used in clinical preparations were from bacterial sources. However, there was no report on the toxic side effects of L-asparaginase from fungal sources.

DNA sequences of ITS regions (including 5.8S gene) was 599 base pair in length. The GenBank accession number of BR438 was EU668994. Blast search showed that BR438 had high sequence similarities with species of

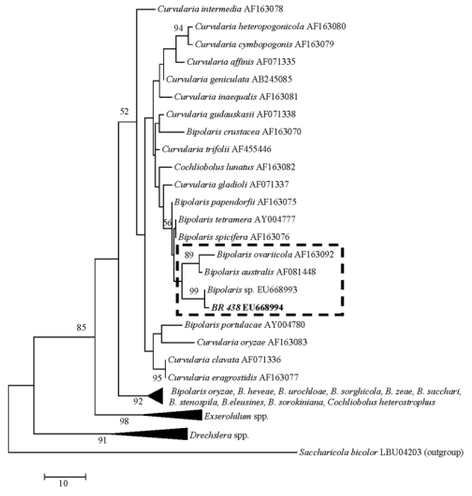

Figure 2. Maximum-parsimony tree generated from ITS sequences of 49 taxa showing the relationships of BR438 with reference taxa. The tree was rooted with *Saccharicola bicolor* (TL = 379, CI = 0.504, RI = 0.795). Bootstrap values higher than or equal to 50% (1000 replicates) are shown at branches.

Pleosporaceae. Maximum parsimony analysis revealed that isolate BR438 belonged to the Genus *Bipolaris* and clustered with *Bipolaris australiensis* and *B. ovariicola* (Figure 2). *Bipolaris* is a widespread genus and common fungi in *Graminaceae* including rice [24-25].

4. CONCLUSIONS

Several fungal strains were isolated from brown rice and were capable of L-asparaginase production. Among these isolates, *Biporalis* spp. BR438 was found to produce the highest L-asparaginase activity of 6.3 $\pm$ 0.65 U/ml after optimization. *In vitro* testing indicated that its crude extract was safe, therefore this fungal strain has a potential for use in food and feed applications.

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