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Research article

PRODUCTION PURIFICATION AND CHARACTERIZATION OF EXTRACELLULAR ANTI-LEUKAEMIC L-ASPARAGINASE FROM ISOLATED *BACILLUS SUBTILIS* USING SOLID STATE FERMENTATION.

Susmita Shukla and S.K. Mandal

Department of Biochemical Engineering and Food Technology, Harcourt Butler Technological Institute, Kanpur-208002, India.

E-mail: susmita_shukla.@rediffmail.com

ABSTRACT: Bacterial L-asparaginase has been widely used as therapeutic agent in treatment of various lymphoblastic leukemia and food processing aid to reduce the formation of cancer causing acrylamide. The present work deals with production and purification of extracellular L-asparaginase from soil isolates using solid state fermentation. The isolate was characterized by big chemical test and identified as *Bacillus subtilis*. The enzyme production was carried out by solid state fermentation comparing the results with submerged fermentation. The enzyme was purified to near homogeneity by ammonium sulphate precipitation, dialysis, followed by gel filtration on Sephadex G-100 column, CM Sephadex C-50 and SDS-PAGE. The enzyme was purified at 110.2 folds and showed a final specific activity of 1785.7 IU/mg with 26.5% yield. SDS-PAGE of the purified enzyme revealed an apparent molecular weight of 109 kDa. The purified enzyme showed maximum activity at pH 9 when it was incubated at 50˚C for 35 min. The enzyme was activated by Mg^{+2} and strongly inhibited by EDTA.

Keywords: L-asparaginase, *Bacillus subtilis.* solid- state fermentation, extra cellular, purification.

INTRODUCTION

L- Asparaginase, (L- asparaginase amido hydrolase, EC 3.5.1.1) is an enzyme of high therapeutic value due to its use in certain types of cancer therapy mainly in acute lymphoblastic leukemia. It is also active against non-Hodgkin's lymphoma, pancreatic carcinoma and bovine lymphomosarcoma [El-Bessoumy *et al.,*2004]. Microbial L-asparaginases have been particularly studied for their applications as a chemotherapeutic agent in the treatment of human cancer [Gallogher *et al.,* 1989]. But L-asparaginase was not isolated from human source [Muthusivaramapandian *et al.,*2008]. 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 in several fold in future [Pedreschi *et al.,*2008]. It 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 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.

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. In most of the microorganism L-asparaginase accumulates as an intracellular (periplasmic, cytoplasmic and membrane bound) product. The intracellular localization of microbial enzymes has been studied for the production of alkaline phosphate, deoxy ribonuclease , cyclic phosphor-diesterase, 5 '-nucleo-tidase, acid phosphatase , lipase, carboxymethyl cellulose and 17-β-hydroxysteriod dehydrogenase. Enzyme localiza-tion in bacteria has been carried out using various methods. In L-asparaginase producing strains, the existence of both periplasm and cytoplasm enzyme have been reported. The study on the localization of any enzyme plays a vital role in the development of bioprocess (Moorthy *et al*., 2010). L-asparaginase enzyme is produced throughout the world by submerged fermentation (SF). This technique has many disadvantages, such as 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. 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.(Soniyamby *et al*., 2011). Literature reports indicated that the enzyme's biochemical and kinetic properties varies with the genetic nature of the microbial strain used. For example, *Erwinia* L-asparaginase exhibited less allergic reactions compared to *E.coli* enzyme. However Erwinia asparaginase had a shorter half life span than *E.coli*, suggesting the need to discover new L-asparaginase that are serologically different but have similar therapeutic effects (Moorthy *et al.,* 2010).
Extra-cellular asparaginase are more advantageous than intracellular ones, since they can be produced abundantly in the culture broth under normal conditions and purified economically (Amena *et al.*, 2012). Considering the above facts an attempt was made for the production, purification and characterization of an extra-cellular L-asparaginase through solid state fermentation by a new soil isolate.

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). Sephadex G-100, CM-Sephadex C-50 and standard protein for SDS-gel electrophoresis were purchased from Pharmacia.

METHODS

Isolation of microorganism and maintenance of culture.

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.

Submerged fermentation

In order to undertake submerged fermentation 1.0 g of protein rich substrate ground nut cake was taken in a sterile 250 ml Erlenmeyer flasks containing 100 ml of sterilized production medium 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. pH 6.0 then sterilized at 121^{0}C for 15min, cooled to desirable temperature, addition of 2ml ($2x10^6$ cells/ml) inoculum and incubated for 48h at 37^{0}C. The flask was inoculated with 2.0 ml inoculum and incubated for 48 h at 37^0 C in a rotatory shaker at 250 rpm. The enzyme was assayed by direct nesselerization method.

Solid- state fermentation (SSF)

Solid-state fermentation was carried out in a 250 ml Erlenmeyer flask containing 5.0 g groundnut cake as a substrate, moistened with 10 ml 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 0.1M $CaCl_2.2H_2O$; 1.0ml of 20% glucose stock; corresponding to 60% moisture level and at pH 6.0 then sterilized at 121^{0}C for 15min, cooled to desirable temperature, addition of 2.0 ml ($2x10^6$ cells/ml) inoculum and incubated for 48h at 37^{0}C. 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.

Purification of L-asparaginase

The purification was carried out at 4˚C on the crude extract, according to the modified method of (Distasio *et al.*, 1976).

Ammonium sulphate fractionation

Powdered ammonium sulphate was added to the crude extract to 80% saturation. The mixture was left for 12 hrs at 4˚C, followed by centrifugation at 9000 rpm for 20 min at 4˚C. The precipitate was dissolved in a 1.0 M Tris-HCL buffer pH 8.5 and dialyzed overnight against the same buffer at 4˚C.

Sephadex G-100 gel filtration

The dialyzed fraction was applied to a Sephadex G-100 column (100X1.5cm), that was pre equilibrated with 0.05M Tris-HCL buffer (pH8.4). The protein elution was done with 0.05M Tris-HCL buffer (pH8.4), containing 1M KCl at a flow rate of 3ml per 30 min. The active fractions were pooled dialyzed and concentrated.

CM-Sephadex C-50 ion-exchange chromatography

The concentrated enzyme solution was applied to column of CM-Sephadex C-50 that was pre equilibrated with a 0.05M Tris-HCL buffer (pH8.4). It was eluted with 50mM Tris-HCL buffer (pH8.4), containing 0.1M KCl at a flow rate of 3ml per 30 min. The active fractions at each purification step were collected, dialyzed and concentrated and L-asparaginase was assayed by direct nessalerization method.

SDS-PAGE was performed according to the method of Laemmli (1970), with a 10% separating gel and 5% stacking gel containing 0.1% SDS. The gel was strained with coomassie brilliant blue R-250, and destained with a solution of methanol, acetic acid and water in the ratio of 4:1:5.

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^{0}C 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^{0}C 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^{0}C 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.

Protein estimation

Protein estimation was done with Folin-phenol reagent using bovine serum albumin as a standard (Lowry *et al*.,1951).

Determination of Molecular weight

SDS-PAGE was performed according to the method of Laemmli (1970), with a 10% separating gel and 5% stacking gel containing 0.1% SDS. The gel was strained with coomassie brilliant blue R-250, and destained with a solution of methanol, acetic acid and water in the ratio of 4:1:5. The molecular weight of the purified L-asparaginase was determined in comparison with standard molecular weight markers phosphorylase b (97.4 kDa), Bovine serum albumin (66kDa), Ovalbumin (43 kDa), carbonic anhydrase (29 kDa), lactoglobulin (18.4 kDa) and lysozyme (14.3 kDa).

Enzyme characterization

Effect of pH temperature and incubation time on purified L-asparaginase

The activity of L-asparginase was evaluated at different levels of pH, temperature and incubation time. Purified enzyme was incubated with 0.05 M buffers of pH4-12 under assey conditions and the amount of ammonia liberated was determined. The buffers used were potassium phosphate (pH4.0-7.0), Tris-HCL (pH 8.0-9.0), and glycine-NaOH (pH10). Pre-incubation was carried out for 60 min and then the residual activity was measured. Optimum temperature of activity for the enzyme was determined by incubating the assey mixture at temperatures ranging from 30 to 80˚C. Thermo stability studies were carried by pre incubating the enzyme at different temperatures for 60 min. The effect of the incubation time on L-asparginase activity was studied in the range of 5 to 45 min (El-Bessoumy *et al*., 2004). The enzyme characterization experiments were performed in triplicates.

Effect of Metal ions on purified L-asparaginase activity

The enzyme was incubated with various metal ions, $Mg^{2+}Co^{2+}Fe^{2+}Cu^{2+}Zn^{2+}$ and EDTA AT 0.5, 1.0 & 2.0ml concentration for 30 min at 37˚C and the residual activities were determined. Activity of the control not containing any metal ions was also determined.

Purified L-asparginase activity was inhibited by EDTA followed by $Cu^{2+,}$ $Zn^{2+.}$ while a slight stimulation shows by Mg^{2+}.

RESULTS

Screening, crude enzyme production of L-asparaginase positive culture

Preliminary screening of L-asparaginase production by strain of *Bacillus* showed positive results in rapid plate assay and shake flask. A comparison of submerged and solid state fermentation shows a significant difference in enzyme activity. The yield of enzyme was two times greater in solid state (4.9 IU/ml) than submerged (2.1 IU/ml).

Purification of L-asparaginase

The partial purification of L-asparaginase crude extract that was affected by ammonium sulphate (80%) precipitation showed that most of enzyme activity was preserved in precipitate. The total protein decreased from 290 to 37 mg. and specific activity increased from 16.2 to 75.6 IU/mg, at approximately 4.6 folds purity in ammonium sulphate precipitation step. Further purification of the enzyme by Sephadex G-100 resulted in specific activity of 440.4 U/mg with approximately 27.1 folds purity and yield of 39.3%. The final purification of L-asparaginase was achieved by CM Sephadex C-50 column chromatography which resulted in specific activity of 1785.7 IU/mg, approximately 110.2 folds purity, and yield of 26.5% (Table-1).

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Table 1: Purification profile of L-Asparaginase from *Bacillus subtilis*

Purification step	Volume (ml)	Unit/ml	Total activity (U)	Total Protein (mg)	Specific Activity	Purification fold	% recovery
Crude extract	200	23.5	4700	290	16.2	0	100
NH4(SO4)2 precipitation	100	28	2800	37	75.6	4.6	59.5
Sephadex G-100 column	50	37	1850	4.2	440.4	27.1	39.3
CM-Sephadex C50 column	25	50	1250	0.7	1785.7	110.2	26.5

Fig.1 shows the profile of ammonium sulphate fraction purification on Sephadex G-100 gel filtration column chromatography. Although this fraction contained different protein molecules, only one peak showed activity for L-asparaginase. Also the purification of the enzyme rich fractions of the Sephadex G-100 gel filteration on the CM-Sephadex C-50 column is shown in Fig.2. A sharp distinctive peak of L-asparaginase activity, which fits with only one protein peak, was obtained.

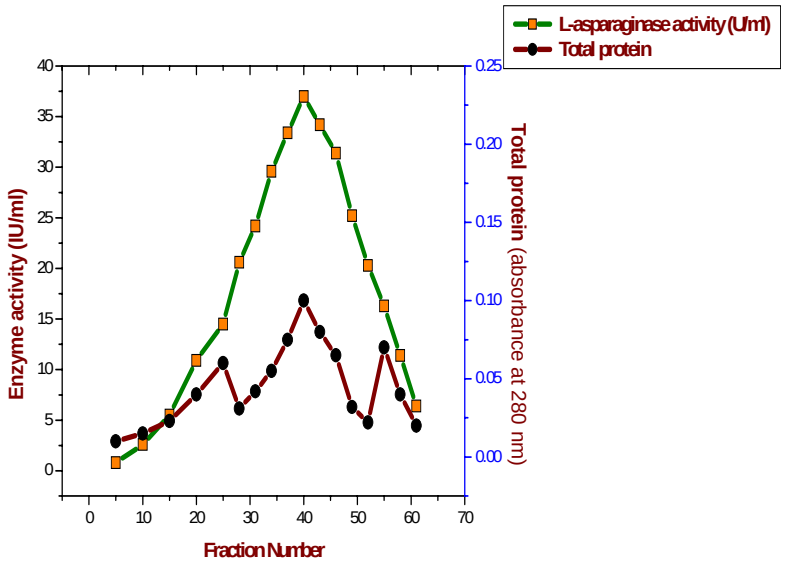

Figure 1: First gel filteration chromatography of L-asparaginase. The dialyzed ammonium sulphate precipitate was chromatographed on Sephadex G-100. Total protein was monitored at 280 nm. The fractions were asseyed for enzyme activity.

Molecular weight of L-asparaginase

SDS-PAGE showed that the enzyme is one band with electrophoretic mobility of 0.48. By using different standard proteins with known molecular weight. The apparent molecular weight of *Bacillus Subtilis* L-asparaginase was 109.0 kDa (Fig.3).

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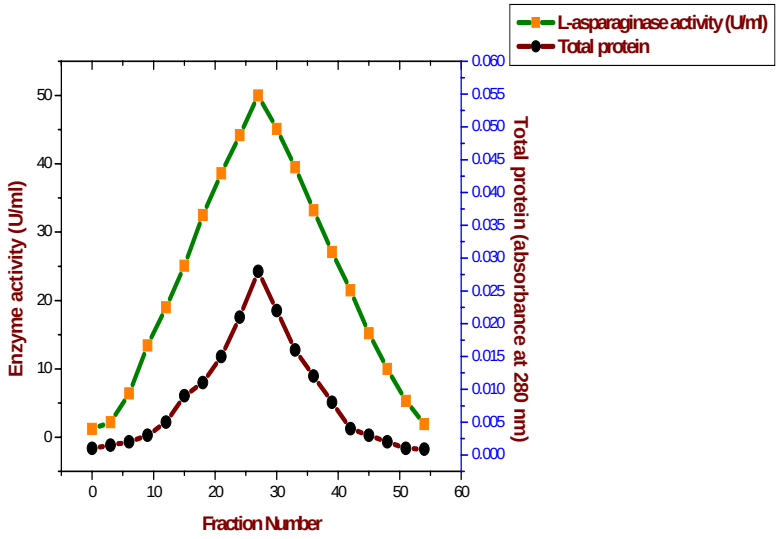

Figure 2: Second CM-Sephadex C50 chromatography of L-asparaginase . the first gel filteration G-100 collected fractions was applied to CM-sephadex C-50. Total protein was monitored at 280 nm. The fractions were asseyed for enzyme activity.

Enzyme characterization

Effect of pH

The pH influence on the L-asparaginase activity was studied by using buffers of different pH values ranging from 4-12. The enzyme activity gradually increased until pH 9, at which time the maximum activity was observed. At higher pH, the enzyme activity decreases. (Fig.4).

Effect of temperature

The effect of temperature on the stability of purified L-asparaginase was determined by incubation of enzyme preparation at different temperature in the range (30-80˚C) for 30 min maximum activity was obtained at 50˚C and enzyme was stable up to 55˚C, beyond this temperature the activity almost lost (Fig.5).

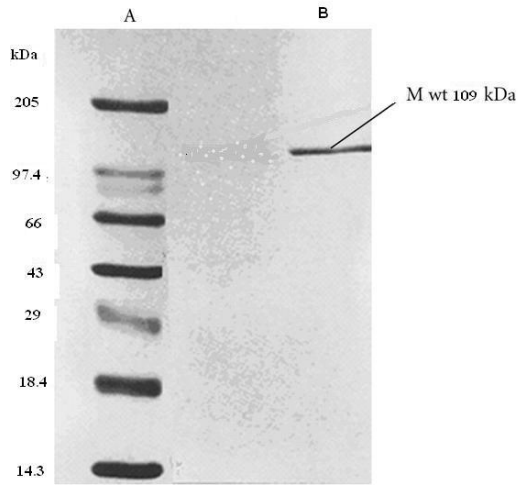

Figure 3: SDS-PAGE of L-asparaginase from *Bacillus Subtilis*. Electrophoresis was carried out on a 10% polyacrylamide containing 0.1% SDS. The gel was stand with coomassie blue R-250. Lane A included the following standard proteins. 1. phosphorylase b (97.4 kDa). 2. Bovine serum albumin (66kDa). 3. Ovalbumin (43 kDa). 4. Carbonic anhydrase (29 kDa). 5. lactoglobulin (18.4 kDa). 6. lysozyme (14.3 kDa). Lane B contained CM Sephadex C-50 column purified enzyme.

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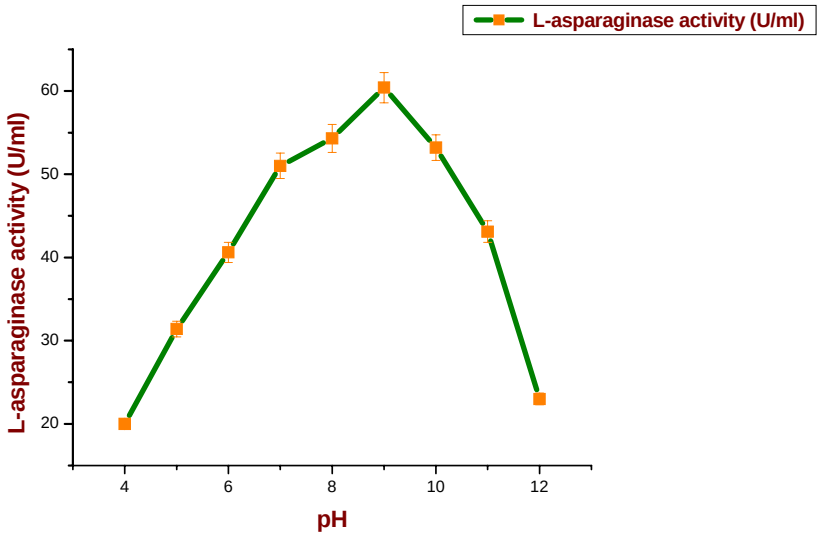

Figure 4: Effect of pH on purified L-asparaginase activity this was studied in the range value from 4~12 using 0.01m phosphate after 30 min. of incubation.

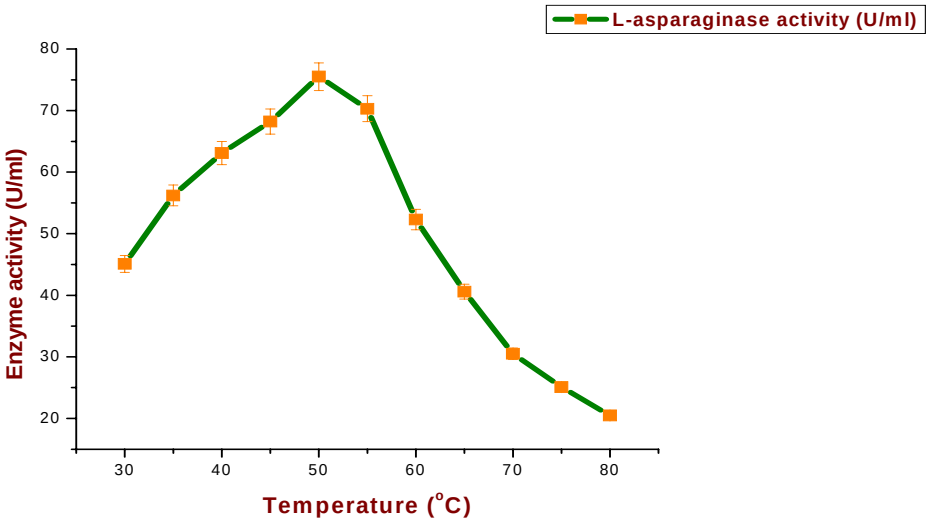

Figure 5: Effect of temperature on purified L-asparaginase activity this was studied in the range of 30 ~80 ˚C in a 0.01 m phosphate buffer pH 9 after 30 min. of incubation.

Incubation Time

The effect of incubation time on L-asparaginase was studied in the ranges of 0-50min (Fig.6). L-asparaginase activity increased as the incubation time increased, the activity ran at maximum for 35 min and decreased as the time increased.

Effect of Metal ion

Purified L-asparaginase activity was strongly inhibited by increasing concentration of EDTA (Mohapatara *et al*.,1995) followed by Cu^{2+}, Zn^{2+}. On the contrary increasing concentration of magnesium increased enzyme activity and maximum activity was observed with 2.0 ml of activator (Fig.7).

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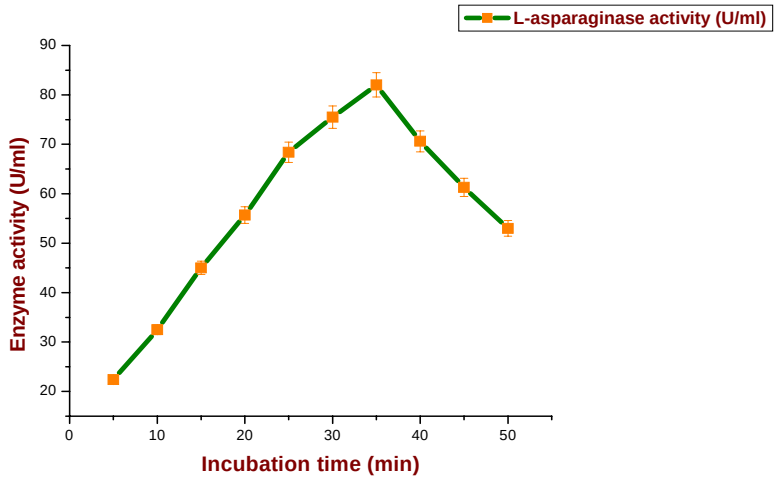

Figure 6: Effect of incubation time on purified L-asparaginase activity this was studied in the range value of 0~50 min. at 50 ˚C and pH 9.

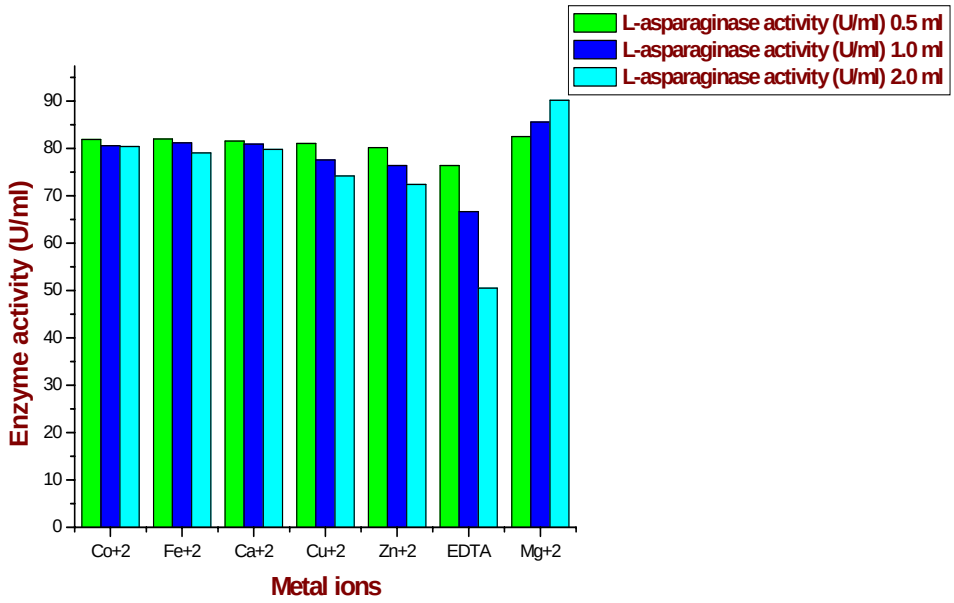

Figure 7: Effect of different Metal ions on purified L-asparaginase activity.

DISCUSSION

Bacillus subtilis was screened for L-asparaginase production by plate assay as well as by submerged fermentation similar screening of L-asparaginase by rapid plate assay were reported in bacterial strain such as *Bacillus circulans* (Prakasham *et al*., 2010), *Streptomyces sp.* PDK-7 (Dhevangi and Poorani, 2006) and *Bacillus sp*. (Moorthy *et al*., 2010). A comparison of submerged and solid state fermentation shows a significant difference in enzyme activity. The yield of enzyme was two times greater in solid state (4.9 IU/ml) than submerged (2.1 IU/ml). This suggests that there may be increased accumulation of intermediate metabolites between substrate and product formation in submerged fermentation. This is also probably due to difference in the physiological state of the microorganism in solid state and submerged fermentation (Basha *et al*,.2009).

Further purification of L-asparaginase enzyme was achieved by using 80% saturated ammonium sulphate precipitation, Sephedex G-100 gel filtration, and CM Spehadex C-50 cation exchange column chromatography. The specific activity increased from 16.2 to 1785.7 IU/mg for crude extract and final preparation respectively, which showed about 110.2 folds purity of L-asparaginase in final purification step. In *streptomyces albidoflavus* L- asparaginase has been purified in CM Sephadex C-50 column up to 99.3 folds (Narayana *et al.,* 2008). In another report, 106 folds purified L-asparaginase was obtained from *Pseudomonas aeroginosa 50071* (El-Bussoumy *et al.,* 2004) by the final CM Sephedex C-50 column chromatography. Recently L-asparaginase purity of about 98.23 folds was reported in *Streptomyces noursei* (Dharmaraj, 2011) and 82.12 folds was reported in S*treptomyces gulbergenesis* (Amena et al., 2010) in final purification.

The purified enzyme was protein profiled by SDS-PAGE for determination of molecular weight of the enzyme which revealed a protein band with molecular weight of 109 kDa. (Fig.3). Purified L-asparaginase from *Streptomyces noursei* (Dharmaraj, 2011), *Streptomyces* sp. PDK-2 (Dhevagi and Poorani, 2006), *Streptomyces albidoflavus* (Narayana *et al.,* 2008), S*treptomyces gulbergenesis* (Amena *et al*., 2010), exhibited a molecular weight of 102, 140, 112 & 85 kDa respectively. Report on production and purification of L-asparganise from *Pseudomonas aeruginosa 50071* revealed, by SDS-PAGE, peptide chain with molecular weight of 160kDa (El-Bessoumy *et al.,* 2004).

Maximum activity of purified L-asparaginase occurred when it was incubated with an optimum substrate concentration at pH 9. A similar pH value was obtained for *Pseudomonas aeruginosa* 50071 (El- Bessoumy *et al.,* 2004). The effect of temperature on the stability of purified L-asparaginase showed maximum enzyme activity at 50˚C, similar results were recorded with *Streptomyces noursei* (Dharamraj, 2011), *E. carotovora* (Maladkar *et al.,* 1993) , *Pseudomonas stutzeri MB 405* (Manna *et al.*,1995). The effect of incubation time on the activity of purified L-asparaginase showed that the activity reached its maximum at 35 min similar results were obtained from *Streptomyces noursei* (Dharamraj, 2011). *Pseudomonas aeruginosa* 50071 showed its maximum activity at 30 min of incubation (El- Bessoumy *et al.,* 2004). Purified L-asparaginase activity was strongly inhibited by EDTA (Mohaptra *et al.,* 1995) followed by Cu^{2+},Zn^{2+}, while a stimulated by increasing concentration of Mg^{2+} (Moorthy *et al.,* 2010) & (Basha *et al.*,2009).

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 agrowaste residue, ground nut cake, as a substrate as compared to submerged fermentation under optimum process conditions. The present study concluded the isolated *Bacillus subtilis* used in this study will be a potential source for extracellular L-asparaginase enzyme. The present study revealed Purification characterization and structure determination of L-asparaginase of this isolated strain. Furthermore high catalytic activity of the enzyme over a wide range of pH and temperature and its considerable stability makes it highly favorable for use as potent anticancer agent and for other application in healthcare industry.

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