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Research Article

PRODUCTION OPTIMIZATION OF EXTRACELLULAR L-ASPARAGINASE THROUGH SOLID- STATE FERMENTATION BY ISOLATED BACILLUS SUBTILIS.

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ABSTRACT: L-asparaginase has been used as anti-tumor agent for the treatment of acute lymphoblastic leukemia and food processing aid to reduce the formation of cancer causing acrylamide. Extracellular L-asparaginase production was optimized through solid state fermentation using ground nut cake by isolated *Bacillus subtilis*. which was not reported in literature.Optimum production of L-asparaginase enzyme (18.4U/ml) was obtained after 48h of incubation at 37^0C moisture content of 70% and at pH 7.
Keywords: L-asparaginase, *Bacillus subtilis.* solid- state fermentation

INTRODUCTION

L- Asparaginase, an antineoplastic agent (L- asparaginase amido hydrolase, EC 3.5.1.1) is a tetrameric enzyme that catalyses the hydrolysis of the non-essential amino acid L-asparagine to L-aspartic acid and ammonia [Fernandes and Gregoriadis, 1997].

The L-asparaginase is present in many animal tissues, bacteria, plants and in the serum of certain rodents but not in man kind. It is produced by a large number of microorganisms that include *E.coli, Erwinia chrysanthemi* [Aghaiypour *et al.,* 2001], *Serratia marcescens, Enterobacter aerogenes* [Mukherjee *et al.,* 1999], *Pseudomonas aeruginosa*[El-Bessoumy *et al.,* 2004], *Pseudomonas stutzeri* [Manna *et al.*, 1995], *Aspergillus terreus, A. tamari*[*De* Moura Sarquis *et al*., 2004], some other filamentous fungi [Ali *et al*., 1994] and a few yeasts have also potential for L- asparaginase production. In general all these organisms are not good producer of this enzyme.

Microbial L-asparaginases have been particularly studied for their applications as a chemotherapeutic agent in the treatment of certain types of human cancer [Gallogher *et al,*. 1989]. L-asparaginase from two bacterial sources (*E. coli and Erwinia carotovora*) is currently in clinical use for the treatment of acute lymphoblastic leukemia [Keating *et al.,* 1993].But l-asparaginase was not isolated from human source [Muthusivaramapandian *et al.,*2008]. It is also active against non-Hodgkin's lymphoma, pancreatic carcinoma and bovine lymphomosarcoma [El-Bessoumy *et al,*.2004]. A partially purified L-asparaginase from *Aspergillus terreus* possesses antitumor property against Ehrlich's ascites in susceptible Swiss mice [*De* Moura Sarquis *et al*., 2004]. L-asparaginase is also used as processing aid in food production to convert asparagines to aspartic acid in order to reduce cancer causing acrylamide formation with the development of new functions, the demand of L-asparaginase may increase several fold in future [Pedreschi *et al,*.2008].

The antitumor activity of the enzyme is based on the dependence of tumor cells to obtain the amino acid L-asparagine (an important nutrient of tumor cells) from body fluids, as these cells are deficient in L-asparagine synthetase. The addition of L-asparaginase in the blood, drastically reduces the level of free L-asparagine in the blood stream leading to starvation in tumor cells for this amino acid as the cancer cells are not able to synthesize asparagine and die in the presence of this asparagine degrading enzyme whereas normal cells are not affected since they can synthesize L-asparagine [Ylikangas and Mononen 2000].L- asparaginase is effective adjunet to standard chemotherapy. The administration of such an enzyme protein for a long duration; produces the corresponding antibody in the tissues resulting in the anaphylactic shock or neutralization of drug effect, therefore, the use of new serologically different L-asparaginase with a similar therapeutic effect is highly desirable.

In view of a wide pharmaceutical importance of L-asparaginase, it is imperative to look for new microbial sources of the enzyme and to work out the biotechnology of the enzyme production using agroresidues and other cheaper materials, resistance to contamination and ease of extraction from the fermented residue. Extracellular asparginese are more advantageous than intercellular since they could be produced abundantly in the culture broth under normal conditions and could be purified economically (Amena *et.al* 2012)

MATERIALS AND METHODS

Chemicals

All chemicals used in this study investigation were of analytical grade and procured from Sigma (USA), Hi – media (India) and Merck (India).

METHODS

Isolation of high yielding L-asparaginase producing strain

The isolation of high yielding microbial strain was carried out from soil samples collected from wheat field, pulse field and leguminous field, in medium M_9 containing (g/l) $Na_2HPO_4.2H_2O$, 6.0; $KH_2PO_4.7\ H_2O$, 3; L-asparagine, 5; agar 20; 2ml of 1M $MgSO_4.7H_2O$; 1.0 ml of .1M $CaCl_2$. $2H_2O$; 1.0ml of 20% glucose stock; pH 6.0, and this medium was supplemented with 0.3ml of 2.5% phenol red prepared in ethanol at pH 7.0 then sterilized at 15 psi for 20 minutes [Gulati *et al* 1997]. The soil samples were inoculated to the isolation medium and poured into sterile Petri dishes and incubated at 37^0C.The control media were also included in which the substrate L-asparagine is not present. The single discrete colonies, which have exhibited clear pink zone surrounding after 48h of incubation, indicated L-asparaginase producing culture. These colonies were picked up and grown in M_9 medium.

Screening of isolates for L-asparaginase production

Strains were screened on the basis of good pink zone of lysis formed around microbial colonies in medium M_9 containing phenol red as colour indicator (0.3ml from 2.5% phenol red stock solution in ethanol) and L-asparagine as a sole nitrogen source. Enzyme production is accompanied by an increase in pH of the medium, which results in the formation of pink zone. The selected strains are maintained on asparagine rich agar slant at 4^0C.

Inoculum preparation

One single slant of culture (24h old slant culture for bacteria) is transferred to 50ml sterile M_9 medium in a 250 ml Erlenmeyer flask and incubated for 24h at 37 ^{0}C. 2.0ml of this cell suspension ($2x10^6$ cells/ml) is used as inoculum.

Solid- state fermentation (SSF)

Solid-state fermentation was carried out in a 250ml Erlenmeyer flask containing 5 g groundnut cake as a substrate, moistened with 10ml of mineral salt solution containing (g/l): $Na_2HPO_4.2H_2O$, 6.0; $KH_2PO_4.7H_2O$, 3; 2ml of 1M $MgSO_4.7H_2O$; 1.0 ml of .1M $CaCl_2.2H_2O$; 1.0ml of 20% glucose stock; corresponding to 60% moisture level and at pH 6.0 then sterilized at 121^0C for 15min, cooled to desirable temperature, addition of 2ml ($2x10^6$ cells/ml) inoculum and incubated for 48h at 37^0C. The enzyme was extracted at the end of the fermentation period by the addition of 90ml of 0.01 M phosphate buffer of pH 7.2 to the fermented medium, shaking for 15 min followed by centrifugation at 8,000 rpm for 20min. The cell free supernatant was used for the estimation of enzyme.

Optimization of L-asparaginase production

The optimization studies included various physico-chemical parameters such as pH of the production medium (5-9); incubation temperature (25-65°C); incubation period (24-144h); moisture content (50-80%); inoculum level (1-5ml); supplementation of different carbon sources(glucose, sucrose, maltose, cellulose, soluble starch,1%w/v) and nitrogen sources (asparagine, glutamine , yeast extract, urea, proline, peptone, NH_4Cl, $(NH_4)_2SO_4$, 1% w/v) for maximum production of L-asparaginase under solid state fermentation by isolated strain *Bacillus subtilis*. All the experiments were carried out in triplicates and averages are reported.

Assay of L-asparaginase

The enzyme is assayed by direct nesselerization method [Imada *et al*. 1973]. One unit of L-asparaginase activity (U) is defined as the amount of enzyme which liberates l μmol of ammonia per min at 37^0C and pH 7.4.

For extracellular enzyme activity, 0.5ml of cell free fermentation broth or extract containing enzyme along with 0.5 ml of .05M tris-HCl buffer (pH 7.4) and 0.5ml of .04 M L-asparagine solution in tris-HCl buffer, then this reaction mixture is incubated at 37^0C for 10 min and the reaction is stopped by the addition of 0.5ml of 1.5M trichloroacetic acid (TCA), precipitated proteins are removed by centrifugation and the clear supernatant is used for Nesseler's reaction. 0.5ml of the above clear supernatant along with 0.5ml of Nesseler's reagent and 3.0 ml of water are incubated for 10 min at 37^0C and the librated NH_3 is determined by spectrophotometer at 480 nm. Suitable blanks of the substrate and enzyme containing samples as well as ammonia standard are included in all assays. The concentration of ammonia in test solution is determined from standard curve of ammonium sulphate as a source of dissolved ammonia.

RESULTS AND DISCUSSION

Isolation of high yielding L-asparaginase producing strain

During isolation of microorganism from different soil samples 12 bacterial isolates. A majority of the isolates were obtained. 5 out of 12, showed appreciable enzyme activity as indicated by the diameter of the zone of lysis around the colony (Table 1). The strain A10 showed highest activity (2.1 U/ml) and identified as *B. subtilis.* Morphological and biochemical characteristics of the strain *B. subtilis* are reported in Table 2.

Table 1.Diameters of zone of lysis and colony of after 48h of isolated bacterial strain and L-asparaginase activity under SmF at pH 6.2 ,temp 37^0C for 48h of incubation

Isolated strain	Colony dia (cm)	Zone dia (cm)	Enzyme Activity (U/ml)
A1	1.2	1.7	0.9
A2	1.4	3	1.7
A3	0.7	1.5	0.7
A4	1.5	3.9	1.9
IA5	1.1	3.2	1.7
A6	1.7	2.7	1.3
A7	1.6	3.5	1.8
A8	0.9	2.3	1.2
A9	1.6	3.5	1.7
A10	2.3	4.7	2.1
A11	1.1	2.4	1.3
A12	0.7	2	1.1

Table 2. Taxonomical properties of *B. subtilis*

Cell shape	rod in short chain
Endospore	+
Gram reaction	+
Colony colour	Creamy white
Gelatin hydrolysis	+
Blue green difusible pigment on King's A	_
Yellowish green diffusible pigment on King's B	_
Oxidase	+
H_2S	+
Levan	+
Citrate utilization	_
Arginine dyhydrolase	_
Catalaze	+
KOH test	_

Optimization of production under SSF

Effect of incubation time on enzyme production under SSF

The effect of incubation period on L-asparaginase production was studied by growing isolated bacterial strain *Bacillus subtilis* in fermentation medium with 5.0 g groundnut cake at different incubation time (24 to 144 h) at 37ºC. Maximum amount of L-asparaginase (4.9U/ml) was obtained in the 90 ml extract of 48 h fermented residue but there was a decrease in enzyme activity with further increase in incubation period (Fig.1). Extended period of incubation might lead to the decomposition of enzyme due to interaction with other component in the media [Ramesh and Lonsane 1987].

Effect of pH

In order to determine the optimum pH for enzyme production under SSF, the fermentation was carried out at nine different pH ranging from 5.0-9.0. The results indicated that the production of enzyme in terms of activity increased till pH 7.0 and further increase in pH, decreased the enzyme production. Maximum enzyme activity (7.2 U/ml) was observed at pH 7.0. Similar pH was reported for the production of L-asparaginase by *Streptomyces plicatus* [Koshy *et al* 1997], *Amycolatopsis* CMU-H002 [Khamna *et al* 2009].

Effect of incubation temperature

The effect of temperature on enzyme production was studied from 25° – 60°C in 250ml Erlenmeyer flask containing fermentation medium (M_9) and 5g of groundnut cake at pH 7 for 48h fermentation. Maximum production of enzyme (7.83 U/ml) in terms of activity was obtained at 37°C (Fig 3). However, the enzyme activity predominantly decreased due to reduced growth of microbes as well as inactivation of enzyme at temperatures higher than that of 37°C. The optimum temperatures for maximum enzyme production was reported at 35°C for *S.albidoflavous* [Narayana *et al*. 2007] , 40°C for *A.nigar* [Mishra 2006] and 30°C for *Pencillium sp.*[Venil *et al*. 2009] respectively.

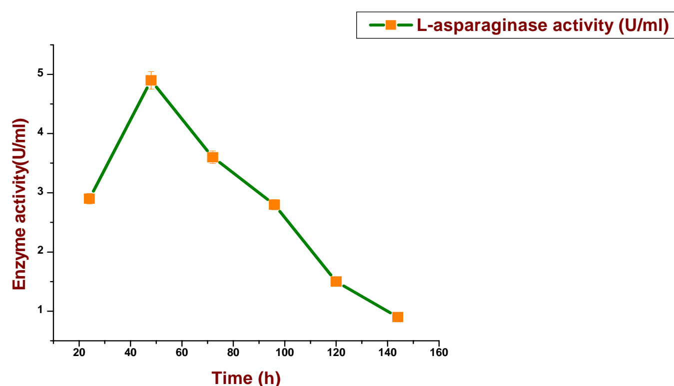

Figure 1. Effect of incubation time on L-asparaginase production by isolated *Bacillus subtilis*.

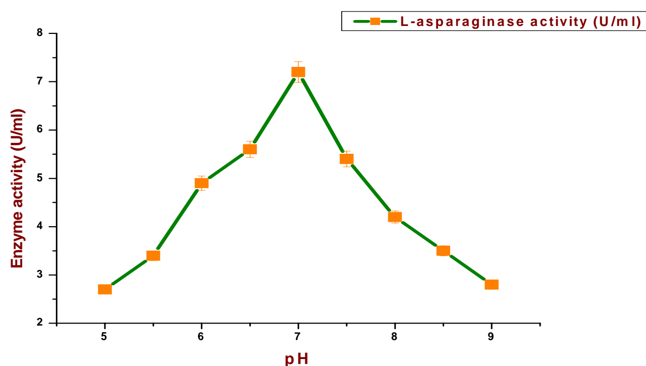

Figure 2. Effect of pH on L-asparaginase production under SSF by isolated *Bacillus subtilis*

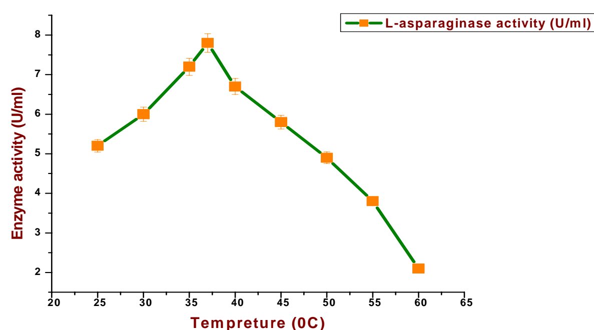

Figure 3. Effect of temperature on L-asparaginase production under SSF by isolated *Bacillus subtilis*

Effect of moisture content

Optimum moisture content of substrate is necessary for proper growth of microbes as well as production of enzymes. Experiments carried out with different moisture levels from 55 to 80 % reveals that enzyme activity increases from 55 to70% moisture content and further increase in moisture content reduces the enzyme activity (Fig.4.). Microbes generally grow near the outer surface of the substrate particle, and water uptake in new biomass and evaporations are thus localized at the surface of the substrate particles due to generation of heat with fermentation causing subsequent evaporation hence the optimum humidity allows the entry of nutrients easily through the cell wall, which favours maximum enzyme production. Any deviation from the optimum humidity results in the decrease in enzyme activity, which may be due to osmotic imbalance inside the cell causing cell lysis. Maximum production of L-asparaginase was obtained (10.8U/ml) at 70% moisture level.

Effect of inoculum level

The size of the inoculum plays a significant role in the production of L-asparagines. Lower inoculum density may give insufficient biomass causing induced product formation, whereas a higher inoculum may produce too much biomass leading to the poor production. The effect of inoculum level on L-asparaginase production was studied by growing isolated *Bacillus subtilis* in fermentation medium (M_9) with 5g ground nut cake, inoculated with different inoculum levels (1-5ml) for 48h.The maximum enzyme activity (13.2 U/ml) was observed with an inoculum of 3ml whereas increase in inoculum size (3-5ml) decreases the enzyme production (Fig.5.).

Effect of different carbon sources .

Effects of different carbon sources (1%w/v) have remarkable influence on enzyme production by isolated *Bacillus subtilis*. Therefore, lactose, sucrose, maltose, cellulose and soluble starch were supplemented to fermentation mediumM_9 by replacing the glucose. The sucrose is best carbon source for L-asparaginase production, followed by glucose and soluble starch (Fig.6.). This is due to the inductive effect of sucrose and its remarkable efficiency in production of enzyme being an inexhaustible source of carbon compared to other carbon sources and it also helps in stabilizing the enzyme [Soniyamby *et al*.2011].

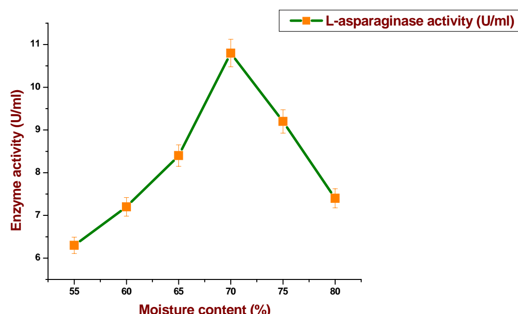

Figure 4. Effect of moisture content on L-asparaginase production under SSF by isolated *Bacillus subtilis*

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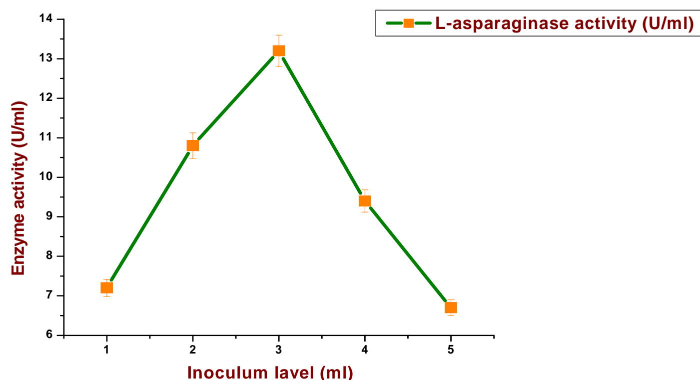

Figure 5. Effect of inoculum lavel on L-asparaginase production under SSF by isolated *Bacillus subtilis*

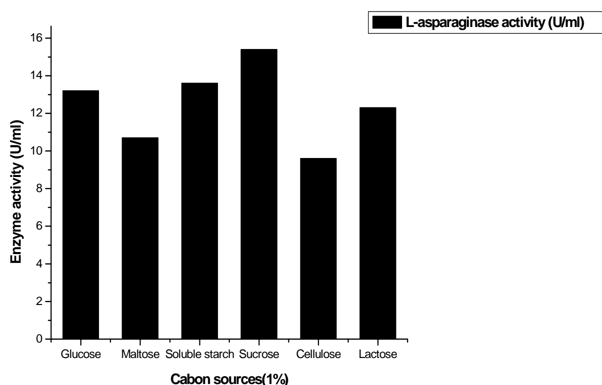

Figure 6. Effect of different carbon sources on L-asparaginase production under SSF by isolated *Bacillus subtilis*

Effect of different nitrogen sources.

Various organic and inorganic nitrogen sources were supplemented (1%w/v) in to medium M_9 for production of enzyme and the result revealed that L-asparagine exhibited maximum L-asparaginase production (18.4U/ml) followed by peptone(16.2U/ml) and yeast extract(15.4U/ml) which indicates that L-asparagine acts as a inducer for L-asparaginase (Fig.7.).

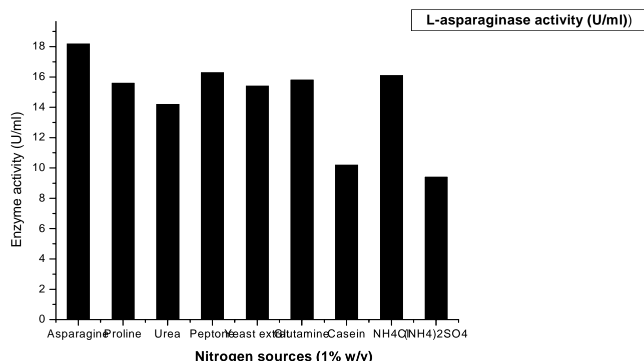

Figure 7. Effect of different nitrogen sources on L-asparaginase production under SSF by isolated *Bacillus subtilis*

CONCLUSION

The isolated *Bacillus subtilis* has the ability to produce a significant amount of extracellular L-asparaginase in solid- state fermentation using a low cost agro waste residue, ground nut cake, as a substrate under optimum process conditions. The present study concluded the isolated *Bacillus subtilis* used in this study will be a potential source for L-asparaginase enzyme. Purification characterization and structure determination of L-asparginase of this isolated strain is needed to prove its potential use. in the large scale production for commercial and pharmaceutical purpose.

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