International Scholars Journals

Advanced Journal of Microbiology Research ISSN 2241-9837 Vol. 12 (1), pp. 001-006, January, 2018. Available online at www.internationalscholarsjournals.org © International Scholars Journals

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Full Length Research Paper

Production, purification and characterisation of extracellular L-asparaginase from a soil isolate of *Bacillus* sp.

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Accepted 09 November, 2017

L-asparaginase is an anti-neoplastic agent used in lymphoblastic leukaemia chemotherapy. Soil microbial isolates were screened for potential producers of L-asparaginase using a phenol red indicator growth medium and the microbe producing the largest hydrolysis zone was selected. The isolate was characterised by biochemical tests and was found to belong to *Bacillus* sp. The enzyme production was carried out by submerged fermentation. Two different carbon sources, glucose and maltose were used for the enzyme production and glucose was found to be the better carbon source. The enzyme was partially purified by ammonium sulphate precipitation. Dialysis was carried out to remove the excess salt and complete purification was achieved by ion -exchange chromatography. The characterised enzyme exhibited maximal enzyme activity at pH 7 and temperature 37°C. The enzyme was activated by $MgCl_2$ and inhibited by EDTA. Protein profiling by SDS-PAGE revealed the molecular weight of the protein to be 45 kDa.

Key words: L-asparaginase, *Bacillus*, submerged fermentation, enzyme, purification.

INTRODUCTION

L-asparaginase (L-asparagine aminohydrolase, EC 3.5.1.1), the enzyme which converts L-asparagine to L-aspartic acid and ammonia, has been used as a chemotherapeutic agent (Fisher and Wray, 2002). It has received increased attention in recent years for its anti-carcinogenic potential (Manna et al., 1995). The clinical action of this enzyme is attributed to the reduction of L-asparagine; tumour cells unable to synthesise this amino acid are selectively killed by L-asparagine deprivation. Though several L-asparaginases of bacterial origin have been developed and their potential usage in clinical trials have been studied to prevent the progress of L-asparagine-dependent tumours, mainly lymphosarcomas, the success hitherto has been rather limited, and most of the treatments must be interrupted due to severe side effects and immunological reactions in the patients.

In most of the microorganisms, 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 phosphatase, deoxy ribonu-clease (Neu and Heppel, 1965), cyclic phosphor-diesterase, 5'-nucleo-tidase, acid phosphatase (Nossal and Heppel, 1966), lipase (Macris et al., 1996), carboxymethyl cellulase (Srinivas and Panda, 1998) and 17- -hydroxysteroid dehydrogenase (Egorova et al., 2004). Enzyme localiza-tion in bacteria has been carried out using various methods (Marr, 1960) . In L-asparagi-nase producing strains, the existence of both periplasm and cytoplasm enzyme have been reported (Schwartz et al., 1966). The study on the localization of any enzyme plays a vital role in the development of bioprocess.

Since the 1970s, several microbial strains like *Aspergillus tamari*, *Aspergillus terreus* (Sarquis et al., 2004), *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),

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Vibrio succinogenes (Radcliffe et al., 1979) and *Staphylococcus* sp. (Prakasham et al., 2007) having potential for L-asparaginase production have been isolated and studied in detail. Among the actinomycetes, several *Streptomyces* species such as *S. karnatakensis*, *S. venezualae*, *S. longsporusflavus* and a marine *Streptomyces* sp. PDK2 have been explored for L-asparaginase production (Narayana et al., 2007). Literature reports indicated that the enzyme's bioche-mical and kinetic properties vary with the genetic nature of the microbial strain used (Eden et al., 1990). For example, *Erwinia* L- asparaginase exhibited less allergic reactions compared to the *E. coli* enzyme. However, *Erwinia* asparaginase had a shorter half-life than *E. coli* (Asselin et al., 1993), suggesting the need to discover new L-asparaginases that are serologically different but have similar therapeutic effects. This may require the screening of soil samples from various sources for isolation of potential microbes, which have the ability to produce the desired enzyme.

Hence, studies are continued and focused on abatement of immune reactivity either by modifying the L-asparaginase or by exploring the exotic environment L-asparaginases with novel properties. The enzyme is produced throughout the world by both submerged and solid-state fermentations. This article details on the trials carried out to isolate a bacterial strain with potent L-asparaginase activity, and to study the enzyme production by purifying and characterising it.

MATERIALS AND METHODS

Isolation of microorganism and maintenance of culture

A known quantity (1 g) of soil sample was collected during January 2010, at a depth of 30 cm from Azyme Biosciences, Bengaluru, India and serially diluted with sterile distilled water and grown on agar-based modified M9 medium (composition (g/l): KH_2PO_4 2.0, L-asparagine 6.0, $MgSO_4.7H_2O$ 1.0, $CaCl_2.2H_2O$ 1.0, glucose 3.0, and agar 20.0) supplemented with phenol red (few drops) as indicator (Prakasham et al., 2010). The inoculated agar plates were incubated at 37°C overnight. A microbial culture that displayed pink red coloured colony was selected for further studies. The pinkish red colony (asparaginase-producing bacterial colony) picked from the plates and was streaked on nutrient agar slant. The isolate (DKMBT10) was grown at 37°C and after growth was stored at 4°C. The bacterium was sub-cultured on fresh nutrient agar slants every fortnight. After isolation as a pure culture, the culture was characterised using morphological (Grams stained, observed under light microscope) and biochemical tests (Carbon utilization, Starch hydrolysis, production of H_2S, liquefaction of gelatin, casein hydrolysis, degradation of urea, citrate utilization, indole production, catalase).

Production of L-asparaginase

L-asparaginase production by the isolate was carried out by submerged fermentation. The sterilised production media (composition (g/l): KH_2PO_4 2.0, L-asparagine 6.0, $MgSO_4.7H_2O$ 1.0, $CaCl_2.2H_2O$ 1.0 and glucose/maltose 3.0) was inoculated with a loop-full of log phase bacterial culture and was incubated in a rotary shaker at 37°C at 200 rpm for 24 h.

Purification of L-asparaginase

The enzyme was purified by the following steps at 4°C. The fermentation broth was centrifuged at 10,000 g for 10 min. The purification was carried out using crude enzyme extract (Distasio et al., 1982). Finely powdered ammonium sulphate was added to the crude extract. The L-asparaginase activity was associated with the fraction precipitated at 70 - 100% saturation. The precipitate was collected by centrifugation at 9,000 g for 15 min, dissolved in sodium borate buffer and dialysed against the same buffer. The dialysed fraction was applied to a DEAE column, an anion ex-changer, pre-equilibrated with Tris-HCl buffer, pH 8.6. The enzyme was eluted (1 ml/min) with NaCl gradient (0.1 - 0.5 M) and 0.1 M borate buffer, pH 7.0. The active fractions were collected, dialysed and concentrated.

ANALYTICAL STUDIES

Estimation of L-asparaginase activity

L-asparaginase enzyme assay was performed by a colorimetric method according to Wriston and Yellin (1973) at 37°C using a UV-Visible spectrophotometer by estimating the ammonia produced during L-asparaginase catalysis using Nessler's reagent. A reaction mixture consisting of 0.01 M L- asparagine and 0.05 M Tris -HCl buffer (pH 8.6) was incubated for 10 min at 37°C. The reaction was stopped by the addition of 0.5 ml of 15% trichloroacetic acid solu-tion. The liberated ammonia was coupled with Nessler's reagent and was quantitatively determined using an ammonium sulphate reference standard. 1 unit of the L-asparaginase (IU) is defined as the amount of enzyme capable of producing 1 mol of ammonia per minute at 37°C.

Estimation of protein

The amount of protein was estimated by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

ENZYME CHARACTERISATION

Effect of pH and temperature

The activity of L-asparaginase was evaluated at different pH and temperature values. The partially purified enzyme was incubated 0.05 M buffers of pH 3 - 11, under assay conditions and the amount of ammonia liberated was determined. Buffers used were potassium phosphate (pH 3.0 - 7.0), Tris-HCl (pH 8.0 - 9.0) and glycine-NaOH (pH 10 - 11). The pre- incubation was carried out for 60 min and then the residual activity was measured. The optimum temperature for the enzyme activity was determined by incubating the assay mixture at temperatures ranging from 4 - 100°C. Thermostability studies were carried out by pre- incubating the enzyme at different temperatures for 60 min (Amena et al., 2010).

Effect of activator and inhibitor

The effects of activator ($MgCl_2$ [1 mg/ml]) and inhibitor (EDTA [1 mg/ml]) on the enzyme activity were evaluated. The partially purified enzyme was incubated with activator/inhibitor at different concentrations ranging from 0.2 - 1.0 ml and the enzyme activity was determined (Ho et al., 1970; Mohapatra et al., 1995).

Figure. 1 Pink colony production by L-asparaginase producing *Bacillus.*

Table 1. Carbon utilization of the *Bacillus* strain (DKMBT10).

Carbon source	Utilization
No carbon source (negative control)	-
Xylose	-
Arabinose	+
Rhamnose	-
Fructose	-
Galactose	+
Raffinose	+
Mannitol	-
Inositol	-
Sucrose	+
Glucose (positive control)	+

Determination of molecular weight

SDS PAGE was performed according to the method of Laemmli (1970), with a separating acrylamide gel of 10% and stacking gel of 5% containing 0.1% SDS. The gel was stained 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 (66 kDa), Ovalbumin (43 kDa), carbonic anhydrase (29 kDa), lactoglobulin (18.4 kDa) and lysozyme (14.3 kDa).

RESULTS AND DISCUSSION

The bacterial strain was isolated from soil samples collected from Azyme Biosciences, Bengaluru. Microbial strains producing L-asparaginase were identified by a pink coloured colony on modified M9 agar medium with phenol red as an indicator for detection of L-asparaginase producing colonies (Prakasham et al., 2010). The microbial strain with the pink coloured colony was selected for further studies (Figure 1). The isolated strain (DKMBT10) was characterized by morphological and biochemical tests. The strain was gram positive and rod shaped colony when observed under microscope. The strain showed positive growth in media containing glucose, arabinose, galactose, raffinose, sucrose and others resulted negative (Table 1). The strain was able to hydrolyze starch and casein, liquefy gelatin, utilize citrate, but could not produce hydrogen sulphide. The strain exhibited positive urease and negative catalase activity (Table 2). According to the morphological and biochemical characteristics of the strain, DKMBT10 was classified to be a species belonging to the *Bacillus* genus.

Table 2. Physiological and biochemical properties of the *Bacillus* strain (DKMBT10).

Parameter	Results
Starch hydrolysis	+
Production of H_2S	-
Liquefaction of gelatin	+
Casein hydrolysis	+
Degradation of urea	+
Citrate utilization	+
Indole production	-
Catalase	-

The selected bacterial strain showed maximum growth at 18 h of submerged fermentation in the presence of carbon source, glucose and maltose. Analysis of the enzyme production pattern during fermentation indicated that maximum enzyme synthesis occurred at 24 h of fermentation. The purification steps, protein concentration, specific activity and yield of L-asparaginase are shown in Table 3. This strain produced a maximum of 0.1 U/mg in the production medium. The protein content in the broth culture was found to be 2.6 mg/l. There are reports on the production of L-asparaginase by submerged fermentation in *Bacillus cereus* MNTG-7 (Sunitha et al., 2010). The production of L-asparaginase in *Bacillus* strains associated with marine alga (*Sargassum* sp.) has also been previously reported (Mohapatra et al., 1995). Enzyme purification by ammonium sulphate frac-tionation and dialysis and its enzyme activity assay were carried out.

The partially purified enzyme showed higher specific activity with glucose as carbon source of 1.09 U/mg when compared with maltose (1.03 U/mg). Similarly, the protein content of the medium containing glucose was high (0.23 mg/l), whereas 0.20 mg/l was recorded in the maltose containing medium. Further purification was carried out

Table 3. Purification steps of the L-asparaginase from *Bacillus* strain (DKMBT10).

Purification steps	Total activity (U)		Total protein (mg)		Specific activity (U/mg)		Recovery (%)		Purification fold	
	Glucose	Maltose	Glucose	Maltose	Glucose	Maltose	Glucose	Maltose	Glucose	Maltose
Crude extract	0.26	0.26	2.60	2.60	0.10	0.10	100.0	100.0	1.0	1.0
Ammonium sulphate and dialysis	0.25	0.21	0.23	0.20	1.09	1.03	96.2	78.8	10.9	10.3
Ion exchange chromatography	0.11	0.05	0.10	0.05	1.12	1.05	43.1	20.0	11.2	10.5

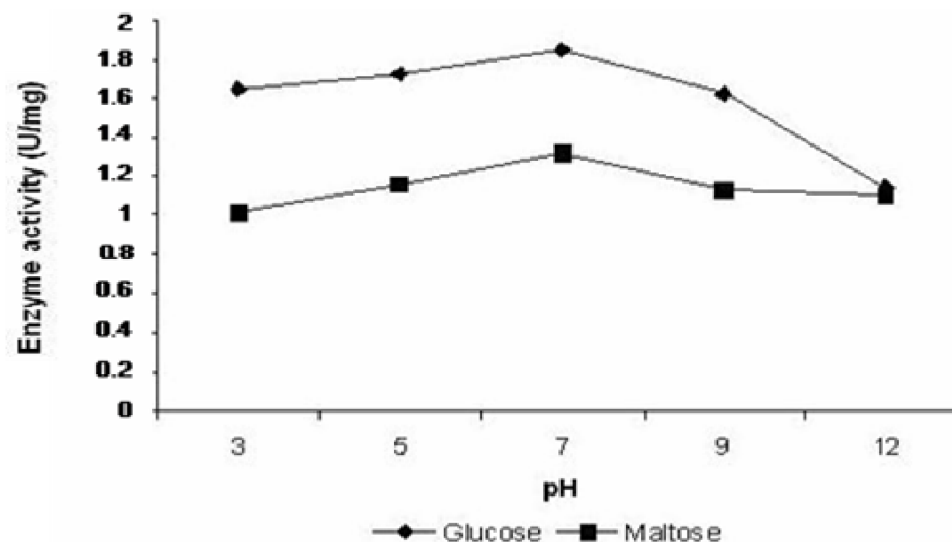

Figure 2. Effect of pH on the activity of L-asparaginase.

by ion-exchange chromatography using DEAE column. The purified enzyme showed higher specific activity with glucose as carbon source (1.12 U/mg) when compared with maltose (1.05 U/mg). The purified enzyme also showed a high protein content of 0.11 mg/l in the medium containing glucose whereas 0.05 mg/l was recorded in the maltose containing medium. Effective production of L-asparaginase by *Serratia marcescens* was observed in the medium containing glucose as carbon source (Sukumaran et al., 1979).

The enzyme thus purified was characterised in terms of optimum pH and temperature and effect of activator and inhibitor. At pH 7 (Figure 2) and at a temperature of 37°C (Figure 3), maximal activity was exhibited by the enzyme produced by *Bacillus* in a medium containing glucose whereas the maltose containing medium showed less activity. Similar pH value was obtained for the actinomycetes isolated from Thai medicinal plant rhizosphere soils (Khamna et al., 2009). *Bacillus subtilis* PS935 showed effective production with the medium containing glucose as sole carbon

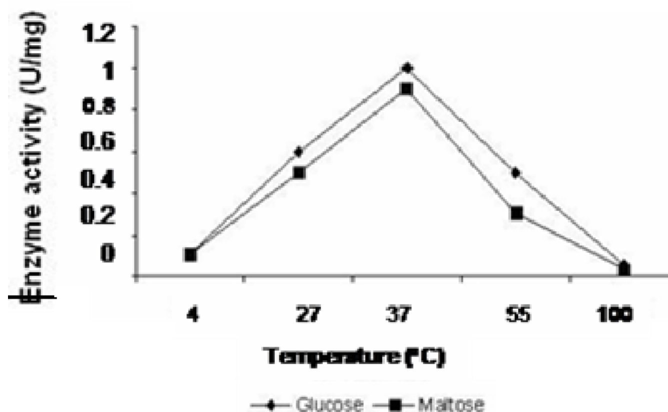

Figure 3. Effect of temperature on the activity of L-asparaginase.

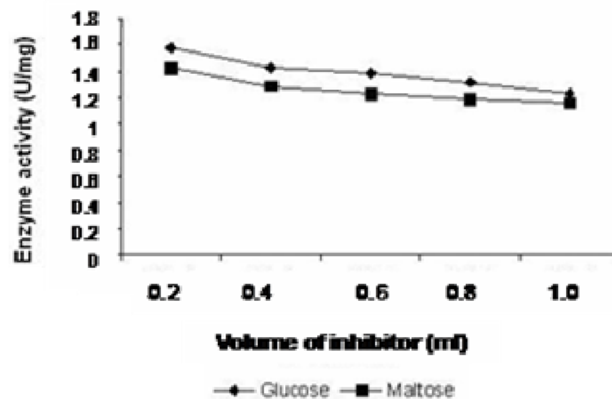

Figure 5. Effect of inhibitor on the activity of L-asparaginase.

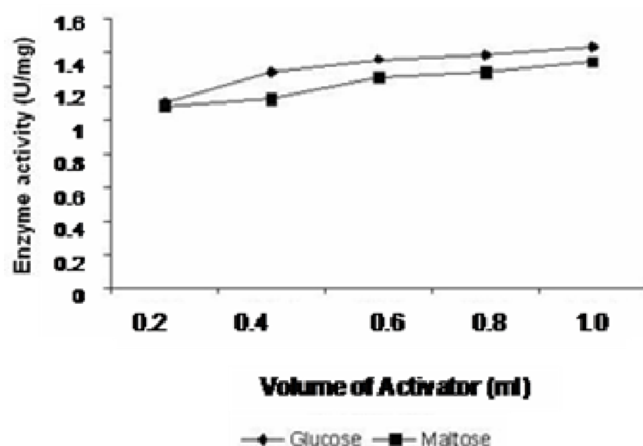

Figure 4. Effect of activator on the activity of L-asparaginase.

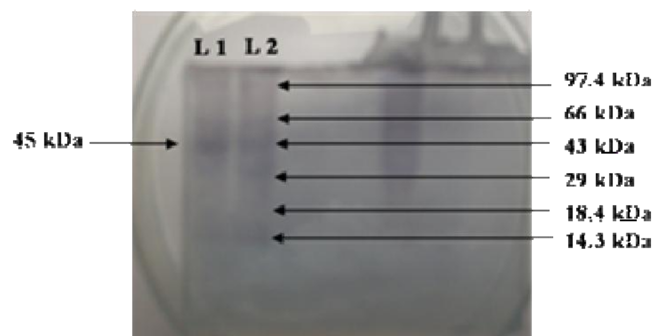

Figure 6. SDS PAGE of the purified asparaginase (L1) and marker (L2).

source at 37°C (Sun and Setlow, 1991).

Increasing concentration of the activator, magnesium chloride, increased enzyme activity and maximum activity was observed with 1.0 ml of activator (Figure 4). On the contrary, increasing concentration of the inhibitor, EDTA, decreased the enzyme activity (Figure 5). In a previous report, the maximum L-asparaginase activity was found to be at 37°C and pH 8.0; the enzyme activity was not affected by the addition of different metal ions (Ca^{2+}, Co^{2+}, Fe^{2+}, Mg^{2+} and Ni^{2+}), but was strongly inhibited by EDTA (Mohapatra et al., 1995).

The purified enzyme was protein profiled by SDS PAGE for determination of molecular weight of the enzyme. The result thus obtained was a protein band of a molecular weight of approximately 45 kDa (Figure 6). Purified L-asparaginase from *Streptomyces gulbargensis* (Amena et al., 2010), *Streptomyces* sp. PDK2 (Dhevagi and Poorani, 2006) and *S. albidoflavus* (Narayana et al., 2007) exhibited a molecular weight of 85, 140 and 112 kDa, respectively. Reports on production and purification of L-asparaginase from *P. aeruginosa* revealed, by SDS PAGE, a peptide chain with molecular weight of 160 kDa (El-Bessoumy et al., 2004).

Conclusion

The present study revealed L-Asparaginase production by submerged fermentation from soil isolate of *Bacillus* (DKMBT10). The enzyme was partially purified by ammonium sulphate precipitation. Dialysis was carried out to remove the excess salt and complete purification was achieved by ion-exchange chromatography. The enzyme was then characterized. The optimum pH of L- asparaginase was found to be 7. The enzyme had its optimum activity at a temperature of 37°C. The enzyme was activated by $MgCl_2$ and inhibited by EDTA. Two different carbon sources, glucose and maltose were used for the production and glucose was found to be the better carbon source. Protein profiling by SDS-PAGE revealed the molecular weight of the protein to be 45 kDa. The high catalytic activity of the enzyme at physiological pH

and temperature and its considerable stability over a wide range of pH and temperature makes it highly favorable to be exploited as a potent anticancer agent. Studies on the enzyme relating to purification and characterisation would open new avenues in the application of the enzyme in the healthcare industry.

ACKNOWLEDGEMENTS

Sumantha wishes to thank Mr. Selvakumar Dharmaraj for his help in the preparation of the manuscript.

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