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FULL LENGTH ARTICLE

Optimization of production of anti-tumor L-asparaginase by free and immobilized marine *Aspergillus terreus*

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Characterization

Abstract L-asparaginase plays a vital role in medical application, particularly in treatment of acute lymphoblastic leukemia as an effective anti-tumor agent. In the present study, twenty-one fungal strains were isolated from marine environment of the Red Sea coasts of Egypt. Screening for fungal L-asparaginase production was done, and only five fungal strains were selected and identified as *Aspergillus*, *Penicillium* and *Fusarium*. The most potent fungal isolate was *Aspergillus terreus* which yielded the highest L-asparaginase specific activity (4.81 U/mg protein). The highest enzyme productivity was observed on the 5th day and the optimized fermentation parameters were pH 6.0, temperature 35 °C. The yield was also high up on using dextrose and asparagine (8.26 U/mg protein) as carbon and nitrogen sources. The cultural conditions were studied using the Plackett–Burman experimental design. Immobilization using *A. terreus* adsorbed on sponge enhanced the enzyme production by 1.33-fold compared to the conventional free-cells. Repeated reuse of the adsorbed cells achieved the maximum enzyme specific activity after three cycles (33.86 U/mg protein).
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Introduction

For over 30 years, L-asparaginase (L-asparagine amido hydrolase E.C.3.5.1.1) has been applied as a chemotherapeutic agent for factually lymphoblastic leukemia treatment in a sense that converts L-asparagine to L-aspartic acid and ammonia. (Savitri et al., 2003). Current researches have been in progress to reduce the carcinogenicity effect of acrylamide on human by reducing its level in foods (Mottram et al., 2002). Elimination of acrylamide formation having minor effect on general formation of Maillard products in baked goods has been achieved by applying L-asparaginase (Hendriksen et al., 2009; Anese et al., 2011).

A great deal of interest has emerged in studying the possibilities of harnessing potential microorganisms that produce this enzyme. Different prokaryotic microorganisms such as *Pseudomonas stutzeri* (Mannan et al., 1995), *Pseudomonas*

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aeruginosa (Abdel-Fattah and Olama, 2002), *Escherichia coli* (Qin and Zhao, 2003) and *Erwinia carotovora* (Warangkar and Khobragade, 2009) are producers of L-asparaginase enzyme. The production of L-asparaginase enzyme from other sources like eucaryotic microorganisms could have minor effects. Fungi and yeasts proved their importance in different fields, particularly in industry, textile, bioremediation, agriculture, beside their vital role in natural cycling as biofertilizer. Fungi genera such as *Aspergillus, Penicillium*, and *Fusarium* have been investigated as L-asparaginase producers (Wade et al., 1971; Sarquis et al., 2004; Soniyamby et al., 2011; Elshafei1 et al., 2012; Dange and Peshwe, 2015 and Vijay1 and Jaya Raju, 2015).

Cell immobilization is considered a promising approach for enhancing the fermentation processes, enzymes production and bioremediation of toxic substances (Beshay, 2003; Kar and Ray, 2008). Advantages of using immobilized cells have been reported in such a way that facilitate continuous operation over a prolonged period, offer possible recycling of immobilized beads and simple way for harvesting the products, reactor productivity, ensures higher efficiency of catalysis (Kar and Ray, 2008) and development of economical methods focusing on lowering the cost of industrial process. Present investigation deals with isolation of L-asparaginase producing marine fungi as a novel source of enzymes in addition to optimization of physical and chemical factors for L-asparaginase production plus using different immobilization techniques.

Materials and methods

Sample collection

Water samples were gathered from different sites of Red Sea coasts, Egypt, in a sterile screw capped bottles, transferred to laboratory in ice box, and stored at 4 °C till analysis. Different dilutions were prepared, 100 µl of each dilution was spread plated on to sterile plates containing modified glucose Czapek's-Dox medium (MGCD). The composition of the medium (g/l): Glucose 2.0; L-asparagine, 10.0; KH_2PO_4, 1.52; KCL, 0.52; $MgSO_4.7H_2O$, 0.52; NaCL, 20.0 traces of $Cu(NO_3)_2.3H_2O$; $ZnSO_4.7H_20$; $FeSO_4.7H_2O$; Agar, 18.0, initial pH 6.0 and supplemented with 0.009% (v/v) of phenol red dye (Saxena and Sinha, 1981). The plates were incubated at 30 °C for 5 days to obtain colonies with pink color. The appeared colonies obtained were further purified by streaking on MGCD medium.

Growth conditions for fungal culture

The fungal isolated were grown on MGCD medium slants for five days at 30 °C. After incubation, conidia were scraped with 5.0 ml of sterile distilled water and the spores were obtained and counted using Hamocytometer. Then, 1 ml (1×10^6 spores/ml) aliquots were used to inoculate 50 ml of sterilized MGCD medium dispensed in 250 ml Erlenmeyer flasks. Thereafter, the flasks were incubated for 5 days at 30 °C under static condition.

Preparation of cell-free extracts

The fungal growth was separated by centrifugation at 6000 rpm for 15 min in a cooling centrifuge and the supernatant was used as the source of the crude enzyme.

Qualitative assay of L-asparaginase

Modified Glucose Czapek's-Dox medium (MGCD) (pH 6.0) was used as an assay medium and the qualitative assay was applied using diffusion technique. Sterilized medium was distributed in the pre-sterilized Petridishes. On the surface of solidified medium each fungal isolate was streaked, and incubated at 30 °C for 48–120 h. Non-inoculated petridish was taken as a negative control. Changing the medium color from yellowish to pink indicated a positive L-asparaginase production. All experiments were done in triplicate.

Quantitative assay of L-asparaginase

L-asparaginase activity was quantitatively estimated by the method of Mashburn and Wriston (1964) and Imada et al. (1973) using Nessler's reagent. The reaction mixture containing 0.5 ml of crude sample, 0.5 ml of 0.5 M buffer (acetate buffer pH 5.4), 0.5 ml of 0.04 M asparagine and 0.5 ml of distilled water was added to make the volume up to 2.0 ml and incubate for 30 min. Thereafter, the reaction was stopped by adding 0.5 ml of 1.5 M TCA (Trichloroacetic acid). The mixture was then centrifuged at 10,000 rpm for 5 min, and 0.1 ml of the supernatant was taken and 3.7 ml of distilled water was added; 0.2 ml of Nessler's reagent (Himedia) was added to the reaction tube. The color reaction was developed through 10 min and the absorbance was measured at 500 nm using spectrophotometer. The ammonia liberated was extrapolated from a curve derived with ammonia sulfate as standard curve. One IU of L-asparaginase is the amount of enzyme which liberates 1 µmol of ammonia per ml per minute [µmole/ml/min].

Estimation of protein

Protein content was determined according to the method described by Lowry et al. (1951) using Bovine Serum Albumin as a standard.

Fungal identification

The fungal isolates were identified according to morphological and cultural features (Watanabe, 2002; CBS, 2006). The isolates belonged to three genera (*Aspergillus, Pencillium and Fusarium*). The identification of fungi was confirmed by Mycological Center, Faculty of Science, Assiut University, Egypt.

Effect of temperature and pH on the L-asparaginase production

A. terreus was cultivated in MGCD broth at different pH values ranging from 4.0 to 9.0 at 30 °C under static conditions. For optimum temperature, *A. terreus* was incubated at different temperatures (25, 30, 35, 40, 45, 50, 55, and 60 °C) under static conditions for the optimum incubation time.

Effect of different carbon and nitrogen sources

Different carbon sources were added to MGCD broth at equivalent weight. Thereafter, L-asparaginase production was investigated. Carbon compounds, including glucose, lactose, fructose, sucrose, dextran, maltose, galactose and soluble

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