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Isolation, Production and Anti-Tumor Activity of L-Asparaginase of *Pencillium* sp

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Abstract: Fungi isolated form soils were examined for their L-asparaginase activity. Out of 22 genera tested, *Pencillium* sp showed high L-asparaginase activity. Optimum production of L-asparaginase enzyme (15 U/ml) was observed after 96 h of incubation at 30°C, moisture content of 50%. The present study indicated that the L-asparaginase produced from *Pencillium* sp shows antioxidant property, with 64.96% by 1,1-diphenyl-2-picryl-hydrazyl (DPPH\) method.

Key words: L-asparaginase · *Pencillium* · Antioxidant

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]. Asparaginase limited usage for acute myeloblastic leukeamia, chronic lumphocytic leukeamia, Hodgkin's disease, melonosarcoma and non-Hodgkin's lymphoma. Neoplastic cells cannot synthetase L-asparagine due to the absence of L-asparagine synthetase [3].

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 [4].

L-Asparaginase is produced throughout the world by submerged fermentation (SF). This technique has many disadvantages, such as the low concentration production and consequent handling, reduction and disposal of large volumes of water during the downstream processing. Therefore, the SF technique is a cost intensive, highly problematic and poorly understood unit operation [5]. Solid-state fermentation is a very effective technique as the yield of the product is many times higher when compared to that in SF and it also offers many other advantages [6].

L-asparaginase from bacterial origin can cause hypersensitivity in the long-term use, leading to allergic reactions and anaphylaxis. The search for other L-asparaginase sources, like eukaryotic microorganisms, can lead to an enzyme with less adverse effects. It has been observed that eukaryote microorganisms like yeast and filamentous fungi have a potential for L-asparaginase production [4].

The objective of this study was to produce L-asparaginase from soil fungi by solid-state fermentation using different agro industrial waste and to study its antioxidant property.

MATERIALS AND METHODS

Fungal Isolates: Fungi were isolated from soil sample collected from different areas of Nagercoil, Tamilnadu, India.

Preliminary Screening for L-asparaginase Production: The methodology was modified based on Gulati *et al.* [7]. 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

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indicator. Control plates were mCD medium containing $NaNO_3$ as nitrogen source instead of asparagine. The plates were inoculated with the 22 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.

Enzyme Production by Submerged Fermentation: The crude enzyme was prepared by the methods of submerged fermentation (SF). Inoculate the fungal spore in 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) and submitted to an orbital shaker at 160 rpm, 30°C and pH 6.2 for 48 h. Then culture suspension was filtered on Whattman 2 filter paper and cell-free filtrate was used as crude enzyme solution to estimate L-asparaginase activity [8].

Quantitative Assay for L-asparaginase Activity: L-asparaginase activity was measured following method of Imada *et al.* [9]. The cultures were centrifuged at 11,000 x g for 15min. this method utilizes the determination of ammonia liberated from L-asparagine in the enzyme reaction by the Nessler's reaction. Reaction was started by adding 0.5ml supernatant into 0.5ml 0.04 M L-asparagine and 0.5ml 0.05 M tris (hydroxymethyl) aminomethane (tris HCl) buffer, pH 7.2 and incubated at 37°C for 30 min. The reaction was stopped by the addition of 0.5ml of 1.5M trichloroacetic acid (TCA). The ammonia released in the supernatant was determined calorimetrically by adding 0.2 ml Nessler's reagent into tubes containing 0.1 ml supernatant and 3.75 ml distilled water and incubated at room temperature for 10 min and absorbance of the supernatant was read using a UV-visible spectrophotometer at wavelength of 450 nm.

Enzyme Production by Solid State Fermentation: Cultivation was achieved by solid-state fermentation (SSF) as previously reported by Ramesh and Lonsane [10]. The medium that was used for the cultivation of *Pencillium sp* under (SSF) had the following composition: 10 g substrate were moistened with 10 ml of 0.01M phosphate buffer pH 6.2 and placed in 250 ml Erlenmeyer flasks. The fermentation media were sterilized by autoclaving for 15 min at a pressure of 15 Ib/inch to raise the temperature to 121°C. The flasks were inoculated 1 ml (10^6 spores/ml) of spore suspension under sterile conditions and incubated under static conditions at 30°C for 4 d. The extracellular crude enzyme was prepared at the end of the fermentation period by the addition of 50 ml of a 0.01 M phosphate buffer pH 6.2 to the fermented medium, shaking for 30 min followed by centrifugation at 8,000 rpm for 20 min. The cell free supernatant was used as the crude enzyme preparation.

Some of the substrates that have been used included wheat bran (WB), rice bran(RB), sugarcane bagasse (SB), cotton seed oil cake (CSOC), ground nut shell(GNS), ground nut oil cake (GNOC), orange peel (OP), tea waste (TW), pine apple waste (PW) and corn cob (CC).

Optimization of culture conditions on L-asparaginase production by *Pencillium* sp: The *Pencillium* sp, which showed highest activity, was studied optimal condition for L-asparaginase production. Enzyme activity was measured at different parameters by taking one parameter at one time. The factors such as Incubation period, Temperature and moisture content affecting production of L-asparaginase were optimized. The experiments were conducted in 200 ml Erlenmeyer flask containing production medium. After sterilization by autoclaving, the flasks were cooled and inoculated with culture and maintained under various operational conditions separately such as temperature (25, 30, 35, 40, 45 and 50°C), incubation period (7 days) and moisture content (20. 30, 40, 50 and 60%. The culture filtrate was assayed in triplicate for L-asparaginase activity.

Partial Purification of Asparaginase: L-asparaginase was partially purified using the following procedures: The crude enzyme was brought to 45 % saturation with Ammonium Sulphate and kept overnight in a cold room at 4 °C. It was there after subjected to centrifugation at 8000 rpm for 10 min at 4 °C. The precipitate was discarded, while the supernatant was brought to 85 % saturation with Ammonium Sulphate and centrifuged at 8000 rpm at 4 °C for 10 min. The precipitate from this step was collected and stored at 4 °C 6. Dialysis tubes, which were previously soaked in 0.1M Phosphate buffer, pH 6.2, were used for the dialysis of the precipitate. The precipitate was dissolved in 0.1M Phosphate buffer and dialyzed against the same buffer. The enzyme preparation obtained from the above step was further purified by passing through a column of activated DEAE-cellulose previously equilibrated with 0.1M Phosphate buffer, pH 6.2. A total of 30 fractions were collected at the flow rate of 5 ml/30 min. Fractions showing high activity were pooled and used for further studies.

Antioxidant activity (scavenging of DPPH): The antioxidant capacity of the partially purified enzyme samples was studied through their scavenging activity against 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) radicals [11]. DPPH is a stable deep violet radical due to its unpaired electron. In the presence of an antioxidant radical scavenger, which can donate an electron to DPPH, the deep violet color decolorize to the pale yellow nonradical. The bleaching of DPPH was monitored at absorbance of 515 nm.

RESULTS AND DISCUSSION

Isolation of Fungi and L-asparaginase Production: Twenty two fungal species were isolated from the soil sample. According by physiological and morphological identification fungi were identified to the genera *Aspergillus, Penicillium and Mucor* sp. They were examined for L-asparaginase production. Among these, ten 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.

Screening of L-asparaginase Producing Fungi by Submerged Fermentation: All active strains was cultivated in mCD broth. It was found that the range of L-asparaginase production was 0.75-3.75 U/ml. The *Penicillium* sp showed maximum production (3.75 U/ml) was selected for further study.

The preliminary screening of filamentous fungi is based on the semi-qualitative method described by Gulati *et al.* [7]. All the isolated twenty two strains were submitted to this systemic investigation. This preliminary screening showed that only strains from *Pencillium* and *Aspergillus* genera presented asparaginase production. We select *Pencillium* sp for further study; it showed higher enzyme activity of 3.75U/ml. Enzyme activity of some isolates was not correlated to the size of pink zone diameter. This result was similar to Holker *et al.*[13] and Lee *et al.*[14].

Evaluation of Agro-industrial Residues as Substrates for SSF: Among 10 substrates screened (Fig. 1), Sugar cane bagasse gave highest enzyme production (8.2 U/ml), which was higher than that produced by other substrates. The ability of fungi to produce enzyme was different in solid and liquid state fermentations. Different agro industrial wastes were used to find out the best substrate for Asparaginase production by Solid state fermentation.

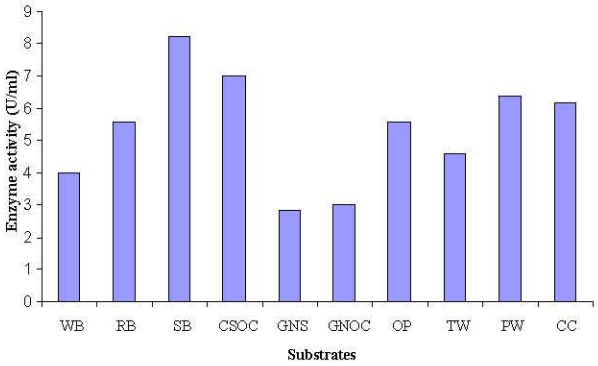

Fig. 1: Screening of Substrates for L-Asparaginase production

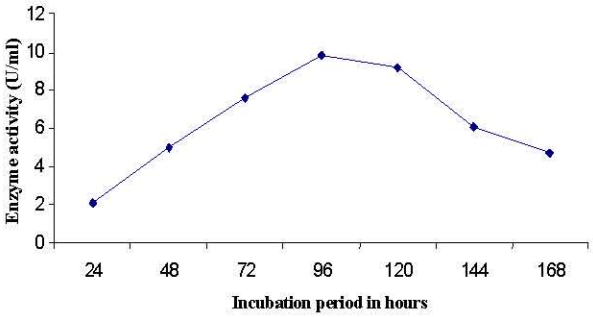

Fig. 2: Effect of Incubation period In enzyme Production

Sugar cane bagasse was found to be the best for this enzyme production. Agro-industrial residues are generally considered the best substrates for the SSF processes and use of SSF for the production of enzymes is no exception to that.

SSF produces a high product concentration having a relatively low energy requirement and appears to be promising for the production of "low-volume and high cost" products such as biopharmaceuticals. Recent evidences indicate bacteria and fungi growing under SSF conditions to be capable of supplying the global demand for various metabolites [15]. The product titers produced in SSF are many-fold higher than that from submerged culture, although the reasons for this are not clear [16].

Effect of Incubation Time, Temperature and Moisture content on L-asparaginase production by *Pencillium* sp: Enzyme activity increased after incubation for 24 to 96 h and slightly decreased after 96 hours when sugar cane bagasse was used as substrate. L-asparaginase production was highest after incubation for 96 h (9.8 U/ml, Figure 2).

The moisture content in sugar cane bagasse tested for maximum L-asparaginase production indicated enhanced enzyme production with increase in

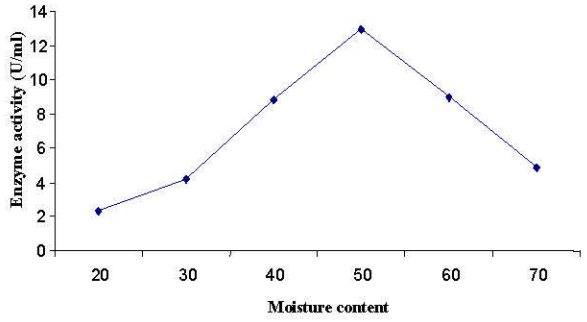

Fig. 3: Effect of Moisture content in Enzyme production

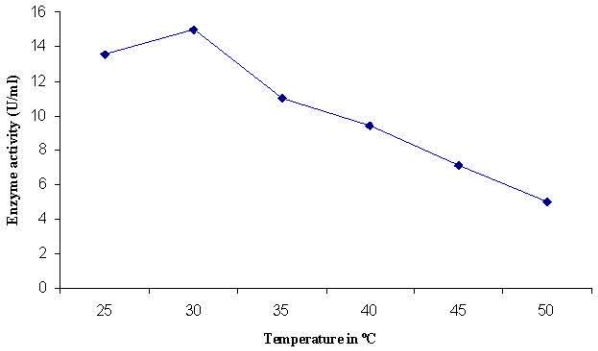

Fig. 4: Effect of Temperature in enzyme production

the substrate moisture content up to 50% beyond which it declined (Figure 3). The highest production of L-asparaginase was obtained at a moisture level of 50% and the maximum L-asparaginase activity of 12.96 U/ml was observed and it declined sharply at lower levels of moisture content. The lowest enzyme activity of 2.34 U/ml at 20% moisture level was observed.

Incubation temperature is another important factor, which affects the enzyme production in SSF. Maximal enzyme production of 15 U/ml was recorded at 30°C and production was reduced at temperatures higher than 30°C (Figure 4). The temperature normally employed in SSF is in the range of 25-35°C and it depends mostly on the growth kinetics of the microorganisms used.

Mishra [12] recommended SSF as an advantage method to increase the yield of L-Asparaginase [12]. Hence, investigation on production of L-asparaginase by solid state fermentation (SSF) using agricultural wastes should be carried out to verify such recommendation.

The media optimization is an important aspect to be considered in the development of fermentation technology. However, there are only a few reports concerning the optimization of media composition especially for fungal strains in enzyme production.

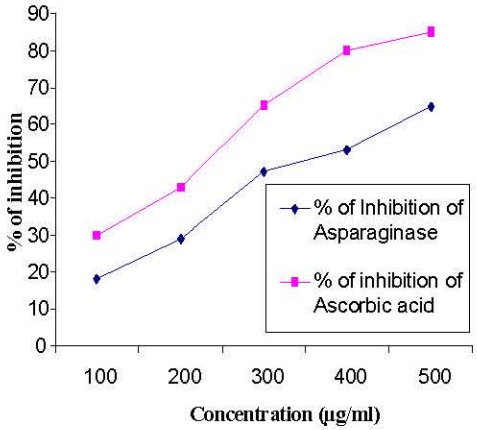

Fig. 5: DPPH Scavenging Activity

The incubation period varies with enzyme productions [17]. Short incubation period offers potential for inexpensive production of enzymes [18]. In the present study the Asparaginase activity increased steadily and reached maximum at 96 h of incubation. This result was similar to Mishra [12] who reported the highest L-asparaginase activity of *A. niger* in solid medium the optimal period for enzyme production was 96 h.

Maximum Asparaginase production was observed at 50% of moisture content. In most of the cases, 40-70% moisture requirements have been reported for maximum growth and substrate utilization. Moisture content is known to be the most studied parameter in solid-state fermentation, because the growth and metabolism of microorganisms in SSF mostly occur in the liquid phase. Optimal moisture content depends on the microorganism and the substrate. Lower moisture levels reduce the solubility of the nutrients in the solid substrate, lower the degree of substrate swelling and increase the water tension. Similarly, higher moisture is reported to decrease porosity, alter the particle structure, develop stickiness and decrease gaseous exchange [19, 20].

Enzyme synthesis occurred between 25-50°C with an optimum at 30°C (Fig. 4). A decrease in enzyme titres was observed when temperature range fell outside the mesophilic range.

DPPH Radical Scavenging Assay: DPPH radical scavenging activity of L-asparaginase was compared with ascorbic acid. It was observed that the enzyme had higher scavenging activity. At a concentration of 300 µg/ml, the scavenging activity of enzyme reached 64.96%, while at the same concentration; the standard was 85% (Fig. 5).

Antioxidant properties, especially radical scavenging activities, are very important due to the deleterious role of free radicals in foods and biological systems. The reduction of DPPH absorption is indicative of the capacity of the extracts to scavenge free radicals, independently of any enzymatic activity. DPPH is a stable non-physiological radical, which could provide a relative figure of the radical scavenging capacity of a tested probe. *Pencillium sp* showed 64.96 % of inhibition. From the present results, it may be postulated that Asparaginase enzyme possess antioxidant activity.

In conclusion, several fungal strains were isolated from soil and were capable of L-asparaginase production. Among these *Pencillium* sp was found to produce the highest L-asparaginase activity of 15U/ml after optimization. Our results clearly showed that the enzyme had strong antioxidant activity.

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