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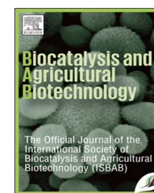

# Solid state fermentation: An effective fermentation strategy for the production of L-asparaginase by *Fusarium culmorum* (ASP-87)

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A B S T R A C T

Production of L-asparaginase by *Fusarium culmorum* (ASP-87) isolated from tropical soil was investigated under solid state fermentation on a laboratory scale using sixty five (65) agro based materials. Among the different agro based materials evaluated, soybean meal supported maximum L-asparaginase production (7.21 U/gds). Various optimization strategies for the production of L-asparaginase were also carried out with soybean meal and it was observed that inoculum size of $1 \times 10^8$ spores/mL, day 6 of incubation period, 3 mm of particle size of the substrate, moisture content of 70%, initial pH of 7.0 and temperature at 30 °C were found to be optimal for L-asparaginase production. Supplementation of glucose (0.1%) and ammonium chloride (0.1%) enhanced L-asparaginase production to 1.7 fold. Mixed substrate fermentation using soybean meal and wheat bran in the ratio (1:1 w/w) further enhanced production of L-asparaginase to 0.5 fold with a final yield of 18.91 U/gds.

## 1. Introduction

Enzymes are proteins with biocatalytic activity that catalyze and speed up rate of various chemical reactions with substrate specificity by lowering the activation energy. Microbes are widely exploited and are the potential source of industrially important enzymes as they synthesize and secrete large amounts of extracellular enzymes. Fungal enzymes account for approximately 60% of commercially available enzymes. Enzymes produced by fungi are widely used in paper and pulp industries, leather industries, textiles, detergents, food and beverages and therapeutics. The therapeutic enzymes produced by fungi have been commonly used as anti-inflammatory, thrombolytics or anticoagulants and oncolytics as replacement for metabolic deficiency. A major application of therapeutic enzymes is in the chemotherapy of cancer.

L-asparaginase (L-asparagine amido hydrolase E.C.3.5.1.1) is an enzyme belonging to an amidase group which catalyzes the hydrolysis of the amino acid L-asparagine to L-aspartic acid and ammonia. It is widely used as a therapeutic agent for the treatment of acute lymphocytic leukemia (ALL) (mainly in children), Hodgkin's disease, acute myelocytic leukemia, chronic lymphocytic leukemia, lymphosarcoma treatment and melano sarcoma (Stecher et al., 1999; Verma et al., 2007). L-asparaginase also has significant applications in food industry to reduce the formation of carcinogenic acryl amides in deep fried potato (Pedreschi et al., 2008).

L-asparaginase is widely distributed among plants, animals and microbes. However, L-asparaginase from fungal source has gained much attention because of its stability, high productivity and easy cultural conditions.

Industrial production of L-asparaginase by bacteria and fungi has been previously achieved by submerged fermentation (SmF). Commercial production of L-asparaginase is carried out by using *Escherichia coli* and *Erwinia chrysanthemi*. Submerged fermentation has several disadvantages like huge volume of waste water generation and difficulties in effluent treatment process. (Datar, 1986). Solid state fermentation (SSF) has gained much interest during recent years and has emerged as an economical, alternative cost effective process for enzyme production by utilizing agricultural and agro industrial residues as substrates which are converted into products with high commercial value (Pandey, 1992). The use of SSF for production of enzymes has many advantages over submerged fermentation such as less production cost with better physiological properties, smaller reactor volume and cheaper fermentation media, higher production rate and easier downstream processing (Pandey et al., 1999; Bhargav, 2008). Solid substrates, mainly agro based materials are reportedly being utilized as substrates for the production of L-asparaginase (Swathi et al., 2014; Kumar et al., 2013; Hymavathi et al., 2009; Mishra, 2006; El-Bessoumy et al., 2004). These substrates act both as physical support and source of nutrients which influences enzyme production. Therefore, screening and selection of an appropriate substrate is an important step for commercial production of an enzyme.

We have previously reported the production strategy of L-

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asparaginase in *F. culmorum* (ASP-87) by submerged fermentation (Meghavarnam and Janakiraman, 2015). In the present study, we explore the possibility of using agro based materials as sole substrates for the better production of L-asparaginase by *F. culmorum* (ASP-87).

## 2. Materials and methods

### *2.1. Substrates and chemicals*

Different agro based substrates were procured from mills and few substrates were purchased from the local market in Bengaluru, Karnataka, India. These substrates were dried and ground into smaller particles in a mill and the particle size of 1 mm was used for the experiment. Other media components used in the experiment were obtained from Hi-media (Mumbai, India). All the chemicals were of analytical reagent grade.

#### *2.1.1. Microorganism and culture conditions*

The fungus *F. culmorum* strain (ASP-87) used in the study was isolated from tropical soil. This fungus was tested for mycotoxin production and found to be negative for fumonisins and trichothecenes. The fungal culture was maintained on Potato Dextrose Agar (PDA) slants by periodically sub-culturing on PDA at 30 °C and stored at 4 °C.

#### *2.1.2. Inoculum preparation*

The culture medium used for inoculum preparation was modified Czapek-Dox medium containing g/L of, Glucose,2.0; L-asparagine,10.0; $KH_2PO_4$,1.52; KCL,0.52; $MgSO_4 \cdot 7H_2O$,0.52; $CuNO_3 \cdot 3H_2O$,trace; $ZnSO_4.7H_20$,trace; $FeSO_4.7H_20$,trace; pH 7.5 (Saxena and Sinha, 1981). Modified Czapek-Dox broth was prepared, sterilized and inoculated with *F. culmorum* (ASP-87) spore suspension ($10^7$ spores/mL). The culture was incubated at 30 °C under static cultivation conditions for 96 h. The mycelial mat obtained was homogenized with water using mortar and pestle under sterile conditions and used as inoculum for further experiments.

### *2.2. Screening and preparation of solid substrates*

In total, sixty five (65) types of agro based materials were used. They are arecanut peel, banana peel, banana stem, barley, black gram husk, bengal gram husk, broken chana, broken red rice, broken wheat, coconut fiber, coconut oil cake, carob pod, coffee husk, colocasia, corn cob, corn flakes, corn flour, corn kernel, corn peel, cotton seed oil cake, dry copra waste, flat bean seed, flat bean peel, gram flour waste, green gram husk, green peas waste, groundnut oil cake, groundnut waste, groundnut shell, jack fruit seeds, jowar husk, lemon peel, linseed powder, musambi peel, mushroom, orange peel, paddy husk, paddy straw, pea husk, pea peel, pomegranate peel, potato peel, ragi bran, ragi flour, ragi straw, rajma seed, red gram husk, rice flour, rice husk, saw dust, seri waste, sesame oil cake, sooji, sorghum milling waste, soybean broken, soybean meal, sugarcane bagassae, sunflower oil cake, sweet potato, tamarind peel, tapioca, tea waste, urad dhal waste, wheat bran and wheat flour were screened for L-asparaginase production.

Five grams of each substrate was weighed separately in a 100 mL Erlenmeyer flask and moistened with distilled water (70%) containing L-asparagine (0.05% w/w). The flasks were sterilized by autoclaving at 121 °C (15 psi) for 15 min. After cooling, the flasks were inoculated with 1.0 mL of spore suspension ($10^7$ spores/mL). The contents of the flasks were mixed thoroughly and incubated at 30 °C for 6 days (Kumar et al., 2013).

#### *2.2.1. Extraction of the enzyme*

After 6 days of incubation, L-asparaginase was extracted from substrates according to the method of Kumar et al. (2013). The substrate with fungal biomass was air dried under shade and fifty milliliter of water was added and kept on orbital shaker at (150 rpm) for 30 min. To optimize incubation period 1g of substrate with fungal biomass was withdrawn periodically at regular intervals in aseptic condition and air dried under shade and taken in a beaker containing 10 mL of distilled water (1:10). The contents of flasks were kept on orbital shaker (150 rpm) for 30 min. The extract was separated by squeezing through a muslin cloth and filtered through Whatman No. 1 filter paper. The filtered extract was centrifuged at 10,000 rpm for 10 min. The resultant supernatant was used as enzyme source for L-asparaginase assay.

#### *2.2.2. Determination of L-asparaginase activity*

L-asparaginase activity in the culture filtrates was determined by the method of Imada et al. (1973). The L-asparagine hydrolysis rate was determined by quantifying the ammonia liberated using Nessler's reagent. A blend of 0.5 mL of 0.04 ML-asparagine, 0.5 mL of enzyme extract, 0.5 mL of 0.05 M Tris-HCl buffer (pH 7.2) and 0.5 mL of distilled water was incubated at 37 °C for 30 min and 0.5 mL of 1.5 M trichloroacetic acid (TCA) was added to stop the reaction. The ammonia liberated in the supernatant was determined spectrophotometrically by adding 0.2 mL of Nessler's reagent into tubes containing 0.1 mL of enzyme and 3.7 mL of distilled water and incubated at room temperature for 20 min. The absorbance was measured at 450 nm and L-asparaginase activity was expressed as the amount of ammonia liberated per mL per minute under the standard assay conditions.

### *2.3. Optimization studies for L-asparaginase production*

Soybean meal was used as the sole source of carbon for L-asparaginase production. The optimization studies were carried out by one factor at a time (OFAT) approach by varying only a single factor at a time and keeping the remaining factors constant. The effect of various physiological and nutritional parameters such as incubation period (4 days to 9 days), initial moisture content of the substrate (40%, 50%, 60%, 70%, 80% and 90%), particle size fine (2 mm), intermediate (3 mm) and coarse (4 mm), inoculum size ($10^6$, $10^7$, $10^8$ and $10^9$ spores/mL), initial pH 4.0–9.0), incubation temperature (25, 28, 30, 33, 35 and 37 °C) on L-asparaginase production has been studied. The influence of supplementation of additional carbon sources such as glucose, maltose, lactose, sucrose, fructose, starch, cellulose, xylose and ribose at 0.1% (w/w) and nitrogen sources such as ammonium chloride, ammonium nitrate, ammonium sulfate, potassium nitrate, sodium nitrate, urea, peptone, yeast extract, beef extract and tryptone at 0.1% (w/w) on L-asparaginase production has been studied. Samples were drawn continuously at 24 h intervals and the enzyme activity was determined. All the experiments were carried out in triplicates.

### *2.4. Effect of combination of mixed substrates on L-asparaginase production*

The substrates soybean meal, carob pod, corn cob, corn kernel, gram husk, green peas, groundnut oil cake, jowar husk, mushroom powder, soybean flour, sugarcane bagassae, sunflower oil cake, urad dhal waste and wheat bran were used for mixed substrate fermentation in various combinations for enhanced production of L-asparaginase. Soybean meal was kept as constant and each substrate was mixed with soybean meal in 1:1 (w/w) ratio and assessed for L-asparaginase production. The substrate combination of soybean mean and wheat bran was evaluated in different ratios (1:4, 2:3, 1:1, 3:2 and 4:1) (w/w) for L-asparaginase production. The fermentation was carried out with the parameters that were optimized by OFTA approach. Five grams of each combination of substrates was weighed separately in a 100 mL Erlenmeyer flask and moistened with distilled water (70%) containing L-asparagine (0.05% w/w). The flasks were sterilized by autoclaving at 121 °C (15 psi) for 15 min. After cooling, the flasks were inoculated with 1.0 mL of spore suspension ($10^8$ spores/mL). The contents of the flasks were mixed thoroughly and incubated at 30 °C for 6 days.

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