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PRODUCTION, PURIFICATION AND CHARACTERIZATION OF L-ASPARAGINASE BY ISOLATED ASPERGILLUS NIGER B4 UNDER SOLID STATE FERMENTATION USING SOYA AS SUBSTRATE

Biological Science

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ABSTRACT

The objective of this investigation was to isolate soil and marine fungal isolates and to screen them for L-asparaginase production. Three isolates out of 48 fungal strains were isolated among which Aspergillus niger B4 was selected for optimization of fermentation parameters employing Soya powder as substrate under solid state fermentation.The fermentation studies indicated that the maximum L-asparaginase production 227.63 U/gds was achieved at 96h fermentation period with pH 6.5, inoculum volume 15%v/w, moisture content 100%, incubation temperature 30oC and particle size 0.6cm. The purified L-asparaginase was used for the characterization.

KEYWORDS:

L-asparaginase, *Aspergillus niger B4,* Solid state fermentation, Soya.

INTRODUCTION

L-asparaginase attracted much attention because of its use as effective therapeutic agent against lymphocytic leukemia and other kinds of cancer in man (1, 2). Cancer cells differentiate themselves from normal cells in diminished expression of L-asparagine (3, 4). Hence, they are not capable of producing L-asparagine, and mainly depend on the L-asparagine from the circulating plasma pools (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 (5, 6, 7, 8, 9)

The SmF technique is a cost intensive, highly problematic, and poorly understood unit operation (10). SSF offers numerous advantages over submerged fermentation (SmF). SSF should not be seen as a technology, which can simply replace SmF. Solid-state fermentation is a very effective technique as the yield of the product is many times higher when compared to that in SmF(11), and it also offers many other advantages (12).

The aim of the present study is to evaluate the suitability and utility of Soya powder as potential for L-asparaginase production using isolated Aspergillus niger B4 under solid state fermentation.

MATERIALS AND METHODS

Fungal isolates and cultural condition

Fungal isolates were isolated from soil and marine sediment samples collected from different regions of Visakhapatnam, AP, Nagarcoil, Kerala, Marina Beach and Chennai, using potato dextrose agar (PDA) medium by serial dilution method. The inoculated agar plates were incubated at 280C for 4 to 7 days. Three isolates out of 48 fungal isolates were selected and tentatively identified as Aspergillus species in the laboratory as described by Rapper and Fennell (13).

Production of L-asparaginase

The production of L-asparaginase was carried out by using 10g of soya (0.6cm) as a substrate under solid state fermentation. The moisture content of the flask is 100% were maintained and inoculated 15%v/w of inoculum (1x107 spores/ml). The content of the flasks were mixed thoroughly and incubated at 30oC for 4 days. The pH 6.5 was maintained throughout the fermentation process.

Optimization of fermentation parameters for L-asparaginase production

Production conditions like incubation time, incubation temperature, inoculum volume, particle size, initial pH, moisture content were optimized.

Extraction of L-asparaginase

The samples were withdrawn periodically at 24 h in aseptic condition 1 gm of powdery substrate was taken into a beaker and distilled water (1:10) was added to it. The contents of flasks were allowed to have contact with water for 1 h with occasional stirring with a glass rod. The extract was filtered through Whatman filter No.1. The clear extract was centrifuged at 2000-3000 rpm for 15 min, supernatant were used as enzyme preparation. Thus prepared crude enzyme was used for assay.

Quantitative assay for L-asparaginase activity

L-asparaginase activity was assayed by Nesslerization, a most common method for estimating L-asparaginase activity. L-asparaginase activity was estimated by quantifying ammonia formation by Nesslerisation using spectrophotometric analysis at 480nm (14).

CHARACTERIZATION OF L-ASPARAGINASE

After fermentation process L-asparaginase was purified using filtration, centrifugation, ammonium sulfate fractionation, Dialysis and Sephadex C 100 gel filtration, this purified enzyme were used for characterization studies which include effect of pH and effect of temperature.

Table - 1: Isolation of L-asparaginase producing fungal cultures from soil samples

No.	Sources	No. of isolates	No. of colonies shown L –asparaginase activity
1	Airport area, Visakhapatnam, (AP)	6	--
2	Sediments from Bheemili beach , Visakhapatnam, (AP)	6	1
3	Sediments from Harbor area, Visakhapatnam , (AP)	11	1
4	Sediments from Marina beach, Chennai, (Tamilnadu)	5	1
5	Drains of Canteen waste Visakhapatnam, (AP)	7	--
6	Sediments from Araku valley, Visakhapatnam, (AP)	5	--
7	Sediments from back waters, Nagarcoil, (Kerala)	8	--

RESULTS AND DISCUSSION

Production of L-asparaginase:

Of the three isolates, B4 shown the maximum enzyme activity hence it was chosen for indepth study after identified the isolate by polyphasic characterization.

Effect of incubation time on L-asparaginase activity

The effect of various incubation periods(24, 48, 72, 96, 120,144h) on L-asparaginase was studied and the results indicated that the enzyme yield was gradually increased (180.53U/gds) was observed at 96h (Fig 1). Relatively a good L-asparaginase activity was observed in the range of 96-120h of incubation time. There after the enzyme production started decreasing could be either due to decrease in nutrient availability in the medium or catabolic repression of enzyme (15)

Effect of incubation temperature on L-asparaginase activity

Different incubation temperatures (22, 24, 26, 28, 30, 32, 34°C) were tested for L-asparginase production. The maximum enzyme production (186.05U/gds) was observed at 30°C (Fig 2) and further increase or decrease of temperature gradually decreases the L-asparginase activity. At higher temperature, the maintenance energy requirement for cellular growth is high due to the thermal denaturation of enzymes of the metabolic pathway results in minimum amount of product formation. At lower temperature, the transport of substrate across the cells is suppressed and lower yield of products are attained (16)

Effect of inoculum volume on L-asparaginase activity

Various inoculum volumes were tested (5%, 10%, 15%, 20%, 25%, 30v/w) of 1X107spores/ml) for their effect on L-asparaginase production(195.23U/gds) was obtained at 15% (v/w) inoculum level as compared to low or high inoculum levels (Fig 3). Similar trend was observed by (17). High inoculum levels are inhibitory in nature and a high inoculum density leads to population overcrowding, higher nutrient competition and rapid exhaustion of nutrients. The lower inoculums density, may give insufficient biomass causing reduced product formation (18)

Effect of initial pH on L-asparaginase activity

The effect of the initial pH of the substrate on L-asparaginase was studied in the range of 5.0-8.0. The results indicated that a gradual increase in L-asparaginase production was observed from the pH 5.0-6.0. Further increase from (6.0-6.5) the initial pH significantly increased the L-asparaginase production followed by a gradual decrease of enzyme yield after pH 6.5 (Fig 4). Similar observations were recorded for the effect of initial pH on the production of L-asparaginase from Aspergillus species in SSF (20)

Effect of moisture content on L-asparaginase activity

The effect of initial moisture content of the substrate on L-asparaginase production was studied in the range of 40-110% v/w. Maximum L-asparaginase production was observed in the range of 50-110% v/w (Fig 5). Further increase in moisture content gradually decreased enzyme production. Increased moisture level is believed to reduce the porosity of the cake thus limiting oxygen transfer (21) whereas low moisture contents causes reduction in the solubility of nutrients of the substrates and low degree of swelling (22).

Effect of particle size on L-asparaginase activity

The effect of particle size on the production of L-asparaginase was studied in the range of 0.2-1cm. Maximum L-asparaginase production was observed with the particle size range of 0.4-0.6cm (Fig 6). Production declined with further increase or decrease in particle size. Similar reports were reported by (19) for the production of L-asparaginase by Pseudomonas aeuroginosa 50071 in SSF.

Characterization of L-asparaginase:

Effect of pH on enzyme activity

The pH 6.5 is optimum for L-asparaginase from Aspergillus niger B4 (Fig.7). The purified L-asparaginase was active over broad pH ranges (3.0 -10.0) and the maximum L-asparaginase activity was observed between pH 7.0-8.0 (Fig.4).

Effect of temperature on enzyme activity

The temperature 30oC is optimum for L-asparaginase from Aspergillus niger B4 (Fig.8). The effect of temperature on L-asparaginase activity was studied in the range of 10-30, 37, 40-100oC. Maximum activity was noticed between 35-40oC. The enzyme activity was gradually declined beyond 50oC.

Effect of modifiers on enzyme activity

The enzyme activity was determined in the presence of different modifiers and a chelator of divalent cations EDTA(5mM) (Table 2). EDTA, DTT and Mercaptoethanol have increased the enzymatic activity and the enzyme completely lost its activity in the presence of iodoacetamide and other chemicals tested did not affect the enzyme suggesting that the enzyme is not a metalloproteinase.

Effect of metal ions on enzyme activity

L-asparaginase was not affected by metal ions like Ca+2, Fe+2, Cu+2 and Mg+2 and inhibited by K+, Na+2, Zn+2 and Hg+2(Table 3).

Substrate specificity of enzyme

The results revealed that the enzyme was100%, 10% and 15% active towards L-asparagine, D-asparagine and L-glutamine respectively (Table 4). The data indicated that the enzyme extracted from A. niger B4 is very much specific to its natural substrate asparagine. This property of the enzyme is very essential on the treatment of patients where incomplete removal of asparagine is required.

From this work we conclude that the novel isolate Aspergillus niger B4 is a promising agent for industrial application since it give significant L-asparaginase using soya as substrate under solid state fermentation(SSF) methodology. Further the high catalytic activity of enzyme at physiological pH and temperature and its considerable stability over a wide range of temperature and pH makes it highly favorable to be explored as potential anticancer agent.

Table – 2: Effect of modifiers on enzyme activity

S.No	Modifiers(5mM)	Relative L-aparaginase activity (%)
1	Control	100
2	Diisopropyl Fluorophosphates	25
3	Iodoacetamide	0
4	Dithiotheritol(DTT)	101
5	Phenyl methyl sulfonyl fluoride	30
6	Mercaptoethanol	108
7	Potassium ferricyanide	36
8	EDTA	118

Table 3: Effect of metal ions on enzyme activity

S.No	Metal ions (2mM)	Relative L-aparaginase activity (%)
1	Control	100
2	Na+	53.3
3	K+	51
4	Mg^{+2}	93.6
5	Fe^{+2}	95.2
6	Hg^{+2}	61.5
	Zn^{+2}	74.5
7	Cu^{+2}	97.6
8	Ca^{+2}	96.5

Table 4: Substrate Specificity of *Aspergillus niger B4* L-asparaginase

S.No	Substrate	Relative activity (%)
1	L – Aspargine	100
2	D – Aspargine	10
3	L – Glutamine	15

Fig.1: Effect of Incubation Time on L-asparaginase Production

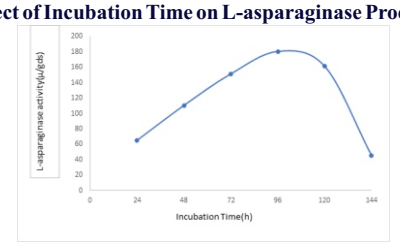


Fig.2: Effect of Incubation Temperature on L-asparaginase Production

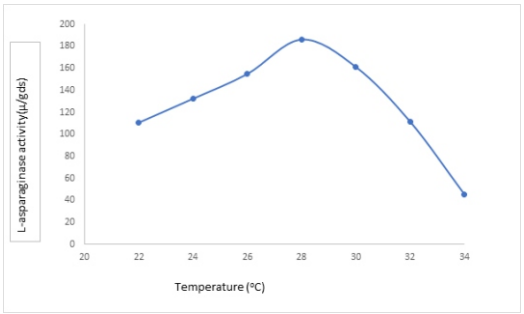


Fig: 3 Effect of Inoculum Volume on L-asparaginase Production

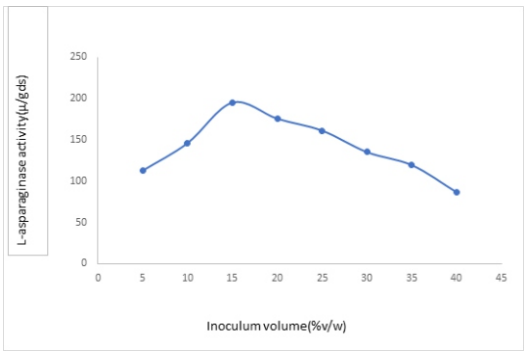


Fig: 4 Effect of initial pH on L-asparaginase Production

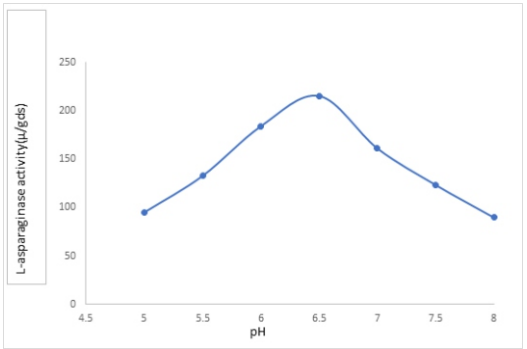


Fig: 6 Effect of Moisture Content on L-asparaginase Production

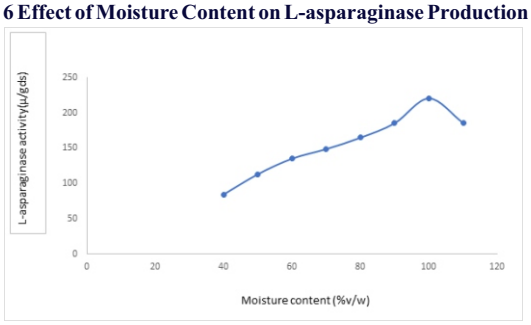


Fig. 6 Effect of Particle Size on L- asparaginase Production

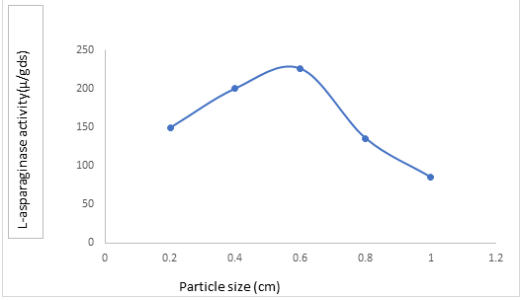


Fig: 7 Effect of pH on L-asparaginase activity

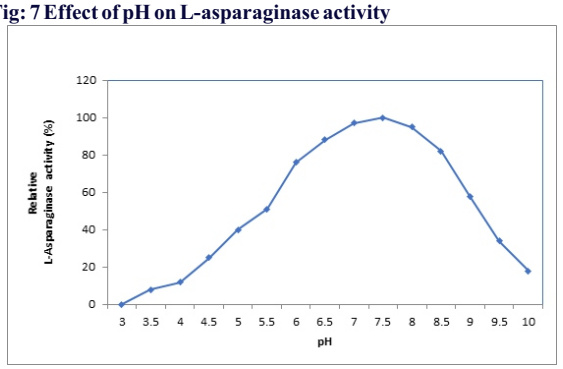
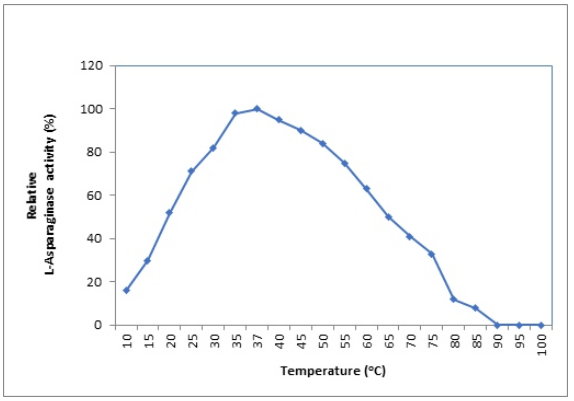


Fig: 8 Effect of Temperature on L-asparaginase activity



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