Int.J.Curr.Microbiol.App.Sci (2017) *6*(9): 784-792

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 6 Number 9 (2017) pp. 784-792**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2017.609.097

Screening and Partial Purification of L-Asparaginase from the Bacteria Isolated from Soil Samples

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Keywords

Isolation, L-asparaginase, Partial purification, Soil samples, *Bacillus.*

Article Info

Accepted:
14 July 2017
Available Online:
10 September 2017

A B S T R A C T

L-asparaginase producing bacteria were isolated from the selected soil samples from different location of Surat district, Gujarat. The primary screening using rapid plate assay determined that out of 120 isolates, 33 strains were producing L-asparaginase. Among 33 L-Asparaginase Producing Bacterial strains (APB), significant pink color zone were observed in APB2 (0.8 mm), APB1 (0.9 mm), APB28 (0.9 mm) and APB26 (1.2 mm) at 72 hrs. Further, these four isolates preliminary screened for fermentation at laboratory level by observing pH, biomass and crude enzyme activity. Among four isolates, APB26 produced substantial L-asparaginase (3.78 unit/ml) followed by APB1 (1.58 unit/ml), APB2 (1.08 unit/ml) and APB28 (0.80 unit/ml) within 72 hrs. Further, the attempt was made to optimize pH and temperature on L-asparaginase production by APB26 within 72 hrs. The results showed that pH 7.0 and 37^{o}C temperature was optimum. Partial purification with ammonium sulfate precipitation showed 2.59 units/ml specific activity with 89.3% yield and 1.16 purification fold while, DEAE cellulose chromatography given 3.57 unit/ml specific activity with 66.3% yield and 1.60 purification fold. Based on morphological, cultural and biochemical test, APB26 was tentatively identified as *Bacillus* species.

Introduction

Enzymes are the selective proteins with high specificity that catalyze all the metabolic reactions in living organisms. Millions of enzymes are produced by prokaryotic and eukaryotic organisms. Use of microbial enzymes as drugs is an important research in the field of pharmaceutical industries. One of the most important discovery in this field is an enzyme L-asparaginase discovered by Clementi in 1922 in serum of guinea pig. L-asparaginase is more promising in the treatments of Hodgkin disease, acute myelocytic leukemia, acute myelomonocytic leukemia, chronic lymphocytic leukemia, lymphosarcoma treatment, reticulosarbom and melanosarcoma (Oettgen, 1967). Administration of guinea pig serum into mice suffered from lymphomas shown regression against cancerous cells was studied by Kidd in 1953. After that, Broome in 1968 conclude the anti-lymphatic activity guinea pig serum was due to L-asparaginase. Partial purification of L-asparaginase from guinea pig sera was carried out by Yellin and

Wriston (1966) but after extraction, insufficient quantity of enzyme boost researcher on microbial source for higher production of L-asparaginase from *Escherichia coli*. It has shown same tumoricidal activity as guinea pig sera. Due to broad diversity, easy to production, recovery and especially its susceptibility towards genetic manipulation for higher production of enzymes, microbes was found to be best alternative for L-asparaginase production. Numerous bacterial species *viz.*, *Escherichia* (Warangkar *et al.,* 2010), *Aerobacter*, *Erwinia*, *Serratia* (Agarwal *et al.,* 2011), *Xanthomonas* (Abbas *et al.,* 2010), *Bacillus* was reported to produce L-asparaginase (Pradhan *et al.,* 2013; Sunitha *et al.,* 2010; Thenmozhi *et al.,* 2011). The present investigation carried out with isolation and screening of L-asparaginase producing bacteria from diversified soil samples, fermentation studies, partial purification of L-asparaginase from potential isolates and preliminary microbial characterization of the isolates.

Materials and Methods

Sample collection

Soil samples collected from the different location of Surat district of Gujarat. Seven different diversified sites (Table 1) were selected to collect the soil samples. Samples were stored in the clean sterile glass bottles and stored at 4°C.

Isolation and primary screening of L-asparaginase producing bacteria

To isolate L-asparaginase producing bacteria, 10 g of each soil samples were inoculated into 100 ml of sterile modified M9 broth (g/l) (2.0 KH_2PO_4, 6.0 Na_2HPO_4, 0.5 NaCl, 1.0 $MgSO_4.7H_2O$, 1.0 $CaCl_2.2H_2O$, 3.0 glucose and 6.0 asparagine, pH 7.2 ± 0.2) and incubated at 30°C for 48-72 hrs (Bhat *et al.,* 2015). Each enriched samples were diluted in the range of 10^{-2} to 10^{-8} and 0.1 ml of diluents was spread on modified sterile M-9 agar medium containing 0.005% phenol red dye and kept at room temperature. Each bacterial colony was picked from the plates and was streaked using quadrate method on nutrient media to get pure culture of bacteria. Pure cultures were preserved on nutrient agar slants at 4°C. Primary screening of L-asparaginase producing bacteria was performed by rapid plate assay (Gulati *et al.,* 1997). For that, each bacterial colony was spot inoculated on modified M-9 agar medium containing 0.005% phenol red dye and kept at room temperature for three to four days. Plates without L-asparagine (use of $NaNO_3$ as nitrogen source) and without dye were used as controls (Jimat *et al.,* 2015). L-asparaginase producing strains were screened based on wide formation of pink zone around the colonies on plates and used for further studies.

Inoculums preparation and L-asparaginase production by isolates

The potential bacterial strains showing large pink halos in rapid plate assay inoculated into 5.0 ml of sterile nutrient medium. It was incubated at 37°C for 24 hrs. Each inoculums (O.D. 0.8±0.2) were suspended into 100 ml of sterile modified M-9 medium (pH 7.0±0.2) in 250 ml Erlenmeyer flasks and kept at 120 rpm for three days along with its control. The un-inoculated medium was used as a control. Further, 10 ml of culture broth was withdrawn after 24, 48 and 72 hrs and used to determine pH, bacterial biomass and crude enzyme activity (Jimat *et al.,* 2015). Each procedure was performed in triplicate.

L-asparaginase assay

To perform L-asparaginase assay, 5.0 ml of culture broth was centrifuged at 10000 rpm for 5 minutes. The 0.1 ml of crude enzyme

(supernatant) was mixed with 0.2 ml of 0.05 M Tris-HCl (pH 8.6) and 1.7 ml of L-asparagine (0.01M). After incubation of 10 minutes, reaction was stopped using 0.5 ml of 1.5 M trichloroacetic acid. To obtain clear solution, it was centrifuged at 10000 rpm for 5 minutes. Further, 0.5 ml of supernatant was diluted upto 7.0 ml using distilled water in a separate tube. It was treated with 1 ml of Nessler's reagent. Color development after 15 minutes was observed using absorbance at 480 nm. L-asparaginase activity of each sample was determined using standard curve of ammonia. One international unit (IU) of L-asparaginase was defined as amount of enzyme that liberates one µ mole of ammonia per minute under the assay condition (Worthington, 2007).

Determination of protein, biomass and pH

Estimation of protein was carried out using Lowry method (1951). For that, one ml of crude extract was mixed with one ml of 0.1 N NaOH solution followed by 5.0 ml of copper reagent. After, 10 minutes incubation at room temperature, 0.8 ml of Folin-Ciocalteu reagent was added and kept for 30 minutes. Absorbance was measured at 660 nm using spectrophotometer. Standard protein assay was in the range of 10-120 (µg/ml) concentration using bovine serum albumin. Bacterial biomass and pH was measured using spectrophotometer (600 nm) and pH electrode, respectively.

Effect of pH and temperature on enzyme production

To study the optimum pH, culturing the strain in production medium with pH range of 4.0 to 8.0 was performed. The optimal pH was used for subsequent study. To determine the optimum temperature, production medium was kept at different temperatures 25°C, 37°C, 45°C and 55°C for 72 hours was carried out (Varma *et al.,* 2016). Strains that showed significant production of enzyme was used for further study.

Partial purification of L-asparaginase from the potential isolates

Ammonium sulfate precipitation

Crude enzyme was subjected to saturate with 45% of ammonium sulfate (pH 8.5). Each saturated sample was kept at 4°C temperature overnight. After incubation, saturated samples were centrifuged at 10,000 rpm for 5 minutes. Supernatant subjected to 80% saturation with ammonium sulfate and centrifuged at 7000 rpm for 10 minutes at 4°C. The collected precipitates subjected to dialysis into pre-treated buffered dialysis bag. After dialysis, the samples were used for enzyme activity and protein estimation (Dhevagi and Poorani, 2006).

DEAE cellulose chromatography

DEAE cellulose column was pre-equilibrated with 50 mM Tris-HCl (pH 8.5) containing 100 mM KCl was loaded with 5 ml of crude enzyme. The column was washed with two-column volume of the above buffer and adsorbed protein was eluted using 50 mM Tris-HCl buffer (pH 8.5). Fractions obtained were analyzed for enzyme activity (Emmanuel *et al.,* 2015).

Preliminary characteristics of potential bacteria

Preliminary microbiological characters like morphological (Gram reaction, capsule and spore staining); cultural (colony characters on Nutrient agar, MacConkey's agar, Tributyrene agar and Blood agar) and biochemical tests (Hydrolysis of starch, casein and gelatin, nitrate reduction, citrate utilization, IMViC test, phenlyalanie

deaminase, oxidase, catalase and utilization of lactose, maltose, sucrose, glucose, xylose, manose, mannitol and fructose) were performed using standard microbiological methods (MacFaddin, 2000).

Results and Discussion

Primary screening of L-asparaginase producing bacteria and determination of enzyme activity

Total 120 distinct bacterial colonies were isolated from organic and polluted soil samples collected from the different locations of Surat, Gujarat. Based on rapid plate assay, it was observed that among 120 isolates, 33 bacterial strains showed pink color zones on modified M-9 medium for L-asparaginase production. The isolates produced zones in the range of 0.1 to 1.2 mm in diameter within 72 hrs. Four L-asparaginase producing bacterial (APB) isolates *viz;* APB 26 (1.2 mm), APB1 and APB 28 (0.9 mm) and APB 2 (0.8 mm) showed significant zone of index and other did not show reproducible results (Table 2). Thus, these four isolates were used for primary fermentation study at laboratory scale level.

Fermentation was performed by inoculating APB1, APB2, APB26 and APB28 bacteria strains into the modified M-9 medium. At specific time interval of 24, 48 and 72 hrs, samples were analyzed for biomass, pH and ammonia production. Data (Table 3) showed that the biomass, pH and enzyme unit were increasing with 24, 48 and 72 hours with all the four bacterial isolates. However, bacterial isolate APB26 gave maximum enzyme activity of 3.78 unit/ml within 72 hrs. All other isolates were found to produce less enzyme activity than APB26. Hence, an isolate APB26 was used for further study.

Effect of pH and temperature on production of L-asparaginase and partial purification

Study of effect of different pH and temperature were performed on production of L-asparaginase produced by APB26. Maximum activity of enzyme was found with pH 7.0 (4.20 units/ml) followed by pH 8.0 (2.50 units/ml) within 72 hrs. Decreased or inhibitory enzyme activity found with too acidic and alkaline condition. Data showed maximum activity of L-asparaginase at 37°C (5.40 units/ml) followed by 25°C (3.50 units/ml). Higher temperature was found to be inhibitory on production of enzyme (Figure 1).

Partial purification of L-asparaginase produced by strain APB26 was performed by two steps; ammonium sulfate precipitation followed by its dialysis and DEAE cellulose chromatography.

Table.1 Soil samples collected from different locations of Surat district, Gujarat

Sr. No.	Soil samples	Location
1	Sugarcane rhizosphere soil	Tarsadi, Bardoli
2	Tulsi rhizosphere soil	Garden of Tarsadi, Bardoli
3	Rice rhizosphere soil	Tarsadi, Bardoli
4	Soil from poultry farm	Poultry farm, Tarsadi
5	Soil treated with compost	Bamroli, Bardoli
6	Soil from the farm located nearby industrial area	Jolwa, Surat
7	Soil enriched with oil from garage	Amroli, Surat

Table.2 Qualitative data of L-asparaginase production during primary screening

Sr. No.	Isolate No.	Zone Index (mm)			Sr. No.	Isolate No.	Zone Index (mm)		
		24 hrs.	42 hrs.	72 hrs.			24 hrs.	42 hrs.	72 hrs.
1.	APB1	0.6	0.7	0.9	18.	APB18	0.3	0.3	0.3
2.	APB2	0.5	0.6	0.8	19.	APB19	0.2	0.4	0.4
3.	APB3	0.1	0.2	0.3	20.	APB20	0.1	0.2	0.2
4.	APB4	0.2	0.3	0.4	21.	APB21	0.0	0.0	0.1
5.	APB5	0.1	0.2	0.4	22.	APB22	0.1	0.1	0.1
6.	APB6	0.3	0.4	0.4	23.	APB23	0.0	0.0	0.1
7.	APB7	0.0	0.1	0.2	24.	APB24	0.0	0.2	0.3
8.	APB8	0.0	0.0	0.1	25.	APB25	0.2	0.4	0.3
9.	APB9	0.2	0.2	0.2	26.	APB26	0.6	0.8	1.2
10.	APB10	0.0	0.1	0.1	27.	APB27	0.0	0.2	0.4
11.	APB11	0.0	0.0	0.1	28.	APB28	0.3	0.6	0.9
12.	APB12	0.2	0.4	0.4	29.	APB29	0.2	0.3	0.4
13.	APB13	0.0	0.1	0.2	30.	APB30	0.0	0.2	0.2
14.	APB14	0.0	0.0	0.1	31.	APB31	0.2	0.2	0.4
15.	APB15	0.0	0.0	0.1	32.	APB32	0.0	0.0	0.2
16.	APB16	0.0	0.0	0.2	33.	APB33	0.2	0.2	0.3
17.	APB17	0.0	0.1	0.1					

Table.3 Data representing the different parameters during fermentation

Sr. No.	Isolates	Biomass (O.D. $_{600}$)			pH			Crude enzyme activity (Unit/ml)		
		24 hrs.	48 hrs.	72 hrs.	24 hrs.	48 hrs.	72 hrs.	24 hrs.	48 hrs.	72 hrs.
1	APB1	0.90	1.20	1.28	7.5	7.8	8.0	0.40	0.52	1.58
2	APB2	0.98	1.32	1.40	7.4	7.7	7.9	0.48	0.55	1.08
3	APB26	1.50	1.84	2.42	7.8	8.2	8.9	2.20	3.50	3.78
4	APB28	0.80	0.95	1.55	7.3	7.5	7.8	0.35	0.49	0.80

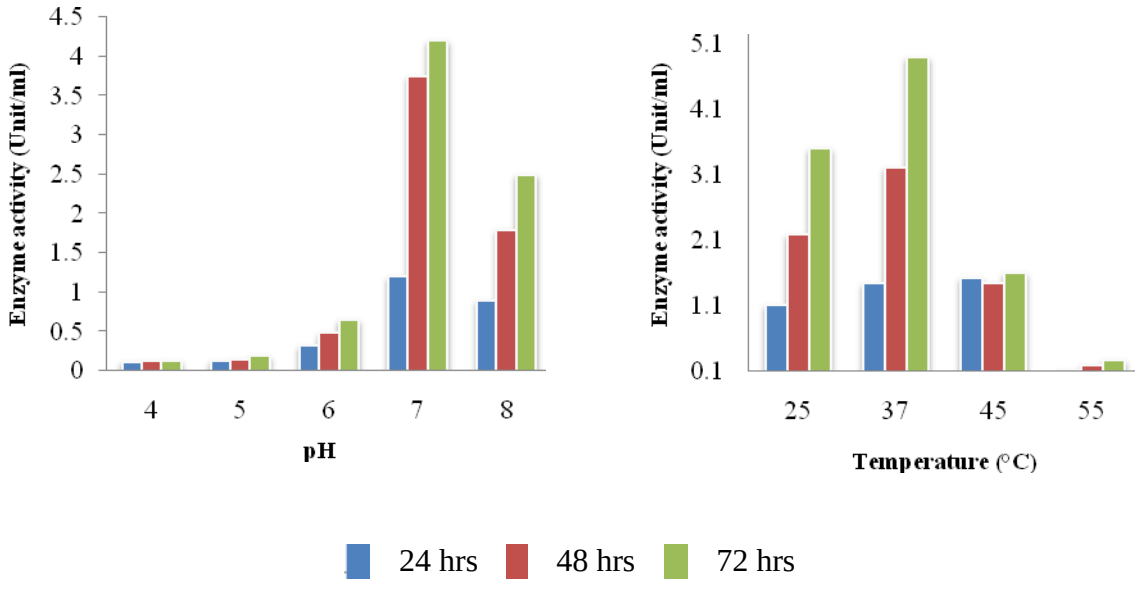
Table.4 Partial purification of L-asparaginase from isolate APB26

Sr. No.	Purification step	Total enzyme activity (units/ml)	Total protein (mg/ml)	Specific activity (units/mg)	Relative Purification Fold	Yield (%)
1	Crude	5.28	2.20	2.22	1.00	100
2	Ammonium sulfate precipitation	4.72	1.82	2.59	1.16	89.3
3	DEAE cellulose chromatography	3.50	0.98	3.57	1.60	66.3

Table.5 Microbiological characteristics of APB26 isolate

Morphological characters					
Gram reaction	Gram positive rods				
Motility	Motile				
Capsule staining	Non-capsulated				
Spore staining	Spore forming				
Biochemical characters					
Indole production	+	Catalase	+		
Methyl red test	+	Oxidase	+		
Voges-Proskauer's test	-	Phenyl alanine deaminase	-		
Citrate utilization	+	**Sugar utilization**			
Ammonia production	+	Lactose	+		
Nitrate reduction	+	Maltose	+		
Starch hydrolysis	+	Sucrose	+		
Casein hydrolysis	+	Glucose	+		
Gelatin hydrolysis	-	Xylose	+		
Urea hydrolysis	-	Mannose	+		
Blood haemolysis on blood agar	+	Mannitol	+		
Lipase	-	Fructose	+		
Cultural characters					
Medium	**Size and Shape**	**Margin**	**Elevation**	**Consistency and Opacity**	**Pigmentation**
Nutrient agar	Medium and Round	Entire	Effused	Smooth and Opaque	Non pigmented
MacConkey's agar	Medium and Round	Entire	Effused	Smooth and Opaque	Lactose non-fermenter

Fig.1 Effect of pH and temperature on production of L-asparaginase from APB 26



Results of ammonium sulfate precipitation showed that specific activity of enzyme was increased from 2.22 to 2.59 units/mg with 1.16 purification fold. Further, DEAE cellulose chromatography results showed 3.57 units/mg specific activity of enzyme with 1.60 relative purification fold and 0.98 mg/ml protein concentration (Table 4).

Microbiological characteristics of isolate

Preliminary morphological, biochemical and cultural characteristics of APB 26 isolate was performed and presented in table 5.

Data showed that APB26 isolate was motile, gram positive rods, spore forming and non-capsulated bacteria. On nutrient media colony was found to be round with medium size with entire margin and effused elevation. It was non-pigmented, opaque and lactose non-fermenter on MacConkey's agar medium. Beta haemolysis was observed on blood agar plate within 24 hrs. Biochemical characters showed that APB 26 was able to utilize lactose, maltose, sucrose, glucose, xylose, mannose, mannitol and fructose; given positive results for indole production, methyl red test, citrate utilization, ammonia production, citrate utilization, hydrolysis of starch and casein, catalase and oxidase enzymes; while negative for Voges-Proskaurer's test, lipase, phenylalanine deaminase, gelatin and urea hydrolysis.

Earlier investigation was carried out either using rhizosphere soil of farms/gardens or mangrove systems (Moorthy *et al.,* 2010; Bhat *et al.,* 2015) for isolation of L-asparaginase producing microorganisms. Rajguru and Deshmukh (2016) had used different ecosystem to get potential L-asparaginase producing microbes. In present study, organic/natural soil and industrially contaminated soil samples were collected to screen the potential L-asparaginase producing bacteria. Total 120 bacterial isolates were isolated from seven different locations as described earlier. Among them, 33 strains were producing pink zone on modified M-9 medium indicated L-asparaginase production during primary screening (Gulati *et al.,* 1997). Based on zone of index four bacterial isolates (APB1, APB2, APB26 and APB28) were used for fermentation study. Further, it was observed that APB1 and APB2 was isolated from sugarcane rhizosphere while, APB26 and APB28 from poultry farm soil. The four selected strains showed gradually increased biomass, pH and ammonia production within 76 hrs. Increased biomass indicated ability of microbes to utilize nutrients of the medium, which used for their growth. According to De Jong (1972) and Shafiq *et al.,* (2013) L-asparaginase production associated with increased pH due to accumulation of ammonia as end product (De Jong, 1972; Shafiq *et al.,* 2013). Based on this, rate of hydrolysis of L-asparaginase was calculated by measuring the release of ammonia by Nessler's method. Data showed that among four strains APB26 (3.78 unit/ml) had given maximum enzyme activity than APB1 (1.58 unit/ml), APB2 (1.08 unit/ml) and APB28 (0.80 unit/ml) within 72 hrs. Thus, APB26 was used for further study. Varma *et al.,* (2016) had studied the effect of different pH and temperature to get maximum L-asparaginase production. Thus, to optimize the pH and temperature of L-asparaginase production, APB26 subjected with different pH (4.0, 5.0, 6.0, 7.0 and 8.0) and temperatures (25°C, 37°C, 45°C and 55°C). Maximum crude enzyme activity was found with pH 7.0 at 37°C. Further, partial purification of L-asparaginase from APB26 was performed using ammonium sulfate precipitation and DEAE cellulose chromatography. Increased specific activity with purification fold observed with both ammonium sulfate (2.59 unit/ml and 1.16 purification fold) and DEAE cellulose chromatography (3.57 unit/ml and 1.60

purification fold). Emmanuel *et al.*, (2015) had observed similar kind of L-asparaginase activity from Hedgehog serum. Further, APB26 isolate was characterized based on its morphological, cultural and biochemical test. Based on results, APB26 isolate was gram positive rods, spore forming and non-capsulated bacteria. Positive results for catalase and oxidase indicated that the isolate was aerobic or facultative anaerobic bacteria (Ahmed, 2012; Acharya, 2012). According to these results, APB26 was able to utilize wide range of carbon source in form of glucose, lactose, sucrose, maltose, mannitol, mannose, fructose and xylose while unable to utilized gelatin. Further, citrate used as a sole carbon and nitrogen source by the isolate. Positive results of starch and casein utilization indicated that it can able to produce amylase and caseinase enzymes. Indole and methyl red positive test indicated fermentative utilization of glucose. APB26 was also able to produce ammonia and nitrate but unable to utilize urea and phenylalanine as a nitrogen source. Beta haemolysis on blood agar indicated haemolysins (erythrocyte lysing enzymes) by APB26. From the above characterization and according to the Bergey's Manual determinative bacteriology the potent APB26 strain might belong to *Bacillus* genus. However, further molecular as well as microbiological studies require for complete identification of the isolate.

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How to cite this article:

Savani, A.V. and Parmar, P.R. 2017. Screening and Partial Purification of L-Asparaginase from the Bacteria Isolated from Soil Samples. *Int.J.Curr.Microbiol.App.Sci.* 6(9): 784-792.
doi: https://doi.org/10.20546/ijcmas.2017.609.097