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Production and optimization of L-asparaginase in *Escherichia coli*

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L-ASPARAGINASE (L-ASNase) has been widely used as a therapeutic agent in the treatment for various lymphoblastic leukemia diseases. This study aimed to isolate and purify local bacterial isolates that are capable of producing L-ASNase, so 150 bacterial isolates from the Nile River where isolated, purified and their ability to produce L-ASNase was assessed. Among these isolates, 32 bacterial isolates showed their ability to produce L-ASNase, so they were selected for further studies. The most active bacterial isolate in the production of L-ASNase was selected, identified as *Escherichia coli* and named MG27. Enzyme localization was determined in cultures grown under aerobic and anaerobic conditions. *E. coli* MG27 was found to produce more L-ASNase in anaerobic conditions compared with that produced under aerobic conditions with 128 folds. Finally, in an attempt to determine the optimum conditions for periplasmic L-ASNase production, the influence of several cultural factors was investigated.

Keywords: *Escherichia coli* MG27, L-Asparaginase, Optimization experiments.

L-asparaginases (L-asparagine amino hydrolase) catalyze the deamination of L-asparagine to L-aspartic acid and ammonia. Both the substrate and the product of this enzymatic reaction play important roles in a number of metabolic processes in all organisms, from bacteria to mammals. The interest in L-asparaginases was greatly enhanced by the fact that some of these enzymes exhibit anti-tumor activity. L-asparaginase is a high value enzyme with intensive chemotherapeutical uses against a wide variety of tumors especially acute lymphoblastic leukemia (ALL). Recently it gained more importance in food industries for reducing acrylamide formation in heat-processed products (Pedreschi *et al.*, 2011).

Screening of L-asparaginase-producing microorganisms is usually conducted on a medium containing L-asparagine and phenol red. L-asparagine is used as

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the sole nitrogen source. The production of L-asparaginase by a microorganism leads to ammonia formation that increases the pH of the medium, hence the pH indicator, phenol red, turns into pink zones around the colonies producing L-asparaginase (Kumar *et al.,* 2010 and Sudhir *et al.,* 2012).

Gulati *et al.* (1997) reported a quick and semi-quantitative plate assay for screening L-asparaginase-producing microorganisms by using phenol red as a pH indicator. Ghasemi *et al.* (2008) described an optimized sensitive medium for detection of trace amounts of L-asparaginase produced by *E. coli*. Similarly, Sarquis *et al.* (2004) used a modified Czapek-Dox medium supplemented with L-asparagine and incorporated with phenol red in a stock solution prepared in ethanol for the selection of fungal stains with the ability to produce L-asparaginase. Jayaveera *et al.* (2008) isolated fifty fungal strains by pH and dye–based fast procedure using modified Czapek-Dox agar medium contains 1% L-asparagine as sole source of nitrogen and phenol red as pH indicator. Pradeep *et al.* (2010) isolated bacteria from tulsi and different commercial compost production units. In addition, Victoria and Krishnaveni (2010) investigated L-asparaginase activities of *Staphylococcus aureus* from bat faeces. Further screening for the most potent isolate was performed by Nesslerization method. Ebrahiminezhad *et al.* (2011) screened moderate halophilic bacteria, isolated from Maharloo Salt Lake, for L-asparaginase production ability.

Furthermore, marine actinomycetes were isolated from marine sediment samples on seawater complex agar (SWC), starch casein agar (SCA) and glucose asparagine agar. The isolates were screened for asparaginase activity using modified M9 medium incorporated with the pH indicator phenol red. L-asparaginase activity was identified by formation of a pink zone around colonies (Basha *et al*., 2009). In addition, Moorthy *et al*. (2010) reported that modified M9 medium supplemented with few drops of phenol red solution was used for isolation and screening of L-asparaginase producing bacteria from soil.

This study targeted to isolate and screen L-ASNase producing bacteria. In order to study localization of L-ASNase, cellular extracts of evaluated isolates were fractionated into extracellular, periplasmic and cytoplasmic fractions. Enzyme localization was determined in cultures grown under aerobic and anaerobic conditions. Finally, in an attempt to determine the optimum conditions for periplasmic L-ASNase production, the influence of several cultural factors was investigated.

Material and Methods

Isolation of coliform bacteria

For isolation of coliform bacteria, water samples (100 ml) were collected from the River Nile at different sites in Cairo and Giza governorates using sterile screw-cap glass bottles. Sterile MacConkey broth purple was inoculated with 1ml of water sample and incubated at 37℃ for 24 hr. Turbid yellow tubes showing gas production were selected and the presence of coliform bacteria was

confirmed by streaking Eosin-Methylene blue (EMB) agar with inocula from positive MacConkey broth purple tubes, and plates were incubated at 37℃ for 24 hr. Dark colonies with metallic green sheen were picked up with sterile inoculating needle and re-streaked on new EMB plates to obtain pure colonies. Single colonies were sub-cultured on Luria-Bertani (LB) slants and maintained at 4℃ for later use.

Screening of L-asparaginase producing bacteria

One hundred and fifty bacterial isolates were evaluated for their ability to produce L-asparaginase according to rapid plate assay procedure (Gulati *et al.*, 1997).

Inoculum preparation and growth conditions

The primary inoculum (starter culture) was prepared by adding a loopful of freshly prepared pure culture into 50 ml of sterile LB broth in a 250 ml flask and incubated at 37ºC and 180 rpm in a shaking incubator for 24 hr. Two percent (v/v) of this starter culture was inoculated into the production medium. Aerobic growth was carried out in 250 ml Erlenmeyer flasks dispensed with 50 ml of the production medium(LB broth) and inoculated with 1 ml (2% v/v) of the starter culture and incubated at 37°C in a shaking incubator (200 rpm).

Anaerobic cultures were grown in test tubes as static cultures in anaerobic jars (Torsion Balance Co., Clifton, N.J.) under a mixture of 90% nitrogen and 10% carbon dioxide gas at a pressure of 3 psi (Cedar and Schwartz, 1968).

Localization of L-asparaginase

In order to determine the localization of L-asparaginase, bacterial isolates were grown for 24 hr in mineral-salts medium A (Davis and Mingioli, 1950) and modified mineral-salts medium A (Gorini, 1961) under aerobic and anaerobic conditions, respectively. Extracellular, periplasmic and cytoplasmic fractions were prepared and assayed for L-asparaginase activity.

Preparation of extracellular fraction

Preparation of extracellular fraction was conducted as described by Kumar *et al*. (2010).

Preparation of periplasmic fraction

The pellet was used for the extraction of periplasmic fluid using osmotic shock method (Hong *et al*., 2007).

Preparation of cytoplasmic fraction

Following periplasmic fractionation, cytoplasmic fractions were prepared by sonication (Rockstroh *et al.*, 2011). Periplasmic proteins were isolated by osmotic

shock as previously described. The periplasmic release method was carried out according to the method of Pierce *et al.* (1997). Finally, Potassium phosphate/solvent permeabilization was performed as the method described by Geckil *et al.* (2005).

L-Asparaginase assay

Enzyme activity was measured using Nesslerization reaction (Prager and Bachynsky, 1968) One unit of L-asparaginase is defined as the amount of enzyme that liberates 1.0 µmole of ammonia from L-asparagine per minute at pH 8.6 at 37°C (Peterson and Ciegler, 1969).

Protein Estimation

Total protein contents were estimated according to method described by Lowry *et al*. (1951).

Rapid confirmation of L-asparaginase by TLC method

Primarily screened isolates were subjected to thin layer chromatography (TLC) for the confirmation of L-asparaginase production according to method described by Arima *et al*. (1972). The enzyme activity was confirmed by detection of the produced aspartic acid by the redness of the spot developed by ninhydrin reagent.

Identification of The most active isolate

The most active isolate in periplasmic L-asparaginase activity was identified according to standard physiological and biochemical identification tests as described in Bergey's Manual of Systematic Bacteriology (Holt *et al*., 1994). Further biochemical tests were performed using API 20E kit (Biomerieux, France) according to the instructions of the manufacturer. The biochemical tests investigated with API 20E were: beta-galactosidase (ONPG), arginine dihydrolase (ADH), lysine decarboxylase (LDC), ornithine decarboxylase (ODC), citrate utilization (CIT), H_2S production (H_2S), urease (URE), tryptophane deaminose (TDA), indole production (IND), Voges-Proskauer (VP), gelatinase (GEL), utilization of glucose (GLU), mannitol (MAN), inositol (INO), sorbitol (SOR), rhamnose (RHA), saccharose (SAC), melibiose (MEL), amygdalin (AMY), and arabinose (ARA). Cytochrome oxidase test (OX) was done separately. Results were analyzed using the bioMérieux apiweb system database.

Optimization of cultural conditions for L-asparaginase II production

All optimization experiments were performed under anaerobic conditions. The production media were inoculated with *E. coli* MG27 starter culture (2% v/v) and incubated statically in test tubes in anaerobic jars at a pressure of 3 psi under a mixture of N_2 and CO_2 (90% and 10%, respectively). At the end of incubation period, cells were collected, periplasmic enzymes were released by osmotic shock method and the specific activity of the periplasmic L-asparaginase II was assayed. All experiments were conducted in triplicates and the mean values were calculated.

Effect of various nitrogen sources

The impact of different nitrogen sources on L-asparaginase II production was performed using modified mineral-salts medium A as basal medium. The basal medium was supplemented with 1% (w/v) asparagine, peptone, corn steep liquor, casein hydrolysate or yeast extract as nitrogen source, separately. *E. coli* MG27 inoculated and the cultures were incubated anaerobically at 37ºC for 30 hr.

Effect of various carbon sources

To test the effect of different types of carbon sources on L-asparaginase II production, glucose, galactose, xylose, maltose or galactose were incorporated into LB broth at final concentration 1% (w/v). *E. coli* MG27 was inoculated and incubated anaerobically at 37ºC for 30 hr, then cells were collected and assayed for periplasmic L-asparaginase activity.

Statistical analysis

The measured data were subjected to the analysis of variance (ANOVA) appropriate to the design. The significant differences between treatments were compared with the critical difference at 5% level of probability by the Duncan's test using PASW 17.0 statistics software (SPSS Inc).

Results

Isolation of coliform bacteria

The presumptive positive turbid yellow tubes showing gas production were selected. Dark colonies with metallic green sheen were picked from EMB agar plates streaked from previously positive MacConkey broth purple tubes. After purification by re-streaking onto new EMB plates, 150 colonies were isolated and maintained on LB slants at 4°C.

Screening of L-asparaginase producing bacteria

One hundred and fifty coliform isolates were screened for L-ASNase production ability. The potential isolates were selected on the basis of pink zone around the colony by plate assay method (Fig. 1). From 150 bacterial isolates, thirty two isolates demonstrated wide pink zone around colonies, indicating good L-ASNase activity, so they were selected for further studies.

Localization of L-asparaginase under aerobic and anaerobic conditions

L-ASNase activity was assayed in extracellular, periplasmic and cytoplasmic fractions prepared from coliform isolates grown under aerobic and anaerobic conditions. Results showed that, no trace amounts of L-ASNase were observed in the culture filtrates of all bacterial isolates indicating that L-ASNase was not produced extracellularly under neither aerobic nor anaerobic conditions (Table 1).

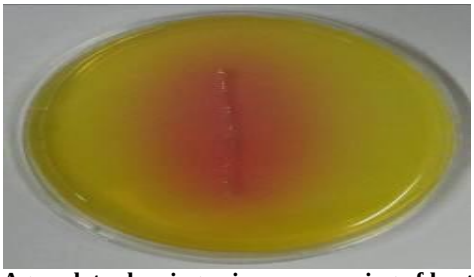

Fig. 1. Modified M9 Agar plate showing primary screening of bacterial isolate for L-asparaginase production. Modified M9 medium, supplemented with phenol red as a pH indicator, was inoculated with bacterial isolate and incubated at 37°C for 24 hr. The isolate that produced pink zone around colony indicated L-ASNase production.

TABLE 1. Localization of L-Asparaginase enzyme under aerobic and anaerobic conditions. Bacterial isolates grown under aerobic conditions (a) and anaerobic conditions (b) at 37°C for 24 hr. Cells were harvested by centrifugation and extracellular, periplasmic and cytoplasmic fractions were prepared and assayed for L-ASNase activity.

Isolate	L-Asparaginase activity (U/mg protein)					
	Aerobic conditions (a)			Anaerobic conditions (b)		
	Extracellular	Periplasmic	Cytoplasmic	Extracellular	Periplasmic	Cytoplasmic
MG1	-	0.0062	3.25	-	0.176	3.68
MG2	-	0.0094	2.84	-	0.618	2.45
MG3	-	0.0260	0.54	-	0.911	0.85
MG4	-	0.0047	2.15	-	0.467	1.95
MG5	-	0.0124	7.65	-	0.883	8.05
MG6	-	0.0115	4.45	-	0.586	4.86
MG7	-	0.0394	3.55	-	1.076	3.72
MG8	-	0.0044	1.66	-	0.094	1.38
MG9	-	0.0087	2.75	-	0.684	3.08
MG10	-	0.0279	2.64	-	1.024	2.29
MG11	-	0.0147	3.22	-	1.313	3.28
MG12	-	0.0098	5.10	-	0.245	4.81
MG13	-	0.0551	6.24	-	1.143	6.56
MG14	-	0.0862	1.38	-	1.266	1.59
MG15	-	0.0146	4.66	-	0.842	4.92
MG16	-	0.0483	1.60	-	0.992	1.83
MG17	-	0.0092	4.78	-	0.484	4.48
MG18	-	0.0028	8.55	-	0.673	8.62
MG19	-	0.0048	3.47	-	0.748	3.41
MG20	-	0.0076	4.86	-	0.668	4.33
MG21	-	0.0209	2.15	-	1.149	2.46
MG22	-	0.0096	7.28	-	0.972	7.17
MG23	-	0.0144	2.63	-	0.525	2.47
MG24	-	0.0068	3.74	-	0.687	3.55
MG25	-	0.0211	4.81	-	0.955	5.19
MG26	-	0.0072	6.14	-	0.274	6.47
MG27	**-**	**0.0146**	**3.11**	**-**	**1.871**	**2.84**
MG28	-	0.0093	4.05	-	0.762	4.66
MG29	-	0.0148	6.44	-	1.088	6.03
MG30	-	0.0071	0.69	-	0.821	0.92
MG31	-	0.0186	1.53	-	1.155	1.79
MG32	-	0.0042	6.24	-	0.281	6.84

Periplasmic proteins were permeabilized using osmotic shock method. Periplasmic L-ASNase was produced in very low amounts under aerobic conditions while it was produced in significant amounts under anaerobic conditions. The specific activity of the periplasmic L-ASNase varied from 0.0028 to 0.0862 µ/mg of protein under aerobic conditions. Whereas, the specific activity of the periplasmic enzymes was much higher in the bacterial isolates that cultivated under anaerobic conditions compared with those grown under aerobic conditions. The isolate designated MG27 cultivated under anaerobic conditions showed 128-fold increase in periplasmic L-ASNase activity when compared to the enzyme activity produced under aerobic conditions (1.871 & 0.0146 µ/mg protein, respectively). Therefore, the isolate MG27 was selected for identification and further studies.

No significant difference was observed in the activity of the cytoplasmic L-ASNase when bacteria were grown under aerobic and anaerobic conditions. Results revealed that, L-ASNase activities in cytoplasmic fractions were almost not affected by the difference in aeration levels which was different from periplasmic fractions.

Rapid confirmation of L-asparaginase by TLC method

The TLC technique was used for the separation and identification of aspartic acid produced by crude extracts prepared from coliform isolates. Aspartic acid was detected roughly by redness of the spot developed by spraying with Ninhydrin reagent (Fig. 2).

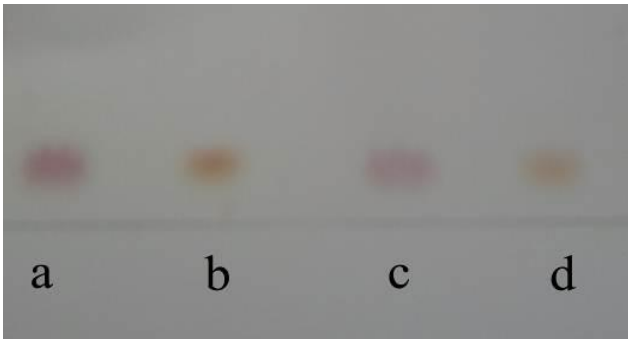

Fig. 2. Rapid confirmation of L-asparaginase by TLC method. a- Positive L-Asparaginase test, b- Negative L-Asparaginase test, c- Positive control, d-Negative control. L-ASNase activities were checked in periplasmic and cytoplasmic extracts by TLC method. Silica gel plates were chromatographed in n-butanol-acetic acid-water solvent system (3:1:1), sprayed with ninhydrin solution and dried in hot air oven at 105°C. The enzyme activity was confirmed by redness of the spots developed by the enzyme product, aspartic acid.

Identification of the most active isolate

Based on the obtained results, the most active isolate in the production of periplasmic L-ASNase (MG27) was selected and identified by extensive phenotypic characterization using methods detailed in Bergey's Manual of Systematic Bacteriology (Holt *et al*., 1994). Microscopic examination of stained smear indicated that this isolate was Gram-negative and rod-shaped. The isolate MG27 showed positive response to indole production, methyl red, lysine decarboxylase. The isolate showed negative results to oxidase, Voges-Proskauer, citrate utilization. Further characteristics for the isolate MG27 are given in Table 2. Depending on its characteristics, the isolate MG27 was identified as *Escherichia coli*.

The identification of the isolate MG27 was confirmed by the standardized API 20E strip System. According to the results presented in Table 3, the isolate was identified as *E.coli*

Optimization of cultural conditions

Effect of incubation period

The organism under test was incubated at 37°C and samples were withdrawn every 2hr and analyzed for the enzyme activity. Results revealed that, no significant production of the enzyme was observed at the first 13 hr but the enzyme activity was increased rapidly after 15 hr of incubation. This increase was continued till 25 hr then no significant increase in the activity level was observed up to 39 hr. During this period maximum activity was achieved. After 39 hr incubation, the enzyme activity decreased significantly (Fig. 3).

Effect of incubation temperature and pH

The results showed that the optimal temperature for periplasmic L-ASNase production was 37°C. Further increase or decrease in the temperature resulted in reduction of enzyme production (Fig. 4). Strong influence of initial pH on enzyme activity was observed. Among the different pHs tested, pH 7.5 was found optimum for L-ASNase activity which was four-fold higher than that obtained at pH 6.0 (Fig. 5). Alkaline pHs were also inhibitory to the enzyme activity with minimum value at pH 9

Effect of various nitrogen sources and concentration

Results represented in (Fig. 6), showed that corn steep liquor and casein hydrolysate were the best nitrogen sources for L-ASNase activity when used separately. More than five-fold enhancement in L-ASNase activity was achieved when corn steep liquor or casein hydrolysate was used as compared to that obtained in the basal medium. The substrate of the enzyme (asparagine) did not induce any significant enzyme activity compared to the control.

TABLE 2. Biochemical tests for identification of isolate MG27 .

Biochemical Test		Reaction
Oxidase		-
Indole production		+
Methyl red		+
Voges-Proskauer		-
Citrate utilization (Simmons)		-
Hydrogen sulfide production		-
Urea hydrolysis		-
Phenylalanine deaminase (24h)		-
Lysine decarboxylase		+
Arginine dihydrolase		-
Gelatin hydrolysis (22ºC)		-
KCN, growth		-
Malonate utilization		-
$_{D-}$Glucose , acid production		+
$_{D-}$Glucose , gas production		+
Acid production	$_{D-}$Adonitol	-
	$_{L-}$ Arabinose	+
	Cellobiose	-
	myo-Inositol	-
	Lactose	+
	Maltose	+
	$_{D-}$Mannitol	+
	$_{D-}$Mannose	+
	Melibiose	+
	α -Methyl-D-glucoside	-
	$_{L-}$Raffinose	+
	$_{D-}$Sorbitol	+
	Sucrose	-
	Trehalose	+
	$_{D-}$Xylose	+
	Mucate	+
Tartrate, Jordans		+
Acetate utilization		+
Nitrate reduction		+
Deoxyribonuclease (25ºC)		-
Lipase		-
ONPG		+
Pigment		-
Catalase production		+
Oxidation-Fermentation		**F**

TABLE 3. Biochemical profile of isolate MG27 using identification system API 20E. The isolate MG27 was identified as *E. coli* belonged to biochemical profile (API code 5044552).

ONPG	ADH	LDC	ODC	CIT	H_2S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	OX
+	-	+	-	-	-	-	-	+	-	-	+	+	-	+	+	-	+	-	+	-
1	0	4	0	0	0	0	0	4	0	0	4	1	0	4	1	0	4	0	2	0
5			0			4			4			5			5			2			

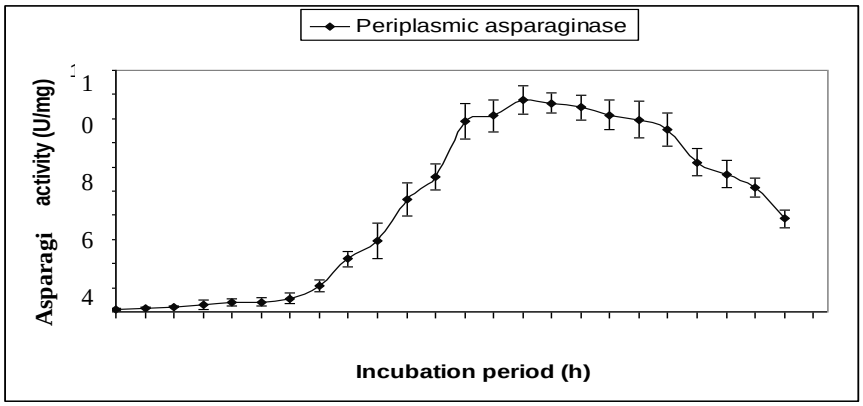

Fig. 3. Effect of incubation period on L-Asparaginase II activity.
- Error bars represent standard deviations (SD).

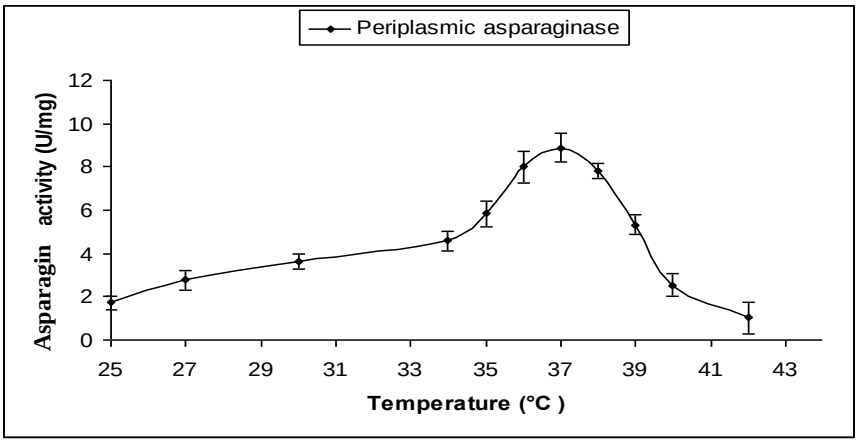

Fig. 4. Effect of incubation temperature on L-Asparaginase II activity.
- Error bars represent standard deviations (SD).

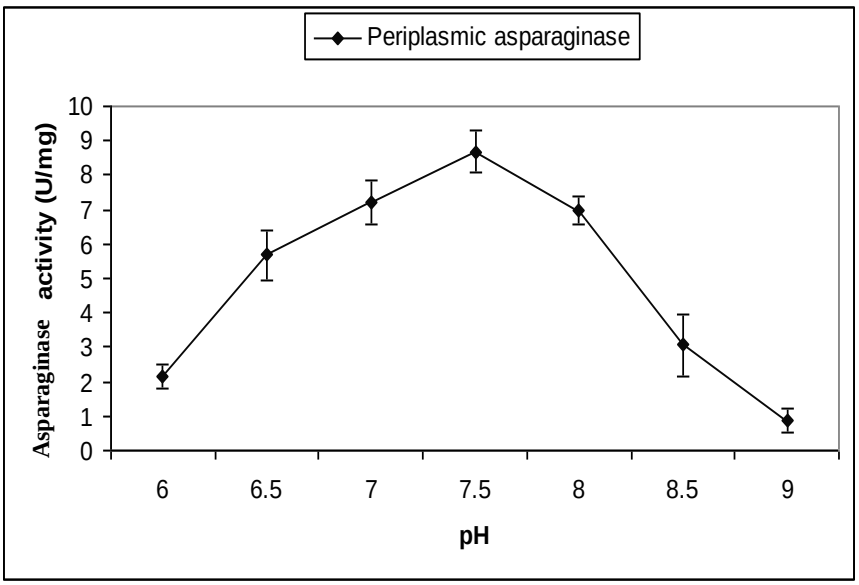

Fig. 5. Effect of initial pH on L-Asparaginase II activity.
- Error bars represent standard deviations (SD).

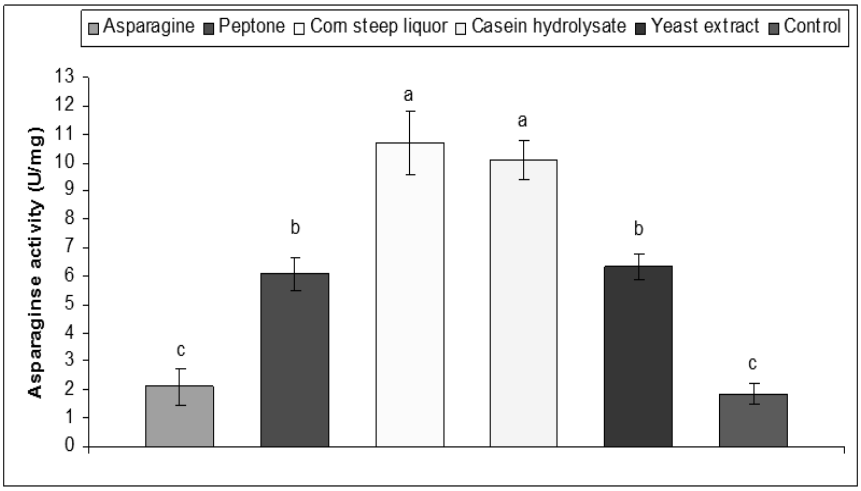

Fig. 6. Effect of various nitrogen sources on L-Asparaginase II activity.
Columns headed by the same letter were not significantly different according to Duncan's multiple range test ($p < 0.05$). Error bars represent standard deviations (SD).

Effect of various carbon sources and concentration: Results represented in Fig. 7 showed that, the enzyme activity was significantly suppressed when either glucose or maltose was used as carbon source. Either glucose or maltose caused more than 60% reduction in the specific activity of L-ASNase II compared to the control culture grown on LB only. This suppression effect was also recorded when xylose was used but with less extent about 38% suppression than the control. Galactose and lactose, however, showed less inhibitory effect on the specific activity of L-ASNase compared to the control. Remarkable decrease in the enzyme specific activity was observed as glucose concentration increased (Fig.8).

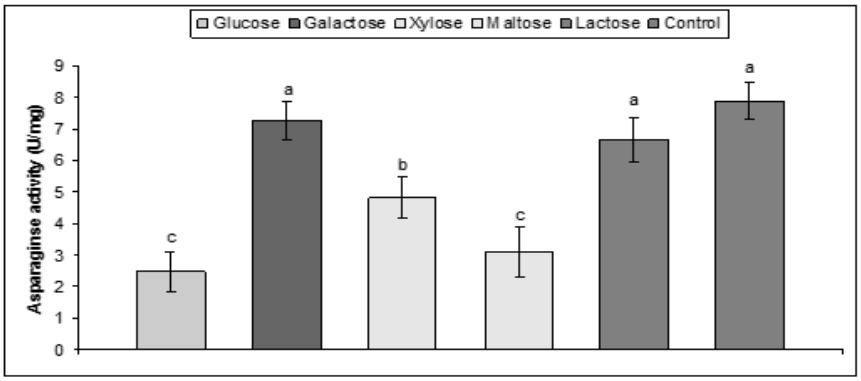

Fig. 7. Effect of various carbon sources on L-Asparaginase II activity.

- Columns headed by the same letter were not significantly different according to Duncan's multiple range test ($p < 0.05$). Error bars represent standard deviations (SD).

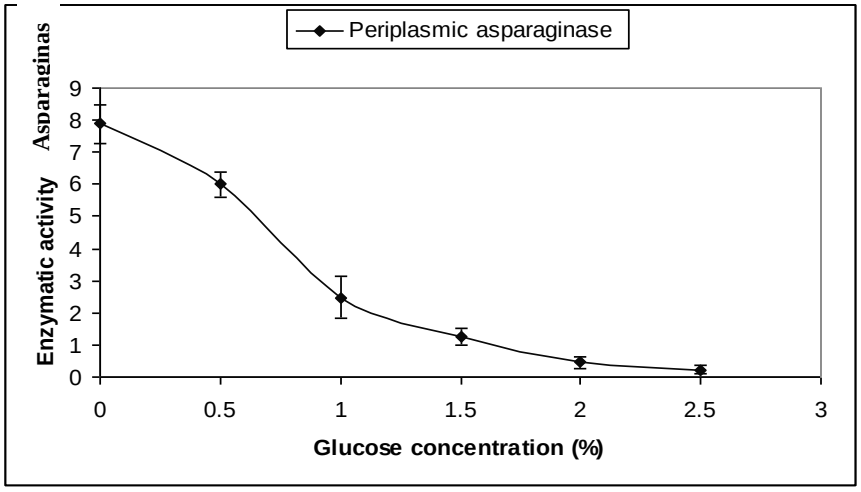

Fig. 8. Effect of glucose concentration on L-Asparaginase II activity.

- Error bars represent standard deviations (SD).

Permeabilization of periplasmic proteins

Figure 9, showed that the maximum level of release was achieved by lysozyme/EDTA method (14.65 U/mg) followed by hexane and toluene methods (11.97 U/mg and 9.95 U/mg, respectively). Xylene treatment released 5.49 U/mg L-ASNase and considered as the poorest enzyme releaser.

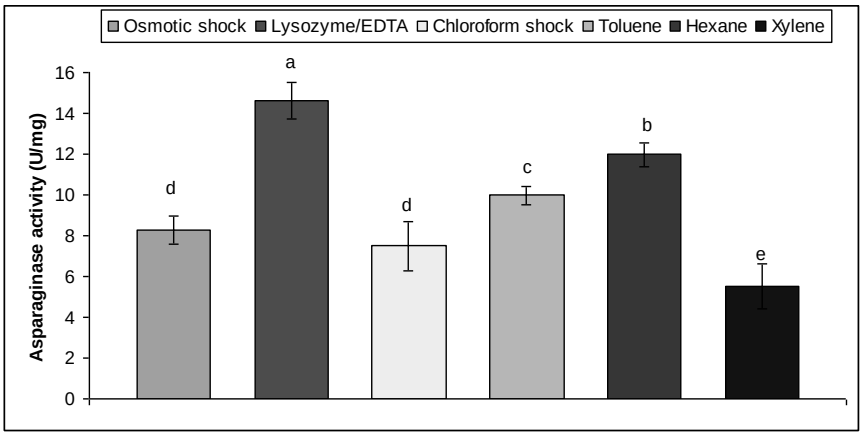

Fig.9. Effect of various permeabilization methods on the release of periplasmic L-asparaginase II.

- Columns headed by the same letter were not significantly different according to Duncan's multiple range test ($p < 0.05$). Error bars represent standard deviations (SD).

Discussion

Studying L-Asparaginase (L-ASNase) has recently gained much attention for its anti-carcinogenic potential. Several authors documented the use of L-ASNase in cancer therapy (Pieters *et al*., 2011 and Tong *et al*., 2013). Although L-ASNases are present in many plants, mammalian and bacterial species, only the enzymes from *Escherichia coli* and *Erwinia chrysanthemii* have been produced on industrial scale as chemotherapeutics in acute lymphoblastic leukemia. This is due to their high catalytic activity and specificity towards L-asparagine (Aghaiypour *et al*., 2001 and Duval *et al*., 2002). In addition to the therapeutic use, L-ASNase has a potent application in food industry to reduce acrylamide formation in heat-processed products (Pedreschi *et al*., 2008 and Kukurová *et al*., 2013).

The first step in this study targeted to the isolation and screening of L-ASNase producing bacteria. From the 150 bacterial isolates screened, 32 had L-ASNase activity. The rapid plate assay was reported for screening of various L-

ASNase producing strains such as *Staphylococcus aureus* (Victoria and Krishnaveni, 2010), *Bacillus circulans* (Prakasham *et al*., 2010), *Streptomyces* sp. ABR2 (Sudhir *et al.*, 2012) and *Streptomyces* sp. (Basha *et al*., 2009).

In order to study localization of L-ASNase, cellular extracts of evaluated isolates were fractionated into extracellular, periplasmic and cytoplasmic fractions in cultures grown under aerobic and anaerobic conditions. These results suggested that the enzyme was located as an intracellular product in all tested isolates. The obtained results were agreed with those reported by Kumar *et al*. (2010). The majority of bacterial L-ASNases are intracellular in location (Kenari *et al*., 2011 and Narta *et al*., 2011). In contrast, extracellular L-ASNases were produced by *Bacillus sp*. BCCS 034 and *Bacillus subtilis* strain hswx88 (Ebrahiminezhad *et al*., 2011 and Pradhan *et al*., 2013).

The results of the present study revealed that periplasmic L-ASNase was better in anaerobic than aerobic conditions. For the isolate designated MG27, anaerobiosis induced about 128-fold increase in periplasmic L-ASNase activity. Jennings and Beacham (1990) reported that the production of *E. coli* periplasmic L-ASNase II is regulated by the oxygen-sensitive FNR protein, which activates a number of genes during anaerobiosis. It has been suggested that the periplasmic L-ASNase II probably has a special function in anaerobic fumarate respiration. It provides aspartate, which is then converted to fumarate that functions as an electron acceptor (Fisher and Wray, 2002).

L-ASNase production was confirmed using TLC method for separation and identification of aspartic acid. Several authors reported TLC method to confirm the production of L-ASNase using ninhydrin as detecting agent (Jayaramu *et al*., 2010 and Shah *et al*., 2010).

Based on screening results, the isolate MG27 showed the highest periplasmic L-ASNase activity compared to the rest of the thirty two isolates. Thus, it was selected for identification and for further studies. The results of classical identification methods suggested that isolates MG27 was identified as *Escherichia coli* on the bases of its morphological, physiological and biochemical characteristics and confirmed by API 20E results. Various authors reported that L-ASNase was produced by different strains of *E. coli* (Chung *et al.*, 2010 and Kenari *et al.*, 2011).

In an attempt to determine the optimum conditions for periplasmic L-ASNase production, the influence of seven cultural factors was investigated. In the current work, incubation period had a significant role in the optimization process for periplasmic L-ASNase activity. Maximum activity was obtained after 25 hr and observed to be stable till the 39^{th} hr of incubation. Previous studies dealing with L-ASNase production indicated that maximum activity in *Citrobacter* sp. and *Erwinia carotovora* was after 26 and 36 hr, respectively (Shah *et al*., 2010 and Deokar *et al*., 2010).

The optimum temperature for periplasmic L-ASNase activity was observed at 37°C. This result is consistent with the results obtained by Mahajan *et al*. (2012) who reported 37°C as the optimum temperature for L-ASNase activity from *Bacillus licheniformis*. Similar results were recorded for L-ASNase activity from *Citrobacter* sp. (Shah *et al*., 2010). Results of the present study also showed a feasible production of periplasmic L-ASNase within pH range of 7.0-8.0 with a maximum production at pH 7.5. These results are in agreement with those obtained by many workers who reported maximum L-ASNase activity at pH 7.5 by *Staphylococcus* sp. – 6A. (Prakasham *et al*., 2007), *Streptomyces albidoflavus* (Narayana *et al*., 2008) and *Bacillus aryabhattai* strain ITBHU02 (Singh and Srivastav, 2013).

As shown in results, more than five-fold enhancement in L-ASNase activity was achieved when corn steep liquor or casein hydrolysate was used, which are in agreement with the findings of Abdel-Fattah and Olama (2002). They reported that casein hydrolysate and corn steep liquor were the most significant nitrogen sources improving L-ASNase activity by *Pseudomonas aeruginosa*. In a similar experiment conducted on *Erwinia carotovora*, the maximum activity of L-ASNase was recorded when corn steep liquor was used, compared to other assessed nitrogen sources as yeast extract, peptone and tryptone (Warangkar and Khobragade, 2009). This stimulatory effect may be because casein hyrolysate and corn steep liquor contain substances other than amino acids as co-factors, vitamins and growth factors that may be involved in L-ASNase activity.

The highest L-ASNase activity was in control medium. Lactose and galactose gave the lowest inhibitory effect (…….% compared to control). The results are in contradictory to the other studies in which lactose was recorded as the best carbon source in various studies in microbial production of L-ASNases (Warangkar and Khobragade and 2009, Kenari *et al.*, 2011). In a similar experiment, *Enterobacter cloacae* grown on galactose had the highest L-ASNase activity (Nawaz *et al.*, 1998). In addition, galactose was found as the best carbon source for production of L-ASNase by *Pectobacterium carotovorum* MTCC 1428 (Sanjeeviroyar *et al.*, 2010).The results clearly showed that glucose had an the maximum inhibitory effect on enzyme activity. This finding is consistent with the results obtained by Geckil *et al.* (2004). They reported that *Enterobacter aerogenes* grown in LB with 1% glucose showed about 20-fold decrease in L-ASNase activity compared with the same strain grown in LB only. Usually, glucose was considered a repressor for L-ASNase production in bacteria (Warangkar and Khobragade, 2009, Kenari *et al*., 2011 and Makky *et al*., 2013). This may be due to catabolite repression and catabolite inhibition of the components involved in lactate transport (Garaev and Golub, 1977). Previous studies suggested that glucose reduced the enzyme activity by lowering the medium pH beside acting as a repressor (Mukherjee *et al*., 2000, Geckil and

Gencer, 2004). Results for the determination the best method for release of periplasmic L-ASNase revealed that lysozyme/EDTA treatment was the most efficient method for recovery of periplasmic L-ASNase. This agreed with Pierce *et al*. (1997) who reported improved release of a periplasmic enzyme in *E. coli* cells treated with lysozyme. Lysozyme catalyzes the degradation of *N*-acetylmuramic acid-*N*-acetylglucosamine bonds (NAM-NAG) in the peptidoglycan layer of cell wall. EDTA chelates Mg^{2+} ions of the outer membrane and destabilizing it. In the presence of EDTA, lysozyme penetrates the outer membrane of *E. coli* cells and hydrolyzes the NAM-NAG bonds of peptidoglycan layer causing breaking up the cell wall peptidoglycan. Hence, the formed spheroplasts release the contents of the periplasm (Witholt *et al.*, 1976).

In this study, periplasmic L-ASNase was successfully recovered by all assessed potassium phosphate/solvent systems but with lower efficacy compared with lysozyme/EDTA method. Hexane was the most efficient solvent system for permeabilization of periplasmic L-ASNase while xylene was the poorest enzyme releaser. Similar results were obtained by Geckil *et al*. (2005). They presented a recovery procedure for periplasmic L-ASNase from two distinctly related Gram-negative bacteria, *Pseudomonas aeruginosa* and *Enterobacter aerogenes*. They showed that the most efficient system for enzyme recovery was 50 mM potassium phosphate with 1% hexane. It has been suggested that hexane is the smallest molecule compared to other evaluated solvents. Thus, it penetrates more efficiently into the outer cell membrane, resulting in disorganization of the outer membrane sufficient enough to cause the secretion of periplasmic proteins. Retained specific activity was also suggested to relate to the dielectric and hydrophobic properties of hexane (De León *et al*., 2003 and Geckil *et al.*, 2005).

In conclusion, isolate MG 27 of *E. coli*, out of 32 bacterial isolates, showed maximum production of periplasmic L-ASNase. Its L-ASNase activity was maximized by optimizing the cultural conditions.

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إنتاج وتعظيم الاستفادة من ل-أسباراجيناز في ألإيشيريشيا كولاي

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لقي الأسباراجيناز إهتماما متزايدا في الأونة الأخيرة و ذلك لقدرتة المضادة للسرطان حيث يعتبر حجرالزاوية في بروتوكولات علاج سرطان الدم الليمفاوي الحاد. هدفت هذة الدراسة إلى عزل و تنقية عزلات محلية قادرة على إنتاج إنزيم الأسباراجيناز، حيث تم عزل و تنقية ١٥٠ عزلة بكتيرية من نهر النيل و تم مسح قدرتها على إنتاج الأسباراجيناز. من بين هذة العزلات، أظهرت ٣٢ عزلة قدرتها على إنتاج الأسباراجيناز فتم إختيارها لمزيدا من الدراسات. تم اختيار العزلة الاكثر نشاطا في إنتاج إنزيم الأسباراجيناز بين الغشائين الخلويين و تم تعريفها على أنها ألإيشيريشيا كولاي و تسميتها MG27 .تم تحديد التواجد الخلوي للإنزيم في المستنبتات البكتيرية تحت الظروف الهوائية و اللاهوائية. وجد أن العزلة المسماة MG27 انتجت ١٢٨ ضعف الإنزيم في الظروف اللاهوائية مقارنة بإنتاجة في الظروف الهوائية..أخيرا تم تقييم الطرق المختلفة لزيادة نفاذية الجدار الخلوي و ذلك لتحديد أكثر الطرق فعالية لإستخلاص الإنزيم من بين الغشائين الخلويين.