0273-2289/1559-0291 (Online)/06/135/0033/$30.00

Production of L-Asparaginase, an Anticancer Agent, From *Aspergillus niger* Using Agricultural Waste in Solid State Fermentation

Abha Mishra

School of Biochemical Engineering, Institute of Technology, Banaras Hindu University, Varanasi-221005, India, E-mail: abha91@yahoo.co.in

Received December 15, 2005; Revised February 18, 2006; Accepted February 21, 2006

Abstract

This article reports the production of high levels of L-asparaginase from a new isolate of *Aspergillus niger* in solid state fermentation (SSF) using agro-wastes from three leguminous crops (bran of *Cajanus cajan, Phaseolus mungo,* and *Glycine max*). When used as the sole source for growth in SSF, bran of *G. max* showed maximum enzyme production followed by that of *P. mungo* and *C. cajan*. A 96-h fermentation time under aerobic condition with moisture content of 70%, 30 min of cooking time and 1205–1405 μ range of particle size in SSF appeared optimal for enzyme production. Enzyme yield was maximum (40.9 ± 3.35 U/g of dry substrate) at pH 6.5 and temperature 30 ± 2°C. The optimum temperature and pH for enzyme activity were 40°C and 6.5, respectively. The study suggests that choosing an appropriate substrate when coupled with process level optimization improves enzyme production markedly. Developing an asparaginase production process based on bran of *G. max* as a substrate in SSF is economically attractive as it is a cheap and readily available raw material in agriculture-based countries.

Index Entries: L-Asparaginase; SSF; *Aspergillus niger*; agro-waste; bran.

Introduction

L-Asparaginase (E.C.3.5.1.1) is present in a wide range of organisms including animals, microbes, plants, and in the serum of certain rodents but not in human beings *(1)*. This enzyme is widely used in chemotherapy of acute lymphoblastic leukemia. However, the toxic side effects of some currently used clinical preparations of bacterial origin have necessitated

the search for alternative sources *(2)*. L-Asparaginase is produced by a number of microorganisms including *Escherichia coli, Erwinia cartovora,* and some species of *Enterobacter (1)*. Production of this enzyme has also been reported from *Corynebacterium glutamicum (3)*, *Cylindrocarpon obtusisporum (4)*, *Pseudomonas stutzeri (5)*, *Rhodosporodium toruloids (6)*, *Tetrahymena pyriformis (7)*, *Pseudomonas aeruginosa* 50071 *(1)*, *Aspergillus tamarii,* and *Aspergillus terreus (8)*.

L-Asparaginase production throughout the world is carried out mainly by submerged fermentation (SF). This technique, however, has many disadvantages. For instance, it is cost intensive and has low product concentration. In addition, it generates excess of effluents and consequently needs handling and disposal of large volumes of waste water during downstream processing *(1)*. Solid state fermentation (SSF) has emerged as an effective technique to increase the product yield at low capital cost, low energy input, simple fermentation media, and low water use, and offers simple operational control *(9,10)*. Low water use in SSF results in low production of effluents and reduced growth of contaminating bacteria and yeasts. This in turn, reduces the cost of sterilization. The use of agricultural wastes as source of energy and C-pool makes the SSF environment friendly *(11)*. In addition, the substrates in SSF simulate the natural habitat of filamentous fungi and serve as an anchorage for fungal hyphae. Asparaginase from two bacterial strains (*E. coli* and *E. carotovora*) is in clinical use for the treatment of acute lymphoblastic leukemia *(12,13)*. The filamentous fungi *A. terreus* also possesses antitumor property against Ehrlich's ascites in susceptible Swiss mice and the enzyme may be non-toxic and have myelosuppressive property *(14)*. For the commercial production of the enzyme, selection of a superior strain and substrate is a crucial step. Therefore, an attempt was made to study the production and properties of L-asparaginase from a new isolate of *A. niger* in SSF using bran of pulses, an agricultural waste, used as a feed supplement to cattle in India.

Materials and Methods

Microorganism and Cultivation

The mold considered in this study was a new isolate of *A. niger* obtained from the Institute of Technology, Banaras Hindu University, India. The culture was maintained on modified Czapek Dox medium containing (g/L of distilled water): 2.0 glucose, 10.0 L-asparagine, 1.52 K_2HPO_4, 0.52 KCl, 0.03 $FeSO_4 \cdot 7H_2O$, 0.05 $ZnSO_4 \cdot 7H_2O$, 0.3 $NaNO_3$, and 30.0 Agar at pH 6.2 *(8)*. The bran of pulses containing 19–22% crude protein, 9.8–13% crude fiber, 1.2–1.5% crude fat, and high concentrations of K, P, Ca, Mg, Zn, Fe, and Cu *(15)* were used as substrate.

Plate Assay for Evaluation of L-Asparaginase Production

Modified Czapek Dox's medium was supplemented with 0.3 mL of 2.5% phenol red dye prepared in ethanol at pH 6.5 with L-asparagine incor-

porated in the medium for evaluation of L-asparaginase activity. The media was autoclaved and the plates were inoculated with 3-d-old culture of *A. niger (16)*. The clear zone appeared after 48 h of growth. Uninoculated media served as control.

Enzyme Production

Fermentation was carried out following the method described in Mishra and Das *(17)*. The medium containing 10 g bran of either *Cajanus cajan*, *Phaseolus mungo*, or *Glycine max* with particle size range of 1205–1405 µm, was moistened with 1.0 to 9.0 mL of 0.01 *M* phosphate buffer (pH 6.5) in Roux bottle. The fermentation media were sterilized by autoclaving for 30 min (referred in the text as cooking time) at 15 psi and 121°C. The bottles were inoculated with 3 mL of fungal spore suspension and incubated under stationary condition at 37°C for 96 h. For the extraction of crude enzyme, weighed quantity of fermented substrate was mixed with phosphate buffer (1:5). The mixture was homogenized and filtered. The filtrate was centrifuged and clear supernatant used for enzyme assay.

SSF Substrates and Culture Conditions

Various process parameters such as initial moisture content (10–90%), particle size (710–3353 µ), autoclaving time (15–60 min) at 121°C, initial pH of the medium (3.5–8.5), and fermentation temperature (25–45°C) all influenced the enzyme yield during SSF and were optimized independently over a wide range. To optimize asparaginase activity at a different pH, enzymes extracted in buffers of pH 3.5 to 8.5 were placed in 2.0 mL of 0.01 *M* phosphate buffer (pH 3.5 to 8.5) containing 20 µ*M* of L-asparagine and incubated for 15 min at 40°C in a water-bath shaker. For optimization of temperature, crude enzyme extracts were placed in 2.0 mL of 0.01 *M* phosphate buffer (pH 6.5) containing 20 µ*M* of L-asparagine and incubated for 15 min at 25 to 70°C in water bath shaker. The concentrations of liberated ammonia in the test solutions were determined following Nesselerization method *(18)*.

Enzyme Assay

L-Asparaginase activity was assayed following Nesselerization method *(18)*. One enzyme unit was defined as the amount of enzyme, which liberates 1 µ*M* of ammonia per gram of dry substrate per minute (U/gds) under optimal assay conditions.

Enzyme Purification

The purification of enzyme was carried out at 4°C according to the method as described in El-Bessoumy et al. *(1)*. Finely powdered ammonium sulfate was added to an 80% saturation with gradual stirring, followed by centrifugation at 8000*g* for 20 min under refrigerated conditions. The concentrated enzyme solution was applied to a DEAE cellulose col-

umn (4.2 cm × 25 cm) that was pre-equilibrated with 20 m*M* phosphate buffer, pH 6.5 and eluted with the NaCl gradient (0.1–0.5 *M*). The active fractions were collected, dialyzed, and concentrated. This preparation was used in the subsequent step. The enzyme was assayed by the direct Nesslerization method. The protein concentration was determined by the Lowry's method *(19)*.

Statistical Analysis

The randomized factorial design of the whole experiment consisted of nine factor variables including three bran of pulses and six process determinants. The effect of these variables and their interactions were tested using two-way analysis of variance (ANOVA). Data were homogenized whenever necessary to equalize variance.

Results and Discussion

The study with phenol red dye in rapid plate assay method revealed that the presence of dye did not inhibit the growth of *A. niger* and a clear zone appeared around the colony. A broad zone indicated that the test organism was an efficient producer of L-asparaginase. The plate assay method is quick and enzyme production can be visualized directly from the plates without performing time consuming assays. Gulati et al. *(16)* followed similar experimental protocol for screening asparaginase producing bacteria and fungal strains.

Particle size of the substrates is a crucial factor for enzyme production in SSF. L-Asparaginase production increased with particle size and attained its maxima in the particles size range of 1205–1405 μ (Table 1). Enzyme production declined with further increase in particle size. Two-way analysis of variance indicated significant ($p < 0.01$) effect of particle size on enzyme production. The increase in interparticle porosity with increasing particle size could support mycelium growth and provide better aeration for SSF. However, further increase in porosity with particle size coupled with reduced saturated surface area could reduce nutrient availability for fungal growth. Similar observations were made by Mishra and Das *(17)* for glucoamylase production using wheat bran in SSF.

Optimal conditions for L-asparaginase production from *A. niger* appeared to be 70% initial moisture content (Table 2), 30 min of autoclaving time at 121°C (Table 3), 6.5 as initial pH (Table 4), and 30°C as incubation temperature (Table 5). In all the cases, a decrease or increase in the level of these variables reduced the enzyme yield. With respect to the substrate, *A. niger* showed maximum enzyme production in the bran of *G. max* (39.9 ± 3.92 U/gds) followed by that of *P. mungo* (30.7 ± 3.69 U/gds) and *C. cajan* (26.14 ± 3.67 U/gds) at pH 6.5. Two-way analysis of variance indicated significant ($p < 0.01$) effect of substrate quality (bran of pulses) on enzyme production. The substrates (bran) used in this study are traditional products obtained in the processing of pulses and used as food supplements for

Table 1
Effect of Substrate Particle Size (μ) on Asparaginase Production From *A. niger* Grown on Bran of Pulses in SSF
(30°C; pH 6.5; Fermentation Time, 96 h; Initial Moisture Content, 70%)

Particle size (μ)	Enzyme activity (U/gds)[a]		
	Cajanus cajan	*Phaseolus mungo*	*Glycine max*
<710	5.1 ± 0.31	6.6 ± 0.25	8.54 ± 0.80
710–850	8.8 ± 0.98	10.3 ± 0.98	19.3 ± 1.85
850–1003	11.9 ± 1.31	15.2 ± 1.15	24.1 ± 2.42
1003–1205	20.10 ± 2.20	26.5 ± 3.05	31.5 ± 3.32
1205–1405	26.14 ± 3.67	30.7 ± 3.69	39.9 ± 3.92
1405–2411	23.3 ± 2.61	26.1 ± 2.70	35.7 ± 3.49
2411–2812	19.3 ± 2.24	22.0 ± 2.15	28.3 ± 2.75
2812–3353	14.7 ± 1.75	18.9 ± 2.07	25.4 ± 2.66
>3353	7.4 ± 0.79	10.1 ± 1.12	18.1 ± 1.72

[a]Difference significant: $p < 0.01$ (analysis performed using two-way ANOVA).
Values are mean of triplicates ± SD.

Table 2
Effect of Moisture Content per Gram Bran of Pulses on Asparaginase Production From *A. niger* in SSF
(30°C; pH 6.5; Fermentation Time, 96 h; Particle Size, 1205–1405 μ)

Moisture content (%)	Enzyme activity (U/gds)[a]		
	Cajanus cajan	*Phaseolus mungo*	*Glycine max*
10	5.7 ± 0.53	6.6 ± 0.75	7.9 ± 0.86
20	6.8 ± 1.52	8.3 ± 0.96	11.3 ± 1.41
30	8.6 ± 0.99	12.3 ± 1.18	16.1 ± 1.82
40	11.4 ± 1.92	16.1 ± 1.85	21.1 ± 2.19
50	15.1 ± 1.10	18.6 ± 1.97	26.1 ± 2.81
60	19.9 ± 2.46	22.6 ± 2.22	31.7 ± 3.20
70	24.8 ± 3.06	29.9 ± 2.76	38.7 ± 3.48
80	20.1 ± 2.17	23.9 ± 2.51	30.1 ± 3.18
90	17.1 ± 1.92	19.8 ± 2.12	24.5 ± 2.41

[a]Difference significant: $p < 0.01$ (analysis performed using two-way ANOVA).
Values are mean of triplicates ± SD.

cattle in India. The observed significant difference in asparaginase titres, when *G. max* bran was used as a substrate, may be attributed to the presence of some additives (nutrients/activators), favorable C:N ratio, and degradability of this substrate as a carbon source *(1)*.

L-Asparaginase production increased with moisture content of the medium, supporting maximal yield at 70% moisture content (Table 2). In addition to the problem associated with the lowering of oxygen transfer

Table 3
Effect of Cooking Time on Asparaginase Production
From *A. niger* Grown on Bran of Pulses in SSF
(30°C; pH 6.5; Fermentation Time, 96 h;
Particle Size, 1205–1405 µ; Initial Moisture Content, 70%)

Cooking time (min)	Enzyme activity (U/gds)[a]		
	Cajanus cajan	*Phaseolus mungo*	*Glycine max*
15	16.8 ± 1.75	20.3 ± 2.49	28.3 ± 2.80
30	24.8 ± 3.05	27.9 ± 2.82	38.7 ± 3.63
45	18.4 ± 2.26	21.1 ± 2.95	32.5 ± 3.45
60	10.0 ± 1.12	13.6 ± 1.12	21.9 ± 1.72

[a]Difference significant: $p < 0.01$ (analysis performed using two-way ANOVA).
Values are mean of triplicates ± SD.

Table 4
Effect of pH on Asparaginase Production
From *A. niger* Grown on Bran of Pulses in SSF
(30°C; Fermentation Time, 96 h;
Particle Size, 1205–1405 µ; Initial Moisture Content, 70%)

Initial pH	Enzyme activity (U/gds)[a]		
	Cajanus cajan	*Phaseolus mungo*	*Glycine max*
3.5	5.1 ± 0.83	7.6 ± 0.74	10.9 ± 0.96
4.5	10.8 ± 1.20	12.3 ± 1.25	19.3 ± 1.97
5.5	16.1 ± 2.65	21.9 ± 3.02	32.2 ± 2.82
6.5	25.0 ± 3.24	29.5 ± 3.17	37.5 ± 3.84
7.5	18.4 ± 2.10	20.6 ± 2.19	29.6 ± 2.87
8.5	9.8 ± 0.69	11.3 ± 0.78	17.3 ± 1.69

[a]Difference significant: $p < 0.01$ (analysis performed using two-way ANOVA).
Values are mean of triplicates ± SD.

Table 5
Effect of Temperature on Asparaginase Production
From *A. niger* Grown on Bran of Pulses in SSF
(pH 6.5; Fermentation Time, 96 h;
Particle Size, 1205–1405 µ; Initial Moisture Content, 70%)

Fermentation temperature (°C)	Enzyme activity (U/gds)[a]		
	Cajanus cajan	*Phaseolus mungo*	*Glycine max*
25	18.8 ± 1.75	22.3 ± 1.92	32.3 ± 2.75
30	29.1 ± 3.14	31.2 ± 3.06	40.9 ± 3.55
35	24.1 ± 2.96	27.6 ± 2.95	34.4 ± 3.32
40	19.6 ± 2.75	21.9 ± 2.96	28.8 ± 2.91
45	15.4 ± 2.27	17.2 ± 2.45	22.6 ± 2.61

[a]Difference significant: $p < 0.05$ (fermentation temperature); $p < 0.01$ (bran) (analysis performed using two-way ANOVA).
Values are mean of triplicates ± SD.

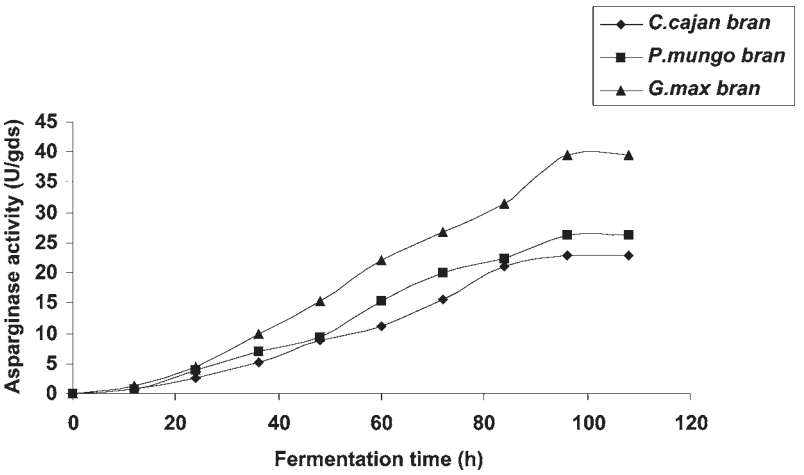

Fig. 1. Effect of fermentation time on asparaginase production (*A. niger* was grown on bran of pulses in SSF at pH 6.5; 30°C; particle size, 1205–1405 μ; initial moisture content, 70%).

at high moisture level *(11)*, the critical importance of moisture content in enzyme production in SSF could also be linked with its effects on other physical properties of the medium. For instance, a reduction in enzyme production at high initial moisture content may be as a result of a reduction in substrate porosity, changes in structure of substrate particles, reduction in gas volume, and reduced fungal growth *(20)*. On the other hand, low moisture level could lower the solubility of nutrients, reduce substrate swelling, and decrease the water retention by the substrate. All these conditions affect fungal growth and ultimately the enzyme production.

The enzyme production was low at 15 min of cooking time (at 121°C) and increased as the time extended (Table 3). However, beyond 30 min, a decreasing trend was observed. It could be that the nutrients present in the bran were broken down incompletely at 15 min of cooking whereas beyond 30 min, low production of enzyme may be attributed to lowered nutrient availability. Regarding temperature, the enzyme yield appeared optimum at 30°C (Table 5). The heat that accumulates in the medium during mesophilic aerobic SSF because of poor heat dissipation could lead to a further drop in the oxygen level and thereby reducing the growth of test organism. The production of enzyme appeared growth dependent and a 96 h of fermentation time supported maximal yield under optimum conditions (Fig. 1).

The influence of pH on L-asparaginase activity and stability was studied using buffers with pH range of 3.5 to 8.5. The enzyme activity increased with pH and attained its maxima at pH 6.5 (Fig. 2). The enzyme appeared pH stable, because no appreciable loss in activity was observed over a wide range of pH under refrigerated condition (data not shown). El-Bessoumy et al. *(1)* observed maximum activity of L-asparaginase from *Pseudomonas aeruginosa* at pH 9.0. Maximum activity of L-asparaginase from *A. niger* at

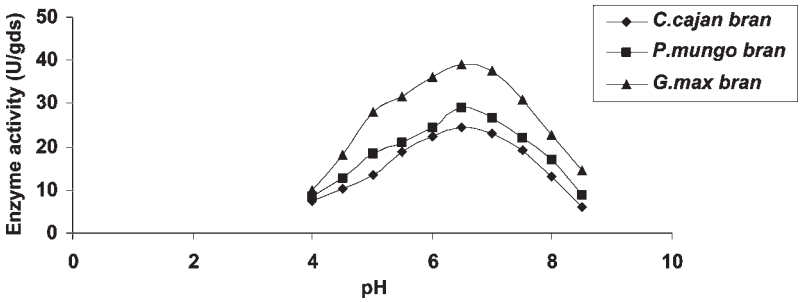

Fig. 2. Effect of pH on asparaginase activity under optimum assay condition (*A. niger* was grown on bran of pulses in SSF at pH 6.5; 30°C; fermentation time, 96 h; particle size, 1205–1405 μ; moisture content, 70%).

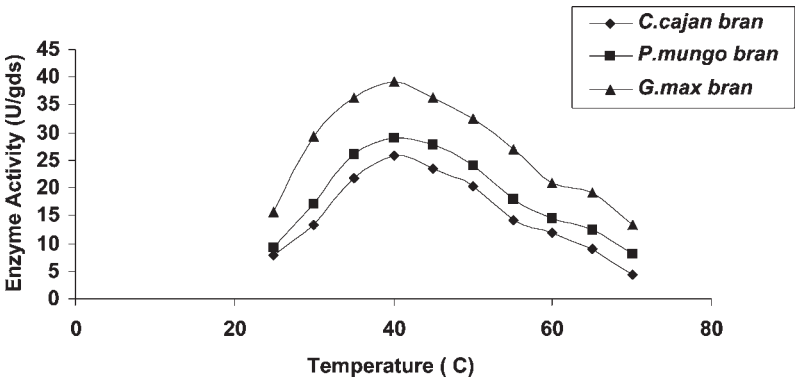

Fig. 3. Effect of temperature on asparaginase activity under optimum assay condition (*A. niger* was grown on bran of pulses in SSF at pH 6.5; 30°C; fermentation time, 96 h; particle size, 1205–1405 μ; initial moisture content, 70%).

near physiological pH makes this enzyme superior to that of bacterial origin as a chemotherapeutic agent in the treatment of leukemia. The reaction rate of L-asparaginase appeared optimal at 40°C. Loss of activity was observed over this temperature, although 50% activity retained even at 60°C (Fig. 3). L-Asparaginase from *A. niger* indicated greater thermal stability than those of bacterial origin *(1)*.

The purification of L-asparaginase crude extract of fermented *G. max* was affected by ammonium sulfate (80%) precipitation and showed that 92% of the enzyme activity was retained after precipitation. The total protein decreased from 70.12 to 35.43 mg/mL in the purification steps. The specific activity increased from 0.57 U/mg (crude extract) to 0.82 U/mg (final preparation) (Table 6).

Most of the L-asparaginase production processes to date follow submerged fermentation exploiting bacterial strains mainly *Serratia marcescens*, *E. cartovora*, and *E. coli*. L-Asparaginase can be produced from low-cost untreated biomass residues without addition of supplements to enhance

Table 6
Purification of L-Asparaginase Produced From *A. niger* Grown on Bran of G. *max* in SSF

Step	Total protein (mg/mL)	Total activity (U/mL)	Specific activity (U/mg)	Fold purification	Yield (%)
Crude enzyme	70.12	40.21	0.57	–	–
Ammonium sulfate (80%)	56.89	37.01	0.65	1.14	92.04
DEAE cellulose	35.43	28.97	0.82	1.44	72.05

growth or production. Therefore, the observations made in this study hold great promise for scale-up production of L-asparaginase from *A. niger* under SSF. Because of the advantage of using concentrated fermentation broth in SSF with low risk of contamination and low recovery cost related to high enzyme titers as compared to submerged fermentation, it is eco-friendly and economically attractive. Maximum enzyme activity at or near physiological pH and temperature, make it extremely valuable in chemotherapeutic treatment of cancer.

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