Screening, Production and Optimization of L-Asparaginase From Soil Bacteria Isolated in Ibadan, South-western Nigeria

Sherifah M. Wakil* and Adesewa A. Adelegan

Department of Microbiology, University of Ibadan, Ibadan, Nigeria

Abstract: L-Asparaginase producing bacteria were isolated from soil samples under optimum conditions using submerged fermentation. Their abilities to produce asparaginase at different pH, temperature, incubation temperature, utilization of different carbon and nitrogen sources as well as their specific conditions for optimal activity were also investigated. *Streptococcus* spp. D1, *Bacillus polymyxa* and *Streptococcus* spp. D2 showed optimum asparaginase production at pH 8 with activities of 11.6 U/ml, 8.8 U/ml and 7.9 U/ml respectively. The pH 7 was observed as optimum pH for *Bacillus firmus* (8.8 U/ml) while optimum pH 6 was observed for *Bacillus circulans* and *Paenibacillus validus*. Maximum L-asparaginase productivity was attained at a temperature of 45°C by *Bacillus firmus*, *Streptococcus sp.* D1 and *Bacillus circulans* with activity of 4.6 U/ml, 5.6 U/ml and 3.8 U/ml respectively while 35°C incubation temperature was optimum for *Paenibacillus validus* and *Bacillus polymyxa* with enzyme activity of 6.2 U/ml and 6.1 U/ml respectively. Mannitol supported the maximum asparaginase production of *Bacillus circulans*, *Streptococcus sp*. D2 and *Bacillus polymyxa* while maltose was observed as the best carbon source for *Streptococcus sp*. D1 and *Bacillus firmus*; and sucrose for *Paenibacillus validus*. The optimum nitrogen source was casein for *Bacillus circulans* (7.57 U/ml) and *Streptococcus* spp. D1 (6.19 U/ml), Yeast extract for *Bacillus* polymyxa (7.037 U/ml) and *Bacillus firmus* (5.368 U/ml) while $NaNO_3$ supported optimum L-asparaginase production for *Streptococcus sp.* D2 (6.006 U/ml) and *Paenibacillus validus* (4.754 U/ml). At optimum conditions, *Bacillus polymyxa* had the highest (4.835 U/ml) while *Bacillus circulans* had the least (2.981 U/ml) asparaginase activity. In all, the bacterial isolates prefers slightly alkaline to alkaline medium (pH 6-8) for optimum asparaginase production.

Keywords: Submerge fermentation, L-asparaginase, enzyme activity, optimum condition.

INTRODUCTION

L-Asparaginase (EC 3.5.1.1) belongs to a group of homologous amidohydrolases family, which catalyses the hydrolysis of amino acid L-asparagine to L-aspartate and ammonia [1]. They are naturally occurring enzymes expressed and produced by animal tissues, bacteria, plants, and in the serum of certain rodents, but not in mankind [2]. Large number of microorganisms that include *Erwinia carotovora* [3]*, Pseudomonas stutzeri* [4], *Pseudomonas aerugenosa* [5] and *E. coli* [6] has been known to produce L-asparaginase. Different types of asparaginase can be used for different industrial and pharmaceutical purposes. Asparaginases are used to reduce the formation of acrylamide, a suspected carcinogen, in starchy food products such as snacks and biscuits [7]. By adding asparaginase before baking or frying the food, asparagine is converted into another common amino acid, aspartic acid, and ammonium.

L-Asparaginase has received increased attention in recent years for its anticarcinogenic potential and is used as a chemotherapeutic agent for acute lymphocytic leukemia and less frequently for acute myeloblastic leukemia, chronic lymphocytic leukemia, Hodgkin's disease, melonosarcoma and non-Hodgkin's lymphoma [7-11]. The reason it is preferred for this purpose is that, it is biodegradable, non-toxic and can be administered at the local site quite easily. The aim of this research is to produce L- asparaginase from soil bacteria.

MATERIALS AND METHODS

Sample Collection

Five different soil samples were collected from different plantation in Moniya and compost site in Kara (Bodija) in Ibadan, South West, Nigeria. The soil samples were collected in polythene bag and transferred to the laboratory for analysis.

Screening and Isolation Of Bacteria

This was carried out with serial diluted sample of 1g of soil sample from each location, plated on sterile M9 basal medium [12] and incubated at 30ºC for 48 hrs. The pinkish-red colony (asparaginase-producing bacterial colony) picked from the plates were purified by subsequent streaking on sterile Nutrient Agar. The asparaginase producers were placed on Nutrient Agar slant for further use.

PRODUCTION OF L-ASPARAGINASE

Half millilitre of the 24hour old inoculum were added to 5millilitre sterile M9 basal medium (KH_2PO_4, 2.0 g, L-

*Address correspondence to this author at the Department of Microbiology, University of Ibadan, Ibadan, Nigeria; Tel: +234 803 412 94 96;
E-mail: shemowak@yahoo.com

ISSN: 1814-8085 / E-ISSN: 1927-5129/15

asparagine, 6.0 g, $MgSO_4.7H_2O$, 1.0 g, $CaCl_2.2H_2O$, 1.0 g, Glucose, 3.0 g; dissolved in 1000 millilitre of water and pH adjusted to 7.0) and incubated at 30°C for 48 hrs. At the end of fermentation period, the crude enzyme was prepared by centrifugation at 1000×g for 20 min in a cold centrifuge (Himac CR21GII). The cell-free supernatant was taken as the crude enzyme and the enzyme assay were performed.

Optimization of Environmental Parameters for L-Asparaginase Production

Effect of pH on L-Asparaginase Production

The effect of pH on L-asparaginase production was studied by growing the isolates in sterile M9 basal medium of different pH (4, 5, 6, 7 and 8) maintained by using phosphate buffer. 0.5 mls of the 24 hour old inoculum was transferred into 5 mls of the basal medium at different pH and incubated at 30°C for 48 hrs [13] and assayed for enzyme activity.

Effect of Incubation Temperature on L-Asparaginase Production

The effect of incubation temperature on L-asparaginase production was studied by growing the bacterial isolates in sterile M9 basal medium. 0.5 mls of the 24 hour old inoculum was transferred into 5 mls of the basal medium and were incubated at 5 different incubation temperatures (25°C - 45°C) for 48 hours and assayed for enzyme activity.

Optimization of Nutritional Parameters for L-Asparaginase Production

Effect of Different Carbon Sources and Concentrations

Filtered sterile glucose, sucrose, maltose, mannitol and lactose at 5different concentrations (1 g – 5 g) were added to the sterile basal medium separately. 0.5 ml of the inoculum was added to 5 ml sterile basal medium with different carbon sources, incubated at 30°C for 48 hrs and assayed for asparaginase activity using the method of Shah *et al*. [13].

Effect of Different Nitrogen sources and Concentrations

Yeast extract, gelatin, Potassium nitrate (KNO_3) and Sodium nitrate ($NaNO_3$) at different concentration (2 g, 4 g, 6 g, 8 g and 10 g) were added to the basal medium separately, autoclaved at 121°C for 15 minutes. 0.5 ml of the inoculum was added to 5 ml sterile basal medium, incubated at 30°C for 48 hrs and assayed for enzymatic activity [13].

Production at Optimum Conditions

Half millilitre of the inoculum was added to 5ml sterile basal medium, which has been incorporated each with different Carbon and Nitrogen sources at optimum concentrations, adjusted to optimum pH and incubated at the optimum incubation temperature for each bacterial isolate.

Estimation of L-Asparaginase Activity

At the end of 48 hrs incubation period, the crude enzyme was prepared by centrifugation at 10,000 rpm for 20 minutes. The cell-free supernatant was taken as the crude enzyme. The L-asparaginase was assayed using the method of Imada *et al*. [14]. Reaction was started by adding 0.5 ml of 0.01 M L-asparagine to 0.5 ml of 0.05 M Tris-HCl buffer (pH7.0) and incubated for 30 minutes at 30±2°C. The reaction was stopped by the addition of 0.1 ml of 15 % Trichloroacetic acid solution and centrifuged in a cooling centrifuge at 10,000 rpm for 10 min. The supernatant was collected and 0.1 ml was taken in a test tube along with 3.7 ml distilled water. The mixture was then incubated for 10 min for colour development. The optical density (OD) was read at 450 nm (Jenway, 6405 UV/Vis, Spectrophotometer). OD was compared with the standard graph and the amount of ammonia in µM was calculated.

RESULTS

Screening and Isolation of Bacteria

The microbial count of asparaginase producing bacteria from different soil samples were presented in Table **1**. The compost soil (COM) has highest (17) asparaginase producing bacteria, while coconut soil (COS) and cow dung soil from site 2 (CS2) had the least (4) asparaginase producing bacteria. Six bacterial isolates were selected based on the zone of inhibition (Plate **1**). Table **2** showed the zones of diameter of L-asparaginase producing bacteria in which isolate D2 (*Streptococcus sp.* D2) has the highest (52 mm) zones of inhibition, followed by isolate D1 (*Streptococcus sp.* D1) while isolate E2 (*Paenibacillus validus*) has the least (30 mm) zones of inhibition.

Effect of Incubation Temperature on L-Asparaginase Production

L-asparaginase activity varies with both temperature and organism (Figure **1**) with *Bacillus circulans* having the least activity at most temperature. The highest

Table 1: Microbial Count of Asparaginase Producing Bacteria from Different Soil Samples

Sample Code	Bacterial count (× 10^6cfu/ml)	Number of isolates	Number of asparaginase bacteria
Cos	10	10	4
GS	9	8	7
CS2	6	10	4
CSK	14	25	8
COM	15	28	17
CD	11	22	8
MS	34	41	6
Com A	8	31	7

Values are mean of triplicate reading.
KEY: Cos- Coconut soil; Com - Compost soil; GS - Garden soil; CS - Cow dung soil from site 2; CD - Cow dung soil from site1; MS - Maize soil; CSK - Coconut soil from site 2; Com A - Compost soil from site 2.

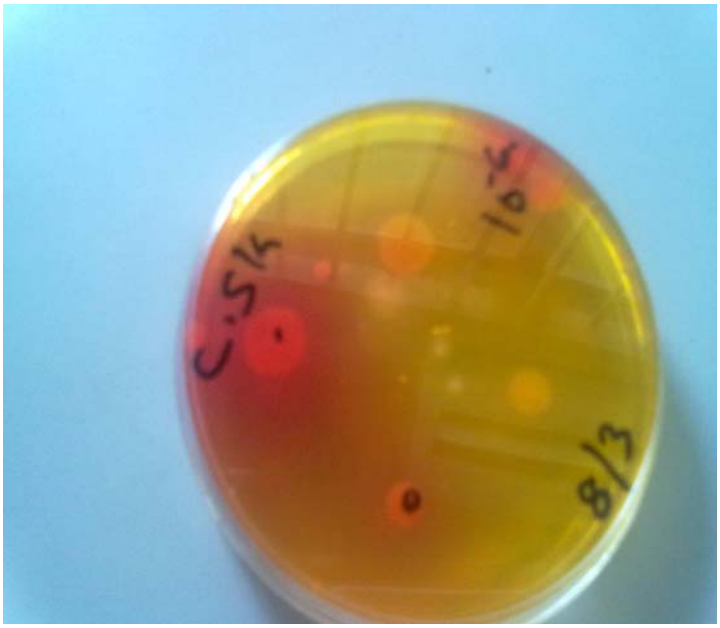

Plate 1: Plate showing L-asparaginase positive bacteria (red) from soil samples.

Table 2: Diameter of Zones of Inhibition for L-Asparaginase Producing Bacteria

Sample code	Bacteria code	Diameter of inhibition zones (mm)	Probable Identity
COM	C5	34	*Bacillus firmus*
COM A	D1	46	*Streptococcus sp. D1*
COM A	D2	52	*Streptococcus sp. D2*
COM A	D3	35	*Bacillus polymyxa*
CD	E1	37	*Bacillus circulans*
CD	E2	30	*Paenibacillus validus*

KEY: C5: Isolate from compost soil site1; D1: Isolate from compost soil site2; D2: Isolate from compost soil site2; D3: Isolate from compost soil site2; E1: Isolate from cow dung soil; E2: Isolate from cow dung soil.

L-asparaginase activity by *Bacillus firmus*, *Streptococcus sp*. D1 and *Bacillus circulans* were observed at 45°C and the least activity observed at 30°C except for *Bacillus circulans* which has its least

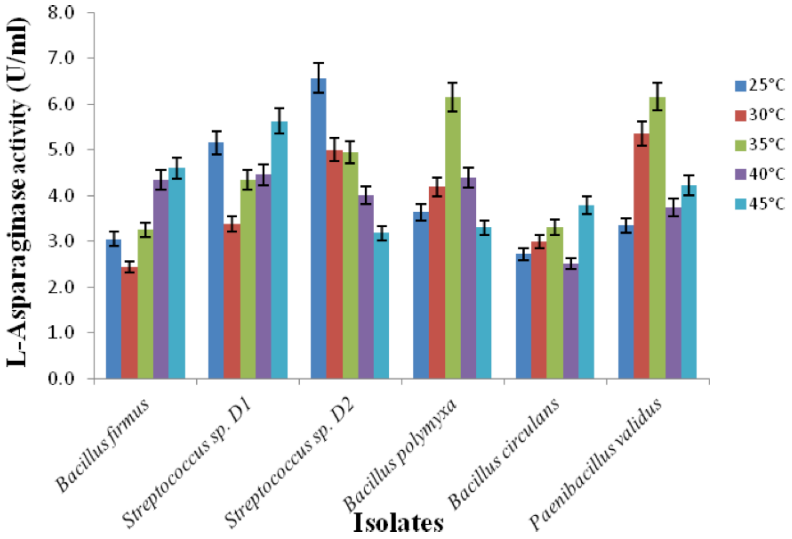

Figure 1: Effect of incubation temperature (°C) on L-asparaginase production.

activity at 40°C. *Streptococcus sp.* D2 had its highest L-asparaginase activity (6.60 U/ml) at 25°C and the least activity (3.2 U/ml) at 45°C. Incubation temperature of 35°C was found to be the best temperature for the asparaginase activity of *Bacillus polymyxa* and *Paenibacillus validus* while the least activity was recorded at 45°C (3.3 U/ml) and 25°C (3.3 U/ml) respectively.

Effect of pH on L-Asparaginase Production

Higher asparaginase activity at all pH was recorded in *Streptococcus sp.* D1 and the least activity by *Paenibacillus validus* (Figure **2**). Asparaginase activity increases with increased pH by all the isolates except *Paenibacillus validus*, *Bacillus firmus* and *Bacillus circulans*. At pH 8, *Streptococcus sp*. D1, *Streptococcus sp*. D2 and *Bacillus polymyxa* had their highest asparaginase activity while *Bacillus circulans* and *Paenibacillus validus* had theirs at pH 6 and *Bacillus firmus* at pH 7.

Influence of Environmental and Nutritional Parameters on Enzyme Production of Selected Bacterial Isolates

Effect of different carbon sources on L-asparaginase production by all the isolates is as shown

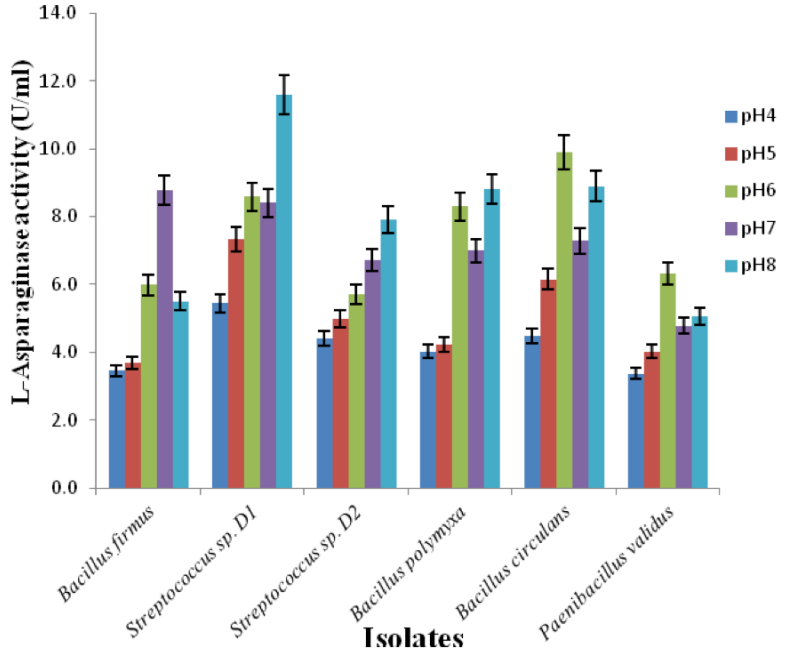

Figure 2: Effect of pH on L-asparaginase production.

Table 3: Effect of Different Carbon Sources (g/l) on L-Asparaginase Production (U/ml)

Bacterial isolates	Asparaginase produced (U/ml)				
	Glucose	Sucrose	Maltose	Lactose	Mannitol
Bacillus firmus	1.521±0.001^{e}	4.002±0.001^{c}	4.962±0.002^{a}	4.060±0.000^{b}	2.832±0.001^{d}
Strep. sp. D1	3.085±0.000^{e}	3.631±0.001^{d}	6.366±0.001^{a}	5.729±0.001^{c}	6.064±0.002^{b}
Strep. sp. D2	4.604±0.001^{d}	5.137±0.000^{c}	5.577±0.001^{b}	5.578±0.002^{b}	6.123±0.001^{a}
B. polymyxa	3.491±0.002^{e}	4.952±0.001^{b}	3.805±0.001^{d}	4.221±0.000^{c}	5.287±0.002^{a}
B. circulans	2.542±0.001^{e}	6.307±0.001^{c}	6.318±0.002^{b}	4.002±0.001^{d}	7.397±0.000^{a}
P. validus	1.847±0.002^{e}	7.373±0.001^{a}	3.400±0.000^{c}	5.623±0.001^{b}	2.948±0.001^{d}

Values are means of duplicate readings ±SD of Asparaginase produced. Means of values on the same row with the same superscript are not significantly different (P≥0.05) from each other.

on Table **3**. For all the isolates, the least asparaginase production was recorded with glucose while the maximum production varies with organism and carbon source. The highest asparaginase activity by *Paenibacillus validus* (isolate E2) was induced by sucrose (7.37 U/ml) while maltose induced highest production of the enzyme in *Bacillus firmus* (4.962 U/ml) and *Streptococcus sp*. D1 (6.366 U/ml). Mannitol was observed to induce highest asparaginase activity/production in *Streptococcus sp*. D2 (6.123 U/ml), *Bacillus firmus* (5.287 U/ml) and *Bacillus circulans* (7.397 U/ml).

For all the isolates, gelatin as nitrogen source induced the least asparaginase production except for *Streptococcus sp.* D2 which had its least activity with casein (4.20 U/ml) (Table **4**). Yeast extract induced highest asparaginase production in *Bacillus firmus* (5.369 U/ml), casein in *Bacillus circulans* (7.571 U/ml) and $NaNO_3$ (6.006 U/ml) in *Streptococcus sp. D*2.

Effect of Different Concentration of Carbon Sources on Enzyme Production

Increase in glucose concentration from 1 g/l to 2 g/l resulted in a drastic decrease in asparaginase production by *Bacillus firmus* while further increase from 2 g/l to 5 g/l has little or no effect on asparaginase production. Similarly, increase in glucose concentration from 1 g/l to 5 g/l was observed to have no significant effect on asparaginase production (Figure **3**). Highest L-asparaginase production (activity) was recorded at 1 g/l glucose concentration by all isolates except *Paenibacillus validus* which recorded highest activity at 4 g/l glucose concentration. Least L-asparaginase production by all the isolates was observed at 3 g/l glucose concentration except in *Bacillus polymyxa* which had its least activity at 5 g/l.

Among all the isolates, *Bacillus firmus* had the least L-asparaginase production at most concentration of mannitol and production decreases with increase in mannitol concentration except at 4 g/l mannitol concentration (Figure **4**). Best concentration of mannitol for highest production of asparaginase among the isolates varies. 1 g/l being the best for *Bacillus firmus* (4.6 U/ml) and *Streptococcus sp. D1* (10.5 U/ml), 2 g/l for *Bacillus circulans* (7.8 U/ml), 3 g/l for *Streptococcus sp.* D2 (6.1 U/ml), 4 g/l for *Bacillus polymyxa* (7.4 U/ml) and *Paenibacillus validus* (5.4 U/ml). *Streptococcus sp. D1* had its highest

Table 4: Effect of Different Nitrogen Sources (g/l) on L-Asparaginase Production (U/ml)

Bacterial isolates	Asparaginase produced (U/ml)				
	KNO_3	$NaNO_3$	Gelatin	Yeast extract	Casein
Bacillus firmus	3.62±0.001^{d}	4.54±0.001^{b}	2.43±0.002^{e}	5.37±0.001^{a}	3.71±0.000^{c}
Streptococcus sp. D1	4.43±0.002^{c}	4.84±0.001^{b}	3.72±0.001^{e}	3.99±0.000^{d}	6.19±0.001^{a}
Streptococcus sp. D2	5.40±0.00^{c}	6.01±0.00^{a}	4.99±0.000^{d}	5.52±0.001^{b}	4.20±0.002^{e}
Bacillus polymyxa	6.64±0.001^{b}	3.85±0.002^{d}	3.28±0.001^{e}	7.04±0.000^{a}	3.91±0.001^{c}
Bacillus circulans	4.27±0.002^{c}	3.56±0.000^{d}	3.48±0.002^{e}	4.35±0.001^{b}	7.57±0.001^{a}
P. validus	3.61±0.000^{b}	4.75±0.002^{a}	3.04±0.001^{e}	3.47±0.001^{c}	3.26±0.001^{d}

Values are means of duplicate readings ±SD of Asparaginase produced. Means of values on the same row with the same superscript are not significantly different (P≥0.05) from each other.

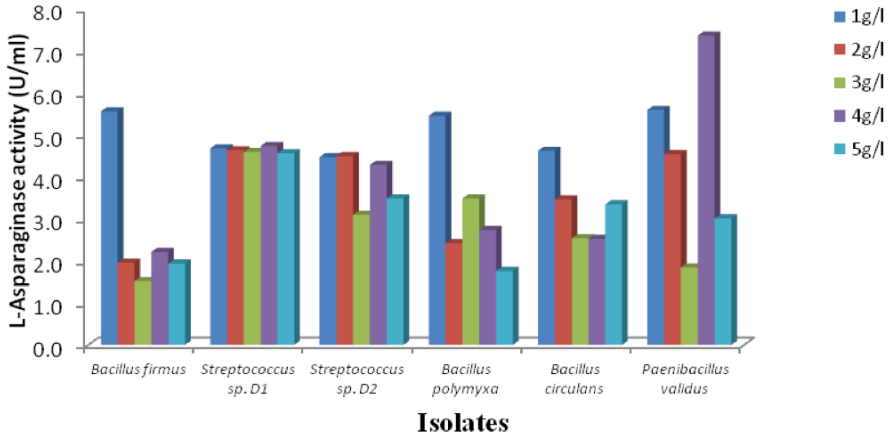

Figure 3: Effect of different glucose concentration(g/l) on L-asparaginase production.

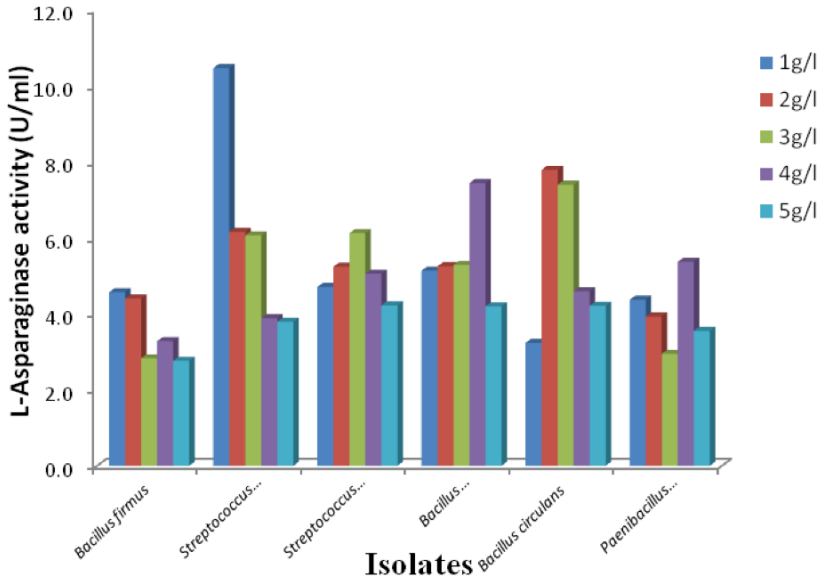

Figure 4: Effect of different mannitol concentration (g/l) on L-asparaginase production.

asparaginase production (10.5 U/ml) at 1 g/l and the least activity (3.8 U/ml) at 5 g/l mannitol concentration. Least asparaginase production by all the isolates, was observed at 5 g/l mannitol concentration except in *Bacillus circulans* and *Paenibacillus validus* which was 1 g/l and 3 g/l respectively.

Effect of different sucrose concentration on L-asparaginase production by all isolates is as shown on Figure **5**. At 3 g/l sucrose concentration, *Bacillus firmus*, *Bacillus circulans* and *Paenibacillus validus* had their highest L-asparaginase production while *Streptococcus sp.* D1 and *Streptococcus sp.* D2 had theirs at 1 g/l and 4 g/l respectively. The least asparaginase activity by all isolates was observed at 5 g/l except *Bacillus firmus*, *Streptococcus sp.* D1 and *Paenibacillus validus* which had theirs at 1 g/l, 3 g/l and 4 g/l sucrose concentration respectively.

Highest activity at all maltose concentration was recorded in *Streptococcus sp.* D1 and the least activity by *Bacillus firmus* (Figure **6**). Highest L-asparaginase production was recorded at 4 g/l maltose concentration by *Bacillus firmus*, *Streptococcus sp*. D2 and *Paenibacillus validus* while *Streptococcus sp.* D1 and *Bacillus polymyxa* had theirs at 1 g/l maltose concentration and *Bacillus circulans* had its highest activity at 3 g/l maltose concentration. The least maltose concentration also varies with the organism.

At 5 g/l lactose concentration, *Bacillus firmus*, *Streptococcus sp.* D1, *Streptococcus sp*. D2 and *Bacillus polymyxa* had their highest L-asparaginase activity while *Bacillus circulans* and *Paenibacillus validus* had theirs at 2 g/l and 3 g/l lactose concentration respectively (Figure **7**). *Bacillus firmus* and *Streptococcus sp*. D1 had their least

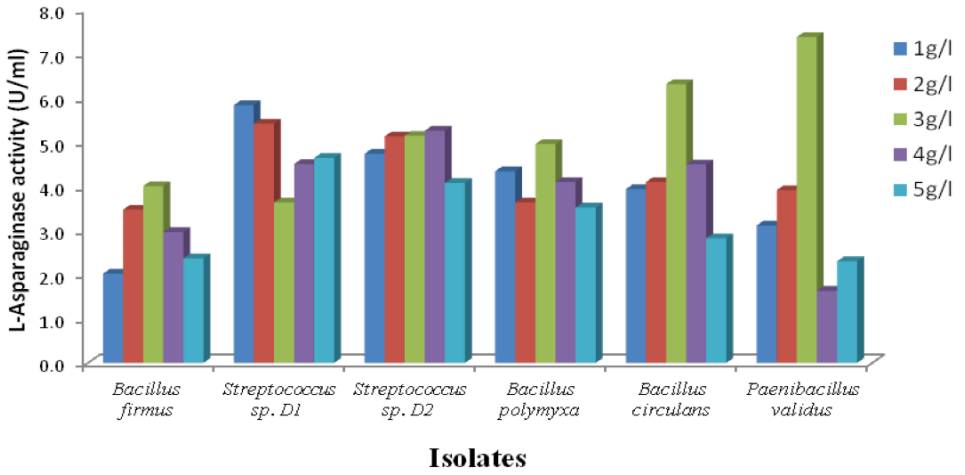

Figure 5: Effect of different sucrose concentration (g/l) on L-asparaginase production.

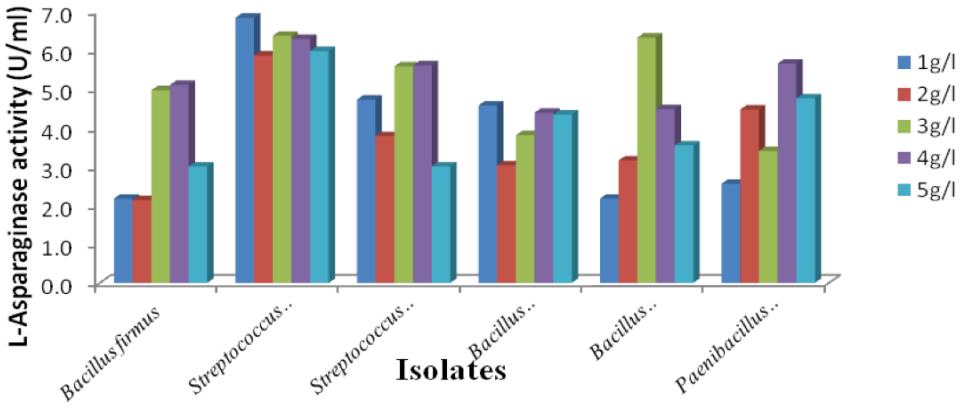

Figure 6: Effect of different maltose concentration (g/l) on L-asparaginase production.

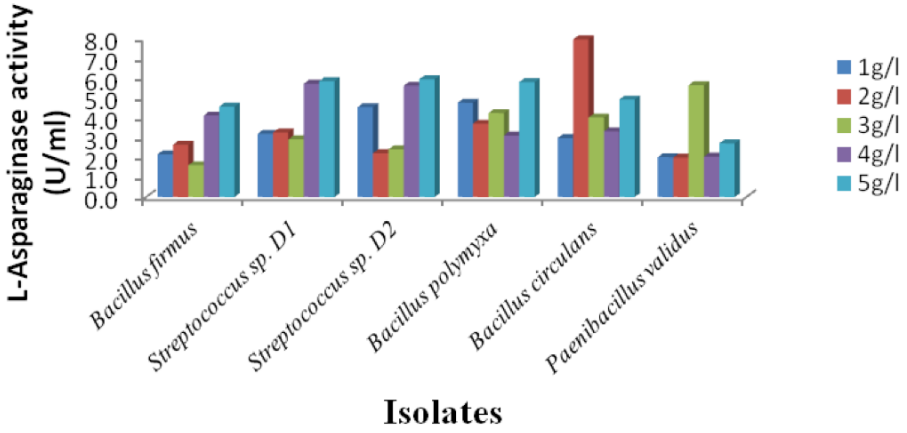

Figure 7: Effect of Lactose concentration (g/l) on L-asparaginase production.

L-asparaginase activity at 3 g/l lactose concentration while *Bacillus circulans* and *Paenibacillus validus* had theirs at 1 g/l and *Bacillus polymyxa* had its at 4 g/l lactose concentration. and *Streptococcus sp*. D2 had its least asparaginase production at 2 g/l lactose concentration.

Effect of Different Concentration of Nitrogen Sources on Enzyme Production

The highest L-asparaginase activity by *Streptococcus sp.* D1 was observed at 10 g/l KNO_3 concentration (6.70 U/ml) and the least activity (4.4 U/ml) was recorded at 6 g/l KNO_3 concentration (Figure

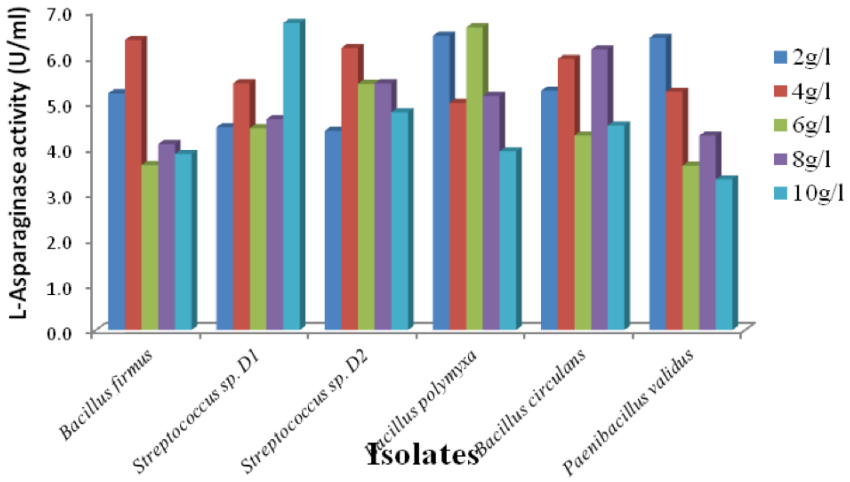

Figure 8: Effect of different KNO_3 concentration (g/l) on L-asparaginase production.

8). 4 g/l KNO_3 concentration was found to be the best for asparaginase activity by *Bacillus firmus* and *Streptococcus sp*. D2 while the least activity was recorded at 6 g/l and 2 g/l KNO_3 concentration respectively. Least L-asparaginase production was observed at 10 g/l for *Bacillus polymyxa* and *Paenibacillus validus* while the highest L-asparaginase production was at 6 g/l and 2 g/l KNO_3 concentration respectively. *Bacillus circulans* had its highest asparaginase production at 8 g/l KNO_3 and the least asparaginase production at 6 g/l KNO_3 concentration.

Increase in $NaNO_3$ concentration concentration from 2 g to 10 g/l resulted in decreased asparaginase production by *Streptococcus sp*. D1 while little or no effect was recorded in *Bacillus circulans* and *Paenibacillus validus* (Figure **9**). Highest L-asparaginase production was recorded at 2 g/l $NaNO_3$ concentration for *Bacillus firmus*, *Streptococcus sp.* D1, *Streptococcus sp.* D2 and *Paenibacillus validus*, while *Bacillus polymyxa* and *Bacillus circulans* had theirs at 10 g/l and 8 g/l respectively. At 8 g/l $NaNO_3$ concentration, *Bacillus firmus* and *Paenibacillus validus* had their least asparaginase activity while *Streptococcus sp*. D2 and *Bacillus circulans* had theirs at 4 g/l $NaNO_3$ concentration and *Streptococcus sp.* D1 had its least asparaginase activity at 10 g/l. *Bacillus polymyxa* had its least asparaginase production at 2 g/l $NaNO_3$ concentration.

Asparaginase activity decreases with increased gelatin concentration by all the isolates except *Streptococcus sp*. D2 and *Bacillus circulans* (Figure **10**). Highest L-asparaginase activity was recorded at 2 g/l gelatin concentration by *Bacillus firmus*, *Streptococcus sp*. D1 and *Paenibacillus validus* while *Bacillus polymyxa*, *Streptococcus sp*. D2 and *Bacillus circulans* had their highest L-asparaginase production at 4 g/l, 6 g/l and 8 g/l respectively. At 10 g/l gelatin concentration, *Bacillus firmus*, *Streptococcus sp.* D1, *Streptococcus sp.* D2 and *Bacillus polymyxa* had their least asparaginase activity while *Bacillus circulans* and

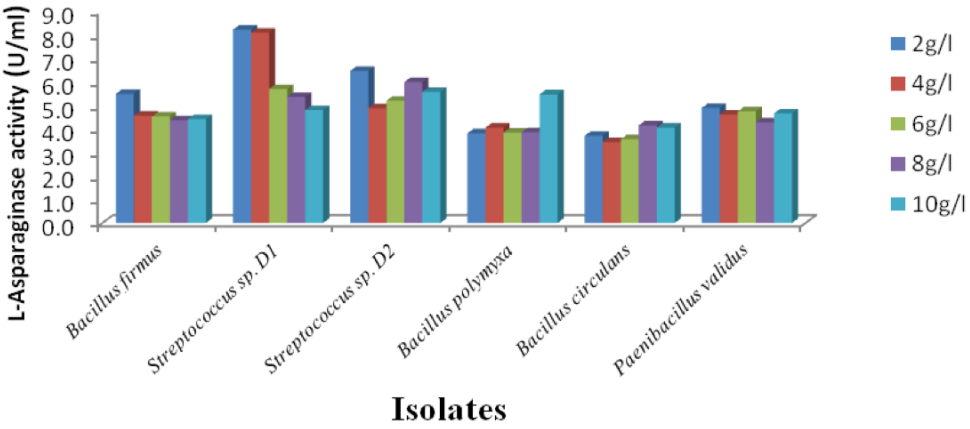

Figure 9: Effect of different $NaNO_3$ concentration (g/l) on L-asparaginase production.

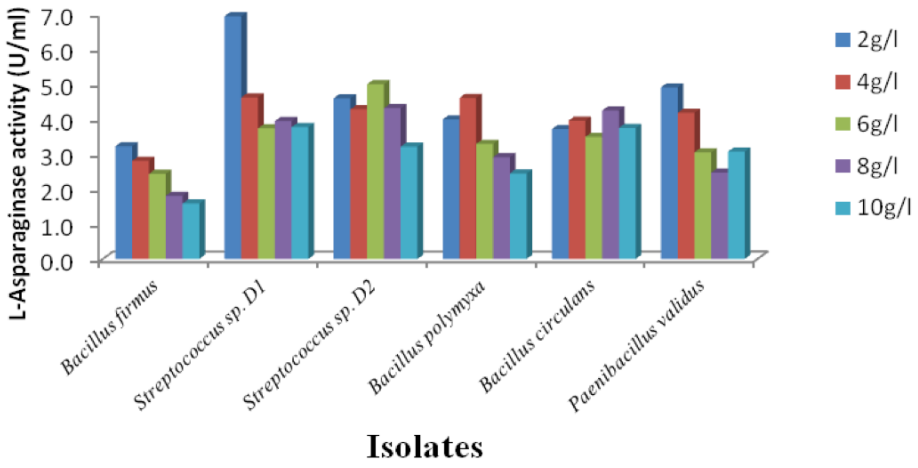

Figure 10: Effect of different gelatin concentration (g/l) on L-asparaginase production.

Paenibacillus validus had theirs at 6 g/l and 8 g/l gelatin concentration respectively.

Effect of different yeast concentration on L-asparaginase production by all isolates is as shown on Figure **11**. *Bacillus firmus* had its highest L-asparaginase production (6.8 U/ml) at 4 g/l yeast extract concentration and least asparaginase production (4.2 U/ml) at 8 g/l. Increase in yeast extract concentration, increases the L-asparaginase production by *Streptococcus sp.* D1 with highest activity (5.3 U/ml) recorded at 10 g/l. *Streptococcus sp.* D2 had its highest L-asparaginase activity (6.6 U/ml) at 8 g/l yeast extract concentration and least asparaginase activity (4.8 U/ml) at 10 g/l yeast extract concentration. *Bacillus polymyxa* had its highest L-asparaginase activity (7.0 U/ml) at 6 g/l yeast extract concentration and least activity (5.0 U/ml) at 8 g/l yeast extract concentration. *Bacillus circulans* had its highest L-asparaginase production (6.0 U/ml) at 2 g/l yeast extract concentration and the least asparaginase production (4.3 U/ml) at 6 g/l yeast extract concentration (Figure **11**). *Paenibacillus validus* had its highest L-asparaginase activity (4.2 U/ml) at 10 g/l yeast extract concentration and least activity at (3.3 U/ml) at 2 g/l yeast extract concentration.

Effect of different casein concentration on L-asparaginase production by all isolates is as shown on Figure **12**. *Bacillus firmus* and *Paenibacillus validus* had highest L-asparaginase production at 4 g/l and 10 g/l respectively. At 6 g/l, *Streptococcus sp.* D1 and *Bacillus circulans* had their optimum L-asparaginase production while *Bacillus polymyxa* and *Streptococcus sp.* D2 had theirs at 4 g/l and 10 g/l respectively. At 8 g/l, *Streptococcus sp.* D1 and *Streptococcus sp.* D2 had their least asparaginase production while *Bacillus polymyxa* and *Bacillus circulans* had theirs at 6 g/l and 10 g/l respectively.

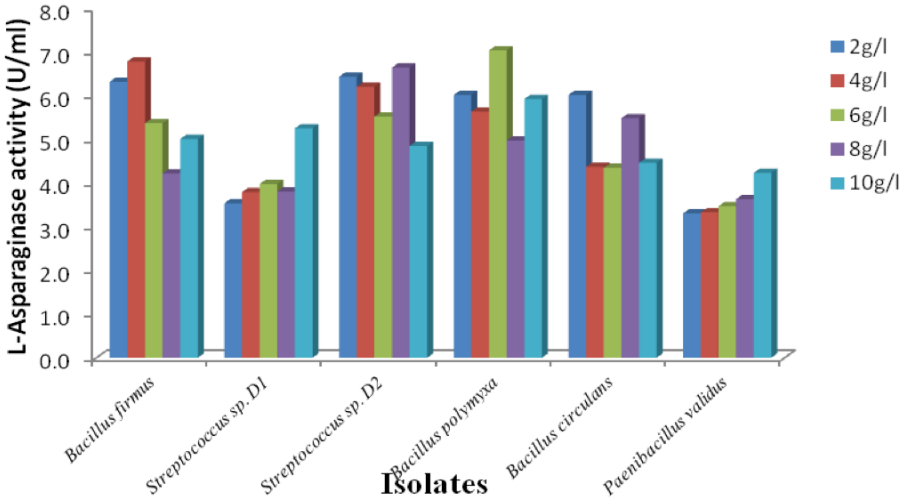

Figure 11: Effect of different yeast extract concentration (g/l) on L-asparaginase production.

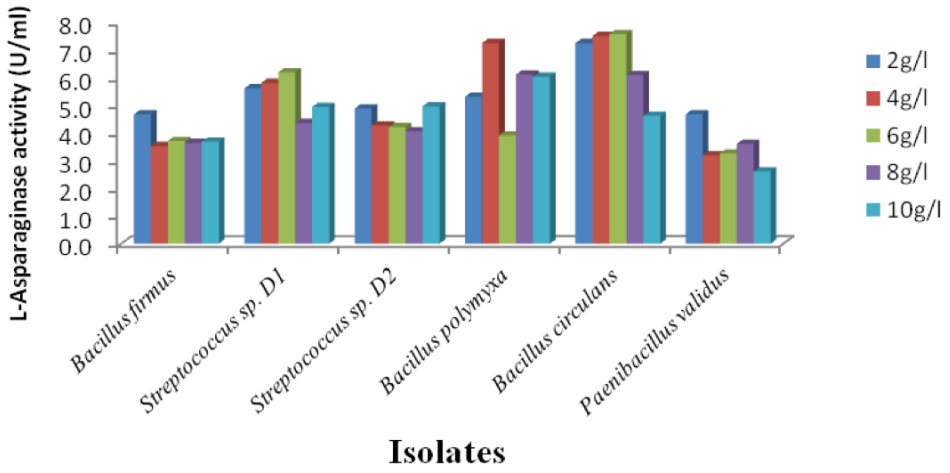

Figure 12: Effect of different casein concentration (g/l) on L-asparaginase production.

Table 5: Activity of L-Asparaginase of Bacterial Isolates at Optimum Conditions

Isolates	Carbon Source	Nitrogen Source	pH	Incubation Temp.	Asparaginase activity(U/ml)
Bacillus firmus	Glucose (1g/l)	Yeast extract (4g/l)	7	45°C	3.028
Streptococcus sp. D1	Mannitol(1g/l)	$NaNO_3$ (2g/l)	8	45°C	3.966
Streptococcus sp. D2	Mannitol (2g/l)	Yeast extract (8g/l)	8	25°C	3.874
Bacillus polymyxa	Mannitol (4g/l)	Casein (4g/l)	8	35°C	4.835
Bacillus circulans	Lactose (2g/l)	Casein (6g/l)	6	45°C	2.981
Paenibacillus validus	Sucrose (3g/L)	KNO_3 (2g/l)	6	35°C	4.221

At optimum conditions, *Bacillus polymyxa* had the highest asparaginase activity of 4.835 U/ml while the least activity (2.981 U/ml) was observed for *Bacillus circulans* (Table **5**). In all, the bacterial isolates prefers slightly alkaline to alkaline medium (pH 6-8) for optimum asparaginase production.

DISCUSSION

Sixty-one asparaginase producing bacteria were isolated from five different soil samples. This may be attributed to the fact that soil is a rich source for potential enzyme producing organisms.

In this study, the result of screening test revealed that asparaginase producing bacteria were able to hydrolyse L-asparagine since they utilized L-asparagine as their substrate and breakdown asparagine to L-aspartate and ammonia which further reacts with water to produce NH_4OH, hence the pH of the medium is basic which subsequently changes the medium from yellow to pink, hence the pinkish zone observed around the colony of asparaginase producing bacteria. This is in accordance with the work of Gulati *et al*. [15] who proved that colour transformation was due to L-asparaginase production. The bacterial isolates with highest asparaginase activity were identified as *Bacillus spp*., *Streptococcus spp*. and *Paenibacillus spp*. This is also similar to the report of Kamble and Khade [16] who reported *Bacillus spp*. and *Paenibacillus spp*. to be a good asparaginase producing bacteria.

The optimum incubation temperature at which *Bacillus firmus*, *Streptococcus spp*. D1 and *Bacillus circulans* showed highest asparaginase yield was 45°C; this was in contrast with the report of Narayana [17] on the optimum incubation temperature for *Streptomyces albidoflavus*. *Bacillus polymyxa* and *Paenibacillus validus* had their highest production at incubation temperature of 35°C, which was similar to the work of Borkotaky and Bezbaruah [18] that reported optimum incubation temperature for L-asparaginase activity by *Erwinia sp.*

Among the physical parameters, pH of the growth medium plays an important role by inducing morphological changes in the microorganisms and in

enzyme secretion. The result of the effect of pH showed that maximum asparaginase yield by *Streptococcus spp*. D1, *Streptococcus spp*. D2 and *Bacillus polymyxa* was at pH8. This observation agrees with the work of Dhevagi and Poorani [19] who reported maximum L-asparaginase by *Streptomyces sp.* PDK7 at pH 8.0 to 8.5.

The effect of various concentration of mannitol (0.1 % to 0.5 %) on L-asparaginase production showed that *Bacillus firmus* and *Streptococcus sp.* D1 had their optimum asparaginase production at 0.1 % while the least asparaginase production by all the bacterial isolates was observed at 0.5 % mannitol concentration except *Bacillus circulans* and *Paenibacillus validus*. This agrees with the work of Thae and Ellaiah [20] who stated that increase in mannitol concentration (from 0.1 %- 0.2 %) resulted in decreased asparaginase production and that increase in mannitol concentration above 0.1% resulted in the accumulation of mannitol in the cultivation medium.

The best sucrose concentration for maximum L-asparaginase production by all the bacterial isolates was 0.3 % except *Streptococcus sp*. D1 which was 0.1 % sucrose concentration. In contrast, Praveen *et al*. [21] observed higher titres of L-asparaginase by *Serratia marcescens* when medium was supplemented with 1.5 % sucrose concentration. Susmika and Mandal [22] have reported sucrose as the best carbon source for L-asparaginase production. This may be due to the inductive effect of sucrose and its remarkable efficiency in asparaginase production, being an inexhaustible source of carbon compared to other carbon sources and it also helps in stabilizing the enzyme [23].

The optimum maltose concentration at which *Bacillus firmus*, *Streptococcus sp*. D2, *Paenibacillus validus* showed optimum asparaginase production was 0.4 % maltose concentration. This agrees with the work of Amena *et al*. [24] who reported 0.5 % maltose concentration as the best concentration for optimum L-asparaginase production for *Streptomyces gulbargensis*. In contrast, Deokar *et al*. [25] observed maximum L-asparaginase production by *Erwinia carotovora* at 1.13 % maltose concentration.

Reports have shown that lactose was the best carbon source for L-asparaginase production [26, 27]. The best lactose concentration for maximum asparaginase production by most bacteria was 0.5%. In contrast, Liu and Zajic [28] observed enhanced L-asparaginase production with 1 % lactose concentration.

The best nitrogen source for maximum asparaginase production by *Bacillus firmus* and *Bacillus polymyxa* was yeast extract. This agrees with the work of Verma [29] who reported yeast extract to be important for cell growth and L-asparaginase synthesis.

The best KNO_3 concentration for extracellular asparaginase production was 0.4 % KNO_3 concentration for *Bacillus firmus* and *Streptococcus sp*. D2. Least L-asparaginase production was observed at 1 % KNO_3 concentration for *Bacillus polymyxa*, *Bacillus circulans* and *Paenibacillus validus*. This agrees with the work of Singh and Srivastava [30] who reported reduction in asparaginase production at 1 % KNO_3 concentration.

Maximum L-asparaginase production for most of the isolates was recorded at 0.2 % $NaNO_3$ concentration, an observation similar to that of Makky *et al*. [31] who observed $NaNO_3$ to be optimum for L-asparaginase activity of *Bacillus spp*. KK2S4.

Bacillus polymyxa had its highest L-asparaginase activity at 0.6 % yeast extract concentration while the least asparaginase activity by all isolates was 0.8 % concentration except *Streptococcus sp*. D2 and *Bacillus circulans*. This agrees with the work of Hosamani and Kaliwal [9] that showed that 0.5 % yeast extract gave optimum L-asparaginase production. This result was similar to the work of Verma [29] who reported the importance of yeast extract at low concentration for cell growth and L-asparaginase synthesis. In contrast, Kavitha and Vijayalakshimi [32] showed that 1.5 % yeast extract concentration was optimum for L-asparaginase production. Also, Deokar *et al*. [25] showed that 1.74 % yeast extract concentration was optimum for L-asparaginase synthesis by *Erwinia carotovora*.

Increase in casein concentration resulted in decreased asparaginase, an observation which agrees the work of Thaer and Ellaiah [20] that showed gradual decline in L-asparaginase production with increased casein concentration. Report have also shown that casein is essential for cell growth and L-asparaginase synthesis, but in high concentration, the production of L-asparaginase is inhibited which might be due to the presence of high substrate concentration and induction of proteolytic enzyme [33].

CONCLUSION

This study clearly indicates that soil can provide a good source of asparaginase producing bacteria and that *Bacillus polymyxa* strains subjected to submerged fermentation at an alkaline pH (pH 6-8) using mannitol (4 g/l) and casein (4 g/l) as carbon and nitrogen sources respectively will produce reasonable amount of L-asparaginase.

REFERENCES

[1] Fernandes AI, Gregoriadis G. Polysialylated asparaginase: preparation, activity and pharmacokinetics. Biochim Biophys Acta 1997; 1341: 26-34. http://dx.doi.org/10.1016/S0167-4838(97)00056-3

[2] Muthusivaramapandian M, Arrivukkarasan S, Aravindan R, Viruthagiri T. Perspectives and Applications of anticancer enzyme L- asparaginase. Asian J Microbiol Biotechnol Environ Sci 2008; 10(4): 851-854.

[3] Cammack KA, Marlborough DI, Miller DS. Physical properties and subunit structure of L-asparaginase isolated from *Erwinia carotovora*. J Biochem 1972; 126: 361-379.

[4] Manna S, Sinaha A, Sadhukhan R, Chakrabarty SL. Purification, characterization and antitumor activity of L-asparaginase isolated from *Pseudomonas stutzeri*. MB-405. Cur Microbiol 1995; 30: 291-298. http://dx.doi.org/10.1007/BF00295504

[5] Abdel-Fatteh Y, Olama ZA. L-asparaginase produced by *Pseudomonas aeruginosa* in solid state culture: evaluation and optimization of culture conditions using factorial designs. Process Biochem 2002; 38: 115-122. http://dx.doi.org/10.1016/S0032-9592(02)00067-5

[6] Qin M, Zhao F. L-asparaginase release from *Escherichia coli* cells with aqueous two-phase micellar systems. Appl Biochem Biotechnol 2003; 110(1): 11-21. http://dx.doi.org/10.1385/ABAB:110:1:11

[7] Bansal S, Gnaneswari P, Mishra P, Kundu B. Structural stability and functional analysis of L-asparaginase from *Pyrococcus furiosus*. Biochem 2010; 75(3): 375-381.

[8] Duval M, Suciu S, Ferster A, Rialland X, Nelken B, Lutz P, Benoit Y, *et al*. Comparison of *Escherichia coli* Asparaginase with *Erwinia* asparaginase in the treatment of childhood lymphoid malignancies. Results of a randomized European organization for research and treatment of cancer-children's leukemia group phase 3 trails. Blood 2002; 99: 2734-2739. http://dx.doi.org/10.1182/blood.V99.8.2734

[9] Hosamani R, Kaliwal BB. L-asparaginase an anti-tumor agent production by *Fusarium equiseti* using solid state fermentation. Inter J Drug Discovery 2011; 3(2): 88-99.

[10] Pieters R, Hunger SP, Boos J, Rizzari C, Silverman L, Baruchel A, Goekbuget N, Schrappe M, Ching-Hon Pui. Cancer 2011; 117(2): 238-249. http://dx.doi.org/10.1002/cncr.25489

[11] Verma N, Kumar K, Kaur G, Anand S. *Escherichia coli* K-12 asparaginase-based asparagine biosensor for leukemia. Artif. Cells Blood Substit. Immobilization Biotechnol 2007; 35: 449-456. http://dx.doi.org/10.1080/10731190701460358

[12] Prakasham RS, Hymavathi M, Subba RC, Arepalli SK, Venkateswara RJ, Kavin KP, Nasaruddin K., *et al*. Evaluation of antineoplastic activity of extracellular asparaginase produced by isolated *Bacillus circulans*. Appl Biochem Biotechnol 2010; 160: 72-80. http://dx.doi.org/10.1007/s12010-009-8679-8

[13] Shah AJ, Karadi RV, Parekh PP. Isolation, optimization and production of L-asparaginase from coliform bacteria. Asian J Biotechnol 2010; 2: 169-177. http://dx.doi.org/10.3923/ajbkr.2010.169.177

[14] Imada A, Igarasi S, Nakahama K, Isono M. Asparaginase and glutaminase activities of microorganisms. J Gen Microbiol 1973; 76: 85-89. http://dx.doi.org/10.1099/00221287-76-1-85

[15] Gulati R, Saxena RK, Gupta RA. Rapid Plate Assay for Screening L-Asparaginase Producing Micro organisms. Lett Appl Microbiol 1997; 24: 23-26. http://dx.doi.org/10.1046/j.1472-765X.1997.00331.x

[16] Kamble KD, Khade PJ. Studies on antineoplastic enzyme producing bacteria from soil. Inter J Pharmaceut Biomed Res 2012; 2: 94-99.

[17] Narayana K. L-asparaginase production by *Streptomyces albidoflavus.* Indian J Microbiol 2007; 48(3): 331-336. http://dx.doi.org/10.1007/s12088-008-0018-1

[18] Borkotaky B, Bezbaruah RL. Production and properties of asparaginase from a new *Erwinia* sp. Folia Microbiol 2002; 47: 473-476. http://dx.doi.org/10.1007/BF02818783

[19] Dhevagi P, Poorani E. Isolation and characterization of L-asparaginase from marine actinomycetes. Indian J Biotechnol 2006; 5: 514–520.

[20] Thaer TA, Ellaiah P. L-asparaginase production by Streptomycete and optimization of production parameters. J Pharmaceut Biomed Sci 2013; 29: 859-869.

[21] Praveen K, Thangabalan B, Venkata R, Vadivel MK, Manohar BS, Srinivasa D. Optimization of parameters for the production of L-asparaginase by *Serratia marcescens*. J Pharmaceut Biomed Sci 2011; 7: 20.

[22] Susmita S, Mandal SK. Production purification and characterization of extracellular Anti-leukaemic L-asparaginase from isolated *Bacillus subtilis* using Solid state fermentation. Inter J Appl Biol Pharmaceut Technol 2013; 4(3): 89-99.

[23] Soniyamby AR, Lalitha S, Praveesh BV, Priyadarshini V. Isolation, production and anti-tumour activity of L-asparaginase of *Penicillium sp*. Inter J Microbiol Res 2011; 2(1): 38-42.

[24] Amena S, Vishalakshi N, Prabhakar M, Dayanand A, Lingappa K. Production, purification and characterization of L-asparaginase from *Streptomyces gulbargensis*. Brazilian J Microbiol 2010; 41: 173- 178. http://dx.doi.org/10.1590/S1517-83822010000100025

[25] Deokar VD, Vetal MD, Rodrigues L. Production of intracellular L-asparaginase from *Erwinia carotovora* and its statistical optimization using response surface methodology (RSM). Inter J Chem Sci Applic 2010; 1: 25-36.

[26] Kenari SLD, Alemzadeh I, Maghsodi V. Production of L-asparaginase from *Escherichia coli* ATCC 11303: Optimization by response surface methodology. Food Bioproduction Process 2011; 86: 315-321. http://dx.doi.org/10.1016/j.fbp.2010.11.002

[27] Savitri NA, Azmi W. Microbial L-asparaginase: A potent antitumour enzyme. Indian J Biotechnol 2003; 2: 184-194

[28] Liu FS, Zajic JE. L-asparaginase Synthesis by *Erwinia aroideae*. Appl Microbiol 1972; 33: 667-668.

[29] Verma N. L-asparaginase: a promising chemotherapeutic agent. Crit Rev Biotechnol 2007; 27: 45-62. http://dx.doi.org/10.1080/07388550601173926

[30] Singh Y, Srivastava SK. Screening and characterization of microorganisms capable of producing antineoplastic drug, L-asparaginase. Inter J Biol Med Res 2012; 3(4): 2548-2554.

[31] Makky EA, Jee-Jian O, Md-Rezaul K, Lee CM. Production and optimization of L-asparaginase by *Bacillus* sp. KK2S4 from corn cob. Afr J Biotech 2013; 12(9): 2654-2658.

[32] Kavitha A, Vijayalakshmi M. A study on L- asparaginase of *Nocardia levis* MK-VL-113. Scientific World Journal 2012; 2012: 1-5. http://dx.doi.org/10.1100/2012/160434

[33] Narayana KJP, Kumar KG, Vijayalakshmi, M. 2008. L-Asparaginase production by *Streptomyces albidoflavus*. Indian J Microbiol 48(3): 331-336. http://dx.doi.org/10.1007/s12088-008-0018-1

Received on 07-11-2014 Accepted on 03-12-2014 Published on 21-01-2015

http://dx.doi.org/10.6000/1927-5129.2015.11.06