ISSN: 0976-3031

Available Online at http://www.recentscientific.com

International Journal of Recent Scientific Research
Vol. 6, Issue, 1, pp.2472-2476, January, 2015

International Journal of Recent Scientific Research

RESEARCH ARTICLE

PRODUCTION, PURIFICATION AND CHARACTERIZATION OF L- ASPARAGINASE FROM SOIL ISOLATE OF *BACILLUS* SPECIES

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ARTICLE INFO

Article History:

Received 5th, December, 2014
Received in revised form 12th, December, 2014
Accepted 6th, January, 2015
Published online 28th, January, 2015

Key word:

L-asparaginase, Precipitation, dialysis, Phenol red indicator.

ABSTRACT

The present investigation focused on L- asparaginase activity by *Bacillus* sp. isolated from soil. L-asparaginase activity was analyzed using phenol red indicator growth medium showed significant zone of hydrolysis. Five different carbon sources such as glucose, maltose, soluble starch, rhamnose and fructose were analyzed for the enzyme production. Glucose was found to be the best carbon source. The partial purification of L-asparaginase was done by two way ammonium sulfate precipitation (45% initial & 85% final saturation respectively). After purification dialysis was carried out by using dialysis membrane-60 to remove excess salt results into the total protein decreased from 400 mg to 220 mg & specific activity increased from 1.18 IU/ml/min to 2.15 IU/ml/min. The characterized enzyme exhibited maximal enzyme activity at pH 7 and 37°C after 24 hrs of incubation. The L- asparaginase activity was increased by 0.97 IU/mg.

INTRODUCTION

The manufacture or processing of enzyme for use as drug is an important facet of today's pharmaceutical industry. Attempts to exploit the advantages of enzymes as a drug are now being done at every pharmaceutical research centre. Interest in L-Asparaginase (EC 3.5.1.1.) has grown considerably since this enzyme was found to have antitumor activity (Broome, 1961). Asparaginase (L- Asparagine amidohydrolase) is an enzyme which responsible for conversion of L- asparagines into aspartic acid and ammonia. The therapeutic use of this enzyme was responsible for remission in most patients suffering from acute lymphoblastic leukemia (ALL) (Verma *et al*., 2007). There was also application of L- Asparaginase enzyme in many other clinical trials of tumor therapy in combination with chemotherapy. L- asparaginase is a oncolytic enzyme that degrade non- essential amino acid like L-asparagine. Tumor cells deficient in synthesizing L-asparagine and extract it from body fluids, by contrast most normal cells can synthesize their own amino acids. The effective depletion of L- asparagine results in cytotoxicity for leukemic cells (Figure 1). L-Asparaginase given parentarally act in this way in many susceptible tumors.

L-Asparaginase is the first enzyme with anti-leukemic activity to be intensively studied in human beings (Savitri *et al*., 2003). Since extraction of L-asparaginase from mammalian cells is difficult, microorganisms have proved to be a better alternative for L-asparaginase extraction, thus facilitating its large scale production.

Since the 1970s, several microbial strains like *Aspergillus tamari*, *Aspergillus terreus*, *Escherichia coli* (Swain *et al*., 1993; Cornea *et al*., 2000), *Erwinia aroideae* (Liu and Zajic, 1973), *Pseudomonas stutzeri* (Manna *et al*., 1995), *Pseudomonas aeruginosa* (Abdel-Fattah and Olama, 2002), *Serratia marcescens* (Sukumaran *et al*., 1979), having potential for L-asparaginase production have been isolated and studied in detail.

The L- asparaginase enzyme is also used as a processing aid to reduce levels of free L- asparagine, which is a major precursor in formation of food contaminant acrylamide. In baking industry baking of starchy food material results into formation of acrylamide which was reduces due to presence of L-asparaginase enzyme. (Jha *et al.*, 2012).

MATERIALS AND METHODS

Sample collection and screening of L- Asparaginase producing bacterial strains

Bacterial strains were isolated from a rhizosphere soil and screened for potent L-asparaginase producer according to the method described by Gulati *et al*., (1997). The culture was maintained in nutrient agar (NA) slant. The slant was incubated at 30°C for 24 hrs and stored at 4°C±1°C. Stock culture was transferred to fresh NA medium every 3-4 weeks.

Identification of isolated bacterial strain

Identification of isolated bacterial cultures was done on the basis of its growth characteristics using different media and biochemical properties such as Grams reaction, motility, lactose fermentation, indole production, methyl red, voges proskauer (VP) reaction, citrate utilization, H_2S production, Catalase and urease test (Buchanan and Gibbons, 1975).

Preparation of inoculum

For inoculum preparation, *Bacillus* sp. was cultivated in nutrient broth having pH 7.0. The cells were cultivated in this medium at 30°C on a rotary shaker at 160 rpm for 24 h (Sunitha M. *et al*, 2010).

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Production of L- Asparaginase

L- Asparaginase production using *Bacillus* sp. was carried out by submerged fermentation. The fermentation media contains (g/ lit)-Na_2HPO_4 6g, KH_2PO_4 2.0g, L- Asparagine 6.0g, 1M $MgSO_4.7H_2O$ 2.0g, NaCl 0.5g, 0.1M $CaCl_2.2H_2O$ 1.0 ml, and 20% glucose stock solution 10 ml, phenol red indicator 0.3ml (2.5% stock in ethanol) with final pH-7. Active bacterial culture (1%) was inoculated in fermentation medium and incubated in rotary shaker at 200 rpm having 37°C for 24 hrs. **(Moorthy *et. al.* 2010)**

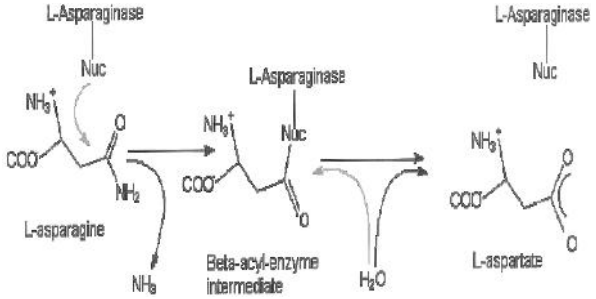

Figure1 Schematic representation of the reaction mechanism of the L-asparaginase (Hill *et al.*, 1967

Analytical studies

Determination of L- Asparaginase activity

L- Asparaginase activity of culture filtrate was determined at the end of cultivation time by quantifying ammonia formation in a spctrophotometric analysis using Nesslers reagent (Wriston and Yellin, 1973). The reaction mixture containing 1.7 ml of 0.01M L-asparagine 0.2ml of 0.05M phosphate buffer (pH 7), 0.1 ml of an enzyme and 0.5 ml distilled water was added to make up the total volume to 2 ml. The tubes were incubated at 37ºC for 30 minutes. The reaction was stopped by adding 0.5 ml of 1.5 M Trichloroacetic acid (TCA). The blank was prepared by adding enzyme after the addition of TCA. To this mixture 0.2 ml of Nessler's reagent was added. After incubating the mixture at 37ºC for 20 minutes the OD was checked at 480 nm with Spectrophotometer. The activity was determined using an ammonium sulfate reference standard. 1 unit of L- asparaginase (IU) is defined as the amount of enzyme capable of producing 1mole of ammonia per minute at 37ºC. **(**Maysa E-Moharam *et al*, 2010)

Estimation of protein

The amount of protein was estimated by the method of Lowry *et al.*, (1951) using bovine serum albumin as standard (Ohnishi S T, 1978).

Effect of different carbon sources and incubation time on growth of organism and enzyme production

The five different carbon sources were utilized as glucose, maltose, rhamnose, fructose and starch to investigate the growth of isolated species and enzyme activity. Along with variation in the carbon sources incubation time was also verified as 24hrs-120hrs **(**Khamna Sutthinan *et al*., 2009**)**.

Enzyme Characterization

The activity of L- Asparaginase was examined at different pH and temperature values. The partially purified enzyme was incubated in 0.05M buffers of pH 5-9, under assay conditions and the amount of ammonia liberated was determined. Buffers used were potassium phosphate (pH 5-7), Tris-HCl (pH 8-9). The preincubation was carried out for 60 min and then the residual activity was measured. The optimum temperature for enzyme activity was measured by incubating the assay mixture at different temperature (25, 30, 37, 40, 45°C) for 60 min (Amena *et al.*, 2010).

Partial purification of L- asparaginase

The crude enzyme extract obtained from submerged fermentation was brought to **20%** saturation with ammonium sulphate and kept overnight in a cold room at 4°C. The ammonium sulphate preparation of enzyme was then subjected to centrifugation at 8000 rpm for 10 min at 4°C. The precipitate was discarded, while the supernatant was brought **to 80%** saturation with ammonium sulphate and centrifuged at 8000 rpm at 4°C for 10 min. The precipitate from this step was collected and stored at 4°c. Dialysis tubes, which were previously soaked in 0.1M phosphate buffer, pH 6.2, were used for dialysis of precipitate. The precipitate was dissolved in 0.1M phosphate and dialyzed against the same buffer.

RESULTS AND DISCUSSIONS

Isolation and screening of microorganisms

A total of five different bacterial cultures were isolated from soil. Out of five isolates, efficient L-asparaginase producing strain was selected in primary screening by rapid plate assay method (Gulati *et al.*, 1994) .The potential strain was selected on the basis of pink zone around the colony using plate assay method (Figure A and B). Screening of soil microbial isolates were performed using phenol red indicator growth medium and microbial strain with highest zone of hydrolysis was selected for further studies (Moorthy *et al.*, 2010)

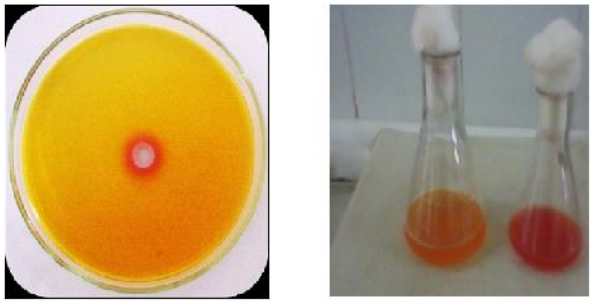

Figure A Plate assay for l- asparaginase activity B. flasks containing M9 Medium showing color change after L-asparaginase production

The isolated strain was identified on the basis of morphological and biochemical characterization according to Bergey's manual of bacteriology (Table 1). On the basis of morphological and biochemical characterization the isolated bacterial strain belongs to *Bacillus* species.

Purification and Characterization of Enzyme

Enzymes that work at extreme pH values or high temperature are of biotechnological interest. Purification of enzymes with these properties contributes to the biotechnology (Kamble *et al*., 2006). In the present investigations, the partial purification of L-asparaginase crude extract was affected by two way ammonium sulfate precipitation. After partial purification it was dialyzed to remove excess salt which results into decreased protein containt of extract from 400 mg to 220 mg & specific activity increased from 1.18 IU/ml/min to 2.15

IU/ml/min. The dialyzed enzyme was characterized in terms of effects of variable temperature and pH .optimum activity was observed at pH 8 and temperature 37°C. In quantitative assay of L-asparaginase performed before and after purification indicates 3-4 fold increase in asparaginase activity resulted due to purification. Characterization of L-asparaginase was done from *Pseudomonas aeruginosa* 50071 (Bessoumy et al., 2004).

Table 1 Morphological and biochemical characterization of isolate

Colony character/Test	Observation/Result
Color	Dirty white
Size	2mm
Shape	Circular
Margin	Entire
Opacity	Opaque
Elevation	Convex
Consistency	Sticky
Surface	Smooth
Motility	Motile
Grams nature	Gram positive
Catalase production	Positive
Oxidase	Positive
Citrate utilization	Positive
Methyl red	Negative
Indole production	Negative
Voges Proskauer	Positive
Nitrate reduction	Positive
Casein hydrolysis	Positive
Starch hydrolysis	Positive
Gas production from glucose	Negative

Effect of variable pH on enzyme activity

The activity of L-asparaginase was evaluated at different pH values from 5-9 and were observed against the isolated *Bacillus* sp. in terms of its growth and enzyme production potential. Partially purified enzyme was incubated with 0.04M L- asparagines and 0.05M buffer of pH 4-9 under assay conditions and the amount of ammonia liberated was determined. To check out stability of enzyme, it was incubated at different pH without substrate. Buffers used in study were potassium phosphate of pH 5-7 and Tris-Hcl buffer of pH 8-9.The incubation was carried out for 60 min and then enzyme activity was measured. In the present investigation at lower pH, growth as well as enzyme production was very poor. The enzyme activity was gradually increased up to pH 8 and it was suddenly decreased at pH 9. The maximum enzyme activity was recorded at pH 8 as 0.66 IU/ml/min. L-asparaginase from *Staphylococcus aureus* shows maximum growth enzyme activity at pH 8 (Kamble *et al.*, 2006).

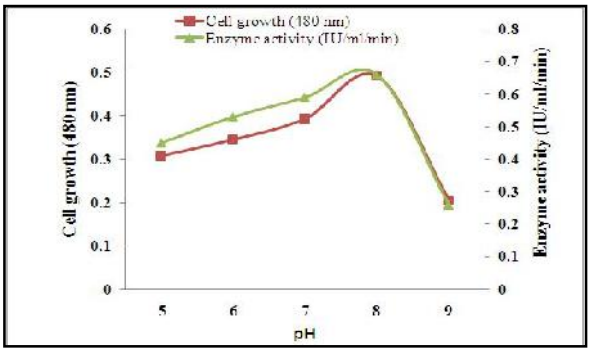

Figure 2 Effect of different pH on enzyme activity and cell growth

Effect of variable temperature on enzyme activity

Evaluation of enzyme activity at different temperature was calculated by incubating the assay mixture containing the enzyme solution with substrate at variable temperature (25, 30, 37, 40, 45°C) for 60 min.

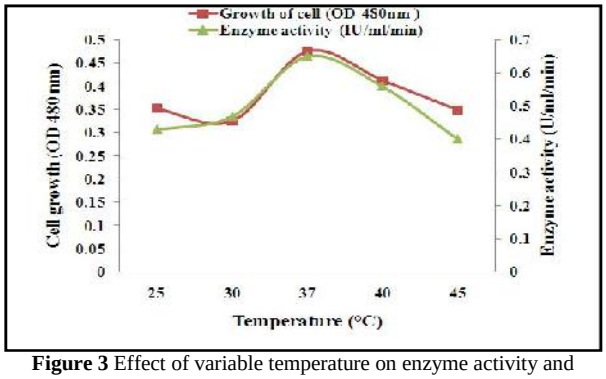

Figure 3 Effect of variable temperature on enzyme activity and cell grow

Evaluation of this showed that maximum enzyme activity appeared as 0.65 IU/ml was at 37°C. The enzyme activity increases up to 37°C and then decreases at 40°and 45°C.Moharam *et al*., (2010) Characterize enzyme in terms of temperature which shows maximum activity at 50°C for native enzyme. According to Kushwaha *et al*., the bacterial L-asparaginase showed maximum activity at 37°C at optimized conditions (Kushwaha *et al*., 2012).

Effect of incubation time on growth and enzyme activity

The analysis of L-asparaginase production was carried out at every 24 hrs interval after inoculation with the maximum activity obtained at 24 h of incubation as 0.68 IU/ml .The isolated bacterial culture was incubated for different incubation time as 24hrs to 120hrs.Along with increase in incubation time there was decrease in enzyme activity due to arrival of starvation condition. Maximum enzyme synthesis by *Bacillus* sp. was observed after 24 hrs of incubation (Moorthy *et al.*, 2010)

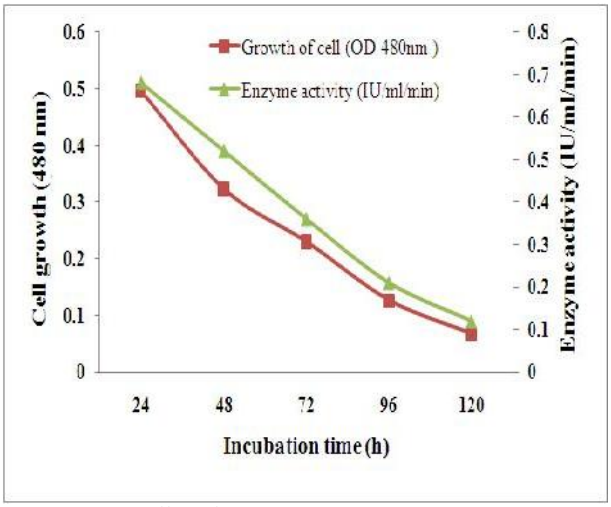

Figure 4 Effect of incubation time on enzyme activity and cell growth

Effect of different carbon sources on enzyme activity

Five different carbon sources were used in production medium to check its effect on growth of organism and enzyme production. Glucose was proven to be the best carbon source and given maximum enzyme activity as 0.58 IU/ml/min. In the presence of fructose very less activity was observed as 0.42 IU/ml/min (Figure 4) (Moorthy *et al*, 2010).

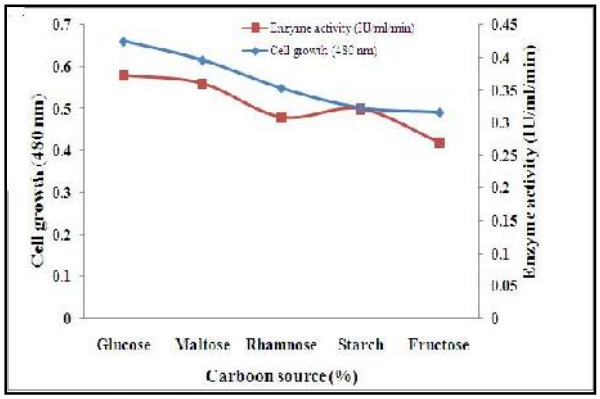

Figure 5 Effect of different carbon sources on enzyme activity and cell growth

CONCLUSION

In conclusive remark, the isolated *Bacillus* sp. showed a prominent L-asparaginase production at large scale after media optimization. The partial purification of enzyme can be done by ammonium sulphate and dialysis membrane which may be useful in therapeutic preparations.

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