Production, Immobilization and Anti-tumor Activity of L-Asparaginase of Bacillus sp R36

Maysa E- Moharam [*1], Amira M. Gamal-Eldeen [2] and Sanaa T. El-sayed [3]

[1] Microbial Chemistry Department, [2] Cancer Biology Laboratory, Center of Excellence for Advanced Sciences, [3] Biochemistry Department, , National Research Center, Dokki, Giza, Egypt
[*]email: maysa12000@hotmail.com

Abstract L-asparaginase is one of the known drugs in the treatment of cancer, especially acute lymphoblastic leukemia. In recent years several bio-conjugation protocols have been developed to improve the pharmacokinetic and immunological properties of anti-leukemic enzyme, L-asparaginase. In this study, fifty two bacilli species were newly isolated in our laboratory and screened for their ability to produce extracellular and intracellular L-asparaginase enzyme. *Bacillus* sp R36 gave the highest intracellular enzyme production. Formation physiology of the enzyme revealed that optimum culture conditions were 9:1 of air::medium ratio, with 55×10^5 CFU/mL inoculums size. The optimum incubation period was found to be 24 hours under shaking growth conditions. The initial pH value 5.6 was favorable for the highest enzyme production. Addition of 1% lactose or 1% raffinose resulted in a doubled enzyme productivity (yielded 204% and 209%, respectively). The enzyme was efficiently immobilized by covalent binding with activated carbon. Immobilized L-asparaginase activity was 33.0 U/g carrier; with immobilization yield of 73.6%. Characterization of the enzyme was performed on native and immobilized forms. Optimum pH value was 7.0 for free and immobilized forms. Optimum reaction temperature was 50 °C for native enzyme, while it was 60 °C for the immobilized enzyme preparation. The immobilization process greatly enhanced the thermal stability of the enzyme. Native L-asparaginase enzyme exhibited thermal stability up to 50 °C, while immobilized form retained 100% of its activity up to 80 °C. Anti-tumor and antioxidant activities were investigated. The enzyme inhibited the growth of two human cell lines including hepatocellular carcinoma (Hep-G2) and colon carcinoma (HCT-116) with IC_{50} value of 112.19 μg/mL and 218.7 μg/mL, respectively. [Journal of American Science 2010;6(8):131-140]. (ISSN: 1545-1003).

Keywords: -asparaginase, *Bacillus*, immobilization, anti-tumor effect.

1. Introduction

L-asparaginases (L-asparagine amido-hydrolase EC 3.5.1.1) hydrolyze L-asparagine to L-asparate and ammonia (Fig. 1a). Bacterial L-asparaginases are enzymes of high therapeutic value due to their use in certain kinds of cancer therapies, mainly in acute lymphoblastic leukemia (ALL) [1-3]. Many bacteria contain two L-asparaginases, a high affinity periplasmic enzyme and a low affinity cytoplasmic enzyme. These enzymes in some bacteria accumulate mainly in periplasmic space [4, 5 and 6]. In *Escherichia coli* and many other bacteria, synthesis of cytoplasmic asparaginase I is constitutive, while expression of periplasmic asparaginase II is activated during anaerobiosis. Further, only the type II enzyme has shown substantial anti-tumor activity [7]. An ideal method for the release of this enzyme would be rapid, inexpensive, gentle and compatible with downstream steps of its purification [8].

The anti-leukemic effect of L-asparaginase is a result of rapid and complete depletion of the circulating pool of L-asparagine as in a great number of patients with lymphoblastic leukemia, the malignant cells depend on exogenous source of L-asparagine to be able to survive, mean while, the normal cells are able to synthesize L-asparagine [3, 9]. The discovery of new L-asparaginase serologically different but having similar therapeutic effects is highly desired [10]. One approach to achieve improved function and properties of the enzyme is its chemical modification (immobilization) with various kinds of biocompatible polymers. Immobilization of enzymes is one of the important trends and goals of biotechnology. Enzymes have an enormous potential as biocatalysts in a wide range of industries and medicine. They offer a distinct advantage due to their specificity, high catalytic efficiency at low temperature and being biodegradable. [11] The use of immobilized enzymes lowers production costs as these can be readily separated from reaction mixture and hence can be used repeatedly and continuously. Several different methods have been employed for enzyme immobilization which includes adsorption onto insoluble materials, entrapment in polymeric gels,

encapsulation in membranes, cross linking with bifunctional or multifunctional reagents and linking to an insoluble carrier [12].

Activated carbon is a form of carbon that has been processed to make it extremely porous and thus to have a very large surface area available for chemical reactions. One gram of activated carbon has a surface area of approximately 500 m^2 with 1500 m^2 being readily achievable. Powdered activated carbon is made in particular form less than 1.0mm in size with an average diameter between 0.15 and 0.25 mm. Activated carbon is used to treat poisonings and overdoses following oral ingestion. It is thought to bind to poison and prevent its adsorption by the gastrointestinal tract. The present work introduces the production (from *Bacillus* sp R36), the physiology, the characterization, and the anti-tumor activity of a new L-asparaginase enzyme (EC 3.5.1.1) in its native and immobilized forms.

2. Material and Methods
Chemicals
Anhydrous L-asparagine, trichloroacetic acid (TCA), Nessler reagent chemicals (HgI2, KI, and sodium hydroxide), Chitin and hexane were purchased from Sigma Chemicals Co. Activated carbon, celite, carboxymethyl cellulose; silica gel and tricalcium phosphate were from Merck Chemicals. All other chemicals used were of analytical grade.

Isolation of Bacteria
One Gram of soil was transferred to a vial containing 10 mL of sterile water and kept on a rotary shaker at 100 rpm for 30 min. The bacterial suspension was pasteurized by heating at 65°C for 15 min. The supernatant was diluted 10-folds and 0.1 mL was spread on pre-solidified nutrient agar medium composed of 5 g peptone, 3 g beef extract and 15 g agar per liter of distilled water. The plates were incubated at 30°C for 48 h and bacterial colonies were purified on nutrient agar. Each of the purified colonies was then sub-cultured on nutrient agar slants.

Culture conditions and membrane permeabliztion
Bacterial isolates were screened for L-asparaginase activity on Luria – Bertani (LB) medium containing (1-1) 10 g peptone, 5 g yeast extract and 10 g NaCl at pH 7.0. A 1/100 inoculum of overnight cultures grown in LB medium was made in 40 mL LB medium in 250 mL Erlenmeyer conical flasks and incubated for 24h at 30°C on a 200 rpm orbital rotary shaker. Cells, cultivated for L-asparaginase production, were harvested by centrifugation (6000 xg for 15 min), washed once with 0.05 mole potassium phosphate buffer pH 8.6,

and re-suspended to A 600 = 5.0 in the same buffer containing n-hexane at 1% (V/V) for L-asparaginase release. The suspensions were incubated at room temperature for 1h, and briefly vortex every 10 min [8].

L-asparaginase assay
The enzyme activity was assayed according to wriston [13]. The reaction mixture contained 0.1 mL permeabilized cells free broth and 0.9 mL of 0.01 mole L-asparagine prepared in 0.05 mole tris- HCl buffer, pH 8.6 and incubated for 30 min at 37°C. The reaction mixture was centrifuged at 6000 xg for 10 min and the ammonia released in the supernatant was determined by Nesslerization reaction. In brief, to 0.5 mL of supernatant, 1.75 mL dist. H2O, 0.25 mL of Nessler reagent was added. After 10 min. absorbance at 480 nm were read with appropriate control. One enzyme unit (U) is defined as the amount of enzyme that librates 1 μmole of ammonia per min at 37°C. Standard curve of ammonium sulphate was used for calculating ammonia concentrations.

Immobilization of L-asparaginase
Different supports were employed for L-asparaginase immobilization according to [14]. Experimentally, 200 mg of each support was shaked in 5 mL tris –HCl buffer (0.01 mole, pH 8.6) containing 2.5 % glutaraldehyde at room temperature for 2h. The carriers were filtered off and washed with distilled water to remove the excess of glutaraldehyde then each treated carrier was incubated with 5 mL of tris HCl buffer containing 1 mL of enzyme. After being shaken for 2h at 30°C, the unbounded enzyme was removed by washing with distilled water until no protein or activity were detected in the wash.

Cell culture
Two human cell lines were used through this work including: hepatocarcinoma (HepG2) and Colon carcinoma (HCT-116), both lines purchased from ATCC, VA, USA. Hep-G2 Cells were routinely cultured in DMEM (Dulbeco's Modified Eagle's Medium), while HCT-116 cells were cultured in Mc Coy's medium. Media were supplemented with 10 % fetal bovine serum (FBS), 2 mmole L-glutamine, containing 100 units/mL penicillin G sodium, 100 units/ml streptomycin sulphate, and 250 ng/ml amphotericin B. Cells were maintained at sub-confluence at 37°C in humidified air containing 5 % CO2. For sub-culturing, monolayer cells were harvested after trypsin / EDTA treatment at 37°C. Tested samples were dissolved in dimethyl sulphoxide (DMSO). All cell culture material was obtained from Cambrex Bioscience (Copenhagen, Denmark). All chemicals were from Sigma/Aldrich,

USA, except mentioned. All experiments were repeated three times, unless mentioned.

Anti-tumor activity

The cytotoxic effect of the samples against Hep-G2 and HCT-116 cells was estimated by the 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay according to Hansen et al., 1989 [15]. The yellow tetrazolium salt of MTT is reduced by mitochondrial dehydrogenases in metabolically active cells to form insoluble purple formazan crystals, which are solubilized by the addition of a detergent. Cells (5 x 104 cells / well) were incubated with various concentrations of the compound at 37 ºC for 48 h in a FBS-free medium, before submitted to MTT assay. The absorbance was measured with an ELISA reader (BioRad, München, Germany) at 570 nm. The relative cell viability was determined by the amount of MTT converted to the insoluble formazan salt. The data are expressed as the mean percentage of viable cells as compared to the respective control cultures treated with the solvent. The half maximal growth inhibitory concentration IC50 values were calculated from the linear equation of the dose–dependent curve of each sample.

Antioxidant activity (scavenging of DPPH)

The antioxidant capacity of the tested samples was studied through their scavenging activity against 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radicals [16]. DPPH is a stable deep violet radical due to its unpaired electron. In the presence of an antioxidant radical scavenger, which can donate an electron to DPPH, the deep violet color decolourize to the pale yellow non-radical. The bleaching of DPPH was monitored at absorbance of 515 nm. The percentage of DPPH bleaching utilized for SC50 (half maximal scavenging concentration) was calculated as follows: 0% is the absorbance of DPPH and 100 % is the absorbance of DPPH with an efficient scavenger (10 mM ascorbic acid, AA).

## 3. Results and Discussion
Screening of bacterial isolates

Fifty two bacilli species were tested for enzyme production. Extracellularly, no appreciable levels of L-asparaginase were detected. After membrane permeabilization with potassium phosphate / 1% hexane system [8], *Bacillus* sp R36 gave the highest enzyme productivity in the cell suspension (20.15 U/mL). The determination of the localization of any enzyme plays a vital role in the development of bioprocess. The existence of L-asparaginase in the membrane fraction of *Tetrahymena pyriformis* and periplasmic space of *Enterobacter aerogenes* and *Pseudomonas.*

aeruginosa has been reported [8, 17]. There are many reports on the production of intracellular L-asparaginase from *E. coli* [18] *Vibrio succinogenes* [19], *Erwinia aroideae* [20], *S. marcescens*[21], *E. aerogenes* [22], and *P. aeruginosa* [10, 23]. Kumar et al [24] that recently carried out a sub-cellular localization of L-asparaginase enzyme using cell fractionation techniques in various organisms. They reported that there was no trace of extracellular activity observed in the culture filtrates, which inferred that the enzyme was secreted as an intracellular product in all microorganisms tested. The maximum L-asparaginase activity was found to be 14.56 U/mL with *Pectobacterium carotovorum* MTCC 1428.

Optimization of L-asparaginase production by Bacillus sp R 36

Some factors affecting L-asparaginase productivity were studied aiming at optimization the anti-leukemic enzyme activity. With respect to the effect of aeration level, 9:1 (25 mL LB medium per 250 mL conical flask) air to medium ratio gave the highest enzyme activity reaching 22.8 U/mL (Fig 1b). Incubation period played an important role in enzyme productivity as maximum enzyme activity was obtained from 20-24 h reaching (20.0 U/mL) after 24 h only of incubation on an orbital shaker at 150 rpm. At 48 h of incubation under the same conditions, the activity decreased to 42% (8.4 U/mL) (Fig 1c). The same incubation period was reported upon production of L-asparaginase by isolated Bacillus circulans [25] and Enterobacter aerogenes and Pseudomonas aeruginosa [8]. Inoculum size in terms of colony forming unit (CFU/mL) had a positively effect on enzyme activity up to 55 x 105 CFU/mL. Higher inoculum sizes were resulting in leveling off the activity (Fig 2a). Prakasham [26] abstracted that incubation temperature, inoculums level and medium pH among all fermentation factors were major influential parameters at their individual level, and contributed to more than 60% of total L-asparaginase production. The effect of initial pH of LB medium was studied. Results revealed that 5.6 initial pH value of production medium (without adjustment) was suitable for enzyme production more than other tested pH values (Fig 2b). In all cases the harvest pH value is highly   alkaline ~ 8.9 due to ammonia production in the fermentation flask. All carbon sources that were tested for L-asparaginase production enhanced enzyme formation only upon adding to Luria– Bertani (LB) medium.
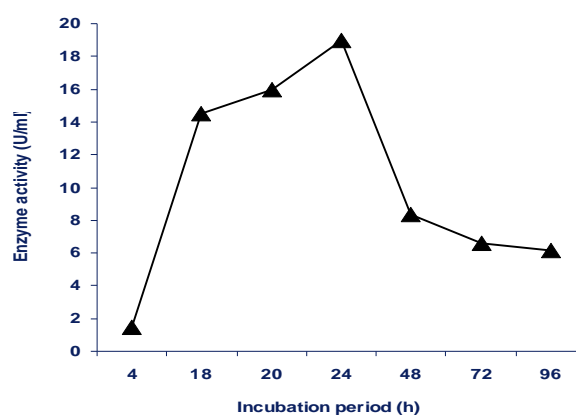
A
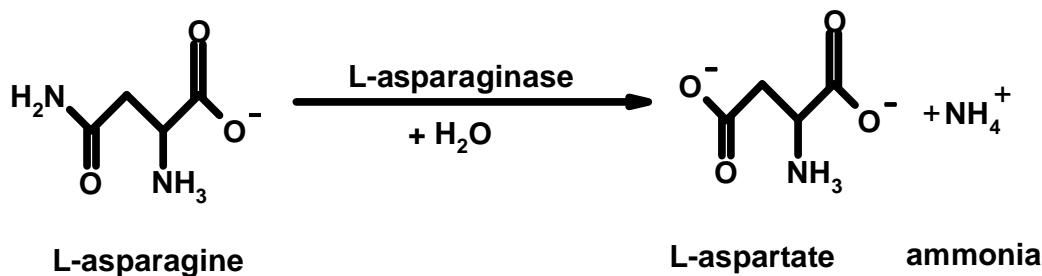
B
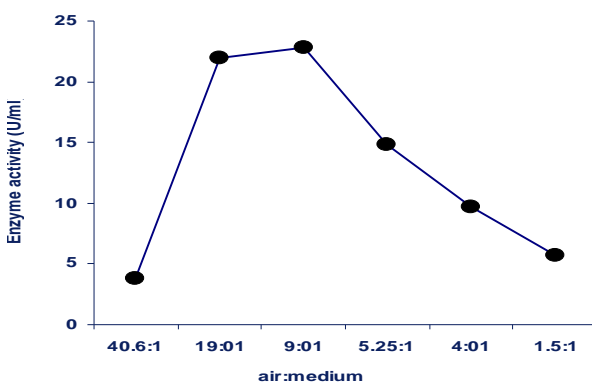
c

Fig (1): a. Role of L-asparaginases in hydrolysis of L-asparagine into L-asparate and ammonia. b. Effect of aeration level on L-asparaginase activity produced by Bacillus sp R36 on LB medium. All data are average value of triplicate measurements. c. Effect of incubation period on enzyme activity. The flasks were incubated on an orbital shaker at 150 rpm at the indicated times. All data are average value of triplicate measurements.
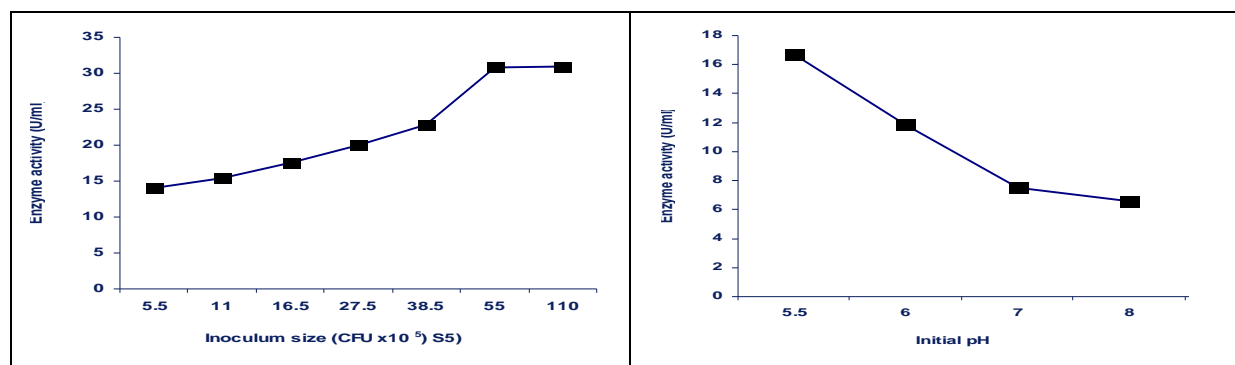

Fig (2): a. Effect of inoculum size on L-asparaginase activity. b. Effect of initial pH value of LB medium on L-asparaginase. All data are average value of triplicate measurements.

On mineral salt medium, Bacillus sp R36 neither could grow nor biosynthesize the enzyme. The obtained results revealed that addition of 1% lactose or 1% Raffinose to LB medium doubled the enzyme activity (204 % and 209 %, respectively). On the other hand, glucose was the most inhibitory carbon source compared with the control LB medium (Fig 3). The effect of carbon sources differ from organism to another, but in general, glucose was regarded as a repressor for L-asparaginase production in bacteria

[27]. Optimization of the culture conditions of L-asparaginase activity was the aim of many studies through which the enzyme productivity increased to many folds. Thus, Abdel –Fattand and olama [10] obtained more than five folds the activity in basal medium using Box-Behnken designing in solid state culture. Kumar et al [28] obtained an over all 8.3 fold in enzyme production compared to the un-optimized medium using the central composite experimental design. Hymavathi et al [29] improved L-asparaginase yield by more than 300% using fractional factorial central composite design (FFCCD).

Immobilization of L-asparaginase

Seven supports, pre-activated with glutaraldehyde, react with different degrees with the terminal amino residues of the enzyme protein. Immobilization of Bacillus sp R36 L-asparaginase by covalent binding was achieved by cross linking between the enzyme and different carriers (i.e. activated carbon, celite, chitin, carboxymethyl cellulose, egg shell, silica gel, tricalcium phosphate and chitosan) throughout glutaraldehyde. The amount of enzyme used for immobilization was 50 U/g carrier. The results (Table 1) indicated that the lowest immobilized activity and immobilization yield 7.9 U/g carrier and 17.8 % were detected with chitosan as a carrier. On the other hand, the highest immobilized activity (33.0 U/g carrier) and highest immobilization yield (73.6%) were obtained with activated carbon as a carrier. Medically activated carbon (activated charcoal) is a supplement used when accidental poisonings have occurred to absorb the poison and carry it out of the body. It is also used to treat high cholesten stomach and gas [30].

Our results are the first report for immobilization on activated carbon of L-asparaginase enzyme. In addition, immobilization of L-asparaginase R36 on activated carbon had a characteristic of simpler processing in comparison with that on silk sericin protein [31], polysaccharide levan [32] and agarose [33]. Kotzia et al [34] immobilizesd L-asparaginase of Erwinia chrysantheni 3937 on epoxy- activated Sepharose CL-6B. They reported that the immobilized enzyme retains most of its activity (60%) and shows high stability at 4°C. More recently, Tabandeh and Aminlari [35] investigated the effect of conjugation with oxidized inulin on the properties of L-Asparaginase (L- ASNase) in the form of Elspar. They found that modified L-asparaginase synthesized at ratio of 2: 1 had activity of 65% of that of native enzyme.

Characterization of L-asparaginase of Bacillus sp R36
a. Effect of pH on free and immobilized enzymes activities.

The effect of pH on L-asparaginase activity of free and immobilized preparations was studied by changing the pH value from 3.0 to 8.6 using a series of buffers namely citrate buffer (pH 3-5), phosphate buffer (pH 6-7), and Tris buffer (pH 8-8.6), (Fig. 4a). Both crude and immobilized L-asparaginase preparations exhibited maximum activity at pH 7. The immobilized form yielded more than 300 % increase in activity (333.5 %). This clearly reflects the suitability of activated carbon as a carrier for this enzyme. Our results are in agreement with that reported by Zhang et al [31] who immobilized E. coli L-asparaginase on micro particles of the natural silk sericin protein. They reported that the optimal range of pH value had no evident changes in comparison with native enzyme. Tabandeh and Aminlari [35] reported that the optimum pH of modified L- ASNase and the native enzyme is at alkaline pH (pH 8) probably due to produced L-aspartic acid acting as competitive inhibitor for enzyme in acidic condition.

b. Effect of Reaction temperature on free and immobilized L-asparaginase.

The effect of the reaction temperature on free and immobilized forms was investigated from 30 ˚C to 80 ˚C (Fig 4b). The optimum reaction temperature of free enzyme was 50 ˚C while the immobilized preparation reacted optimally at 60 ˚C reaction temperature i.e. at 10 ˚C higher than the native enzyme, with an increase of 355.8 % in enzyme activity. The same results were reported by [31], who stated that the optimum reaction temperature of immobilized enzyme was at 60 ˚C while that of free L-ASN ase was at 50 oC.

c. Thermal stability of immobilized enzyme

Residual activities after heating the enzyme at 30-90˚C for 10 min were measured (Fig 4c). An excellent thermal stability was exhibited by the immobilized enzyme preparation. The enzyme retained 100% of its L-asparaginase activity up to 80 ˚C, while 62% of it was retained upon heating for 10 min at 90˚C. The native form, maintained 100% of its activity at 50˚C while at 80˚C it retained 65% of its L-asparaginase activity. At 90 ˚C the native form maintained 57% of its activity. An improvement of thermal stability of immobilized enzyme may be acquired by multiple attachment of activated carbon to the enzyme molecule resulting in greater enzyme rigidity of enzyme conformation and increasing the activation energy for unfolding the enzyme. A marked improvement in thermal stability of modified enzyme was obtained by Tabandeh and Aminlari [35], Amiri et al [36] and Scaman et al [37]. In contrast, [31] reported that the thermo stability of the immobilized ASNase is very

similar to that of the native enzyme and there were no obvious changes in the activities.

Anti-tumor activity

Using MTT assay, the in vitro cytotoxicity effect of Bacillus sp R36 L-asparaginase enzyme on the growth of two tumor cell lines was studied. The IC50 values were calculated from the linear equation of the dose effect of the enzyme against hepatocellular carcinoma Hep G2 cells (y = -0.4069x + 95.648) and against colon carcinoma HCT-116 cells (y = -0.2233x + 98.838). The incubation of Hep G2 with gradual doses of Bacillus sp R36 L-asparaginase enzyme leads to a gradual inhibition in the cell growth as concluded from its low IC50 values 112.19 μg / mL (Fig. 5a). As shown in (Fig. 5b), the treatment of HCT- 116 with the enzyme resulted in a low anti-tumor activity with IC50 value of 218.7 μg / mL compared with the growth of untreated control cells. Cappelletti et al [38] studied in vitro cytotoxicity of a novel L-asparaginase from the pathogenic strain Helicobacter pylori CCUG 17874 against different cell lines. They reported that AGS and MKN 28 gastric epithelial cells being the most affected.
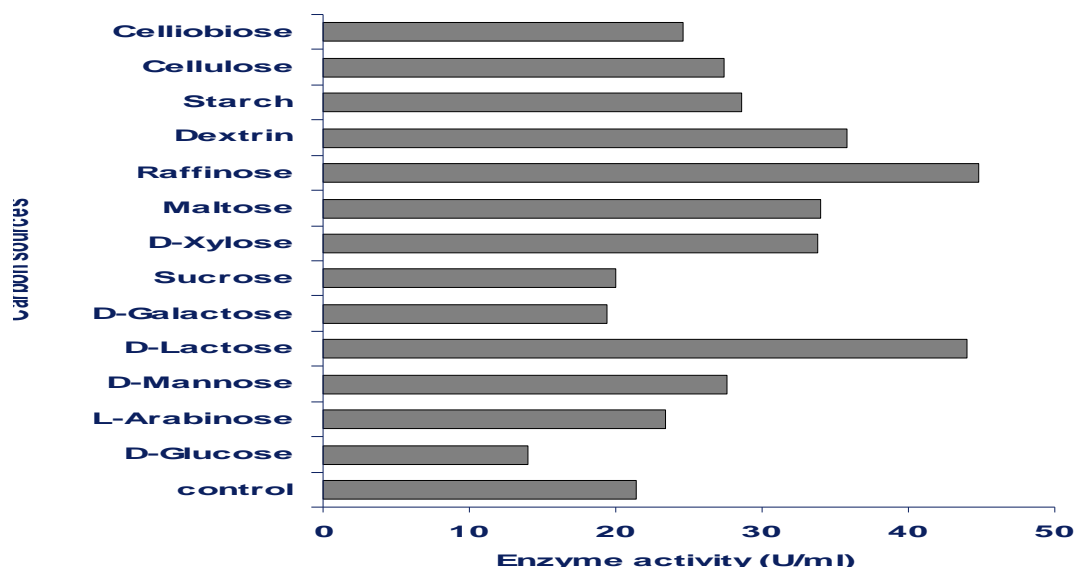

Fig (3): Effect of different carbon sources on L-asparaginase activity (final concentration 1%). All data are average value of triplicate measurements.

Table (1): Immobilization of *Bacillus* sp R 36 L-asparaginase by covalent binding with different carriers.

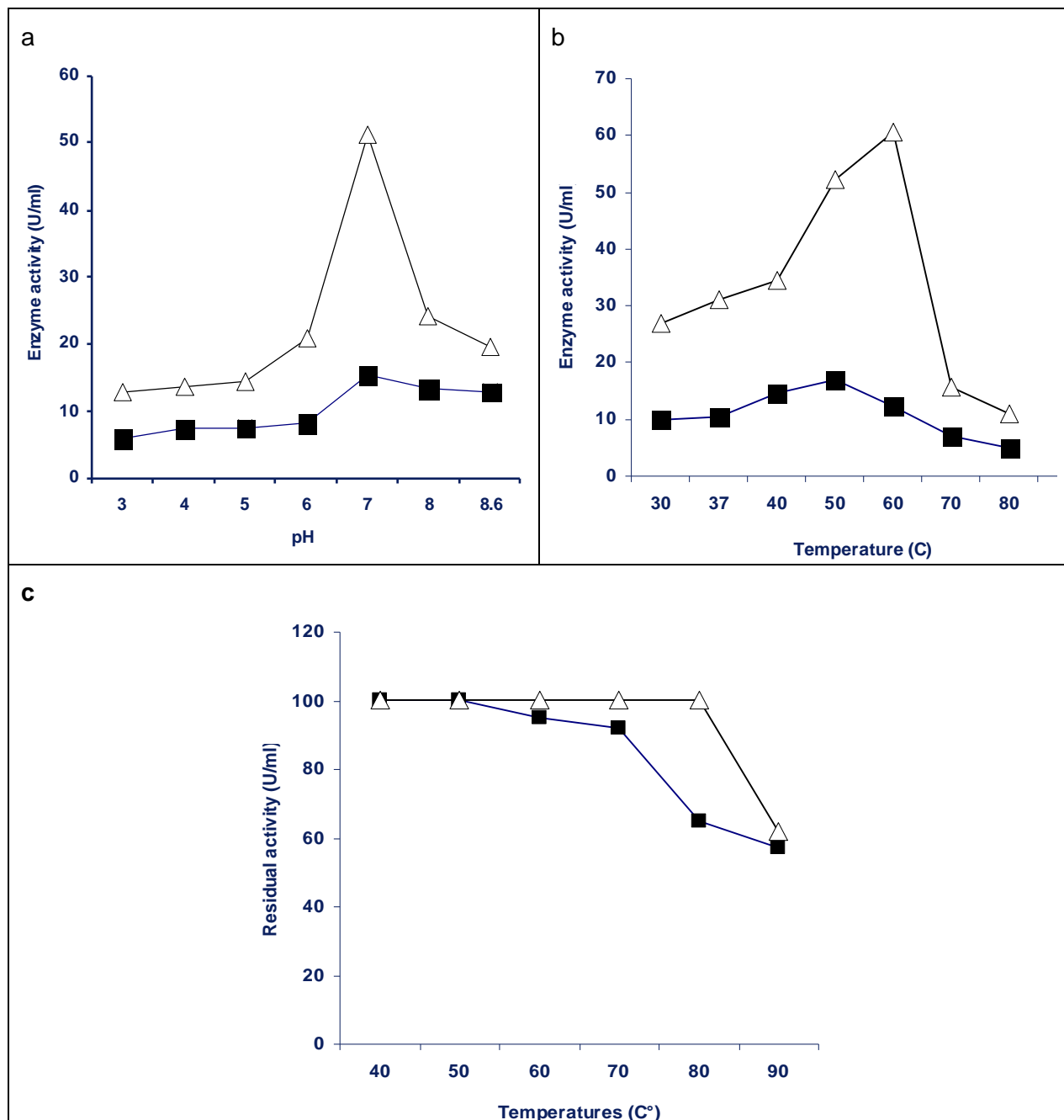| Carrier	Added enzyme (U/g) (A)	Unbounded enzyme (U/g) (B)	Immobilized enzyme (I)	Immobilizati-on yield I / (A-B)%
Activated carbon	50	5.2	33.0	73.6
Celite	50	4.1	27.5	60.0
Chitin	50	3.5	14.3	30.7
CMC	50	11.5	19.8	51.4
Egg Shell	50	2.7	20.3	42.9
SG	50	4.8	28.1	62.1
TCP	50	7.2	8.6	20.1
Chitosan	50	5.5	7.9	17.8

Fig (4): The effect of pH (a) and reaction temperature (b) on free (squared-line) and immobilized enzymes (triangled-line). A series of buffers was used: citrate buffer (pH 3 –

Antioxidant activity

The antioxidant capacity of the enzyme was investigated using DPPH assay. DPPH is a stable non-physiological radical, which could provide a relative figure of the radical scavenging capacity of a tested probe. The DPPH assay showed that Bacillus sp R36 possessed low scavenging activity with high SC50 values of 325.4 µg/mL compared to the scavenging activity of the well-known antioxidant (ascorbic acid, a.a., SC50 8.7 µg/mL).

b

$y = -0.4069x + 95.648$
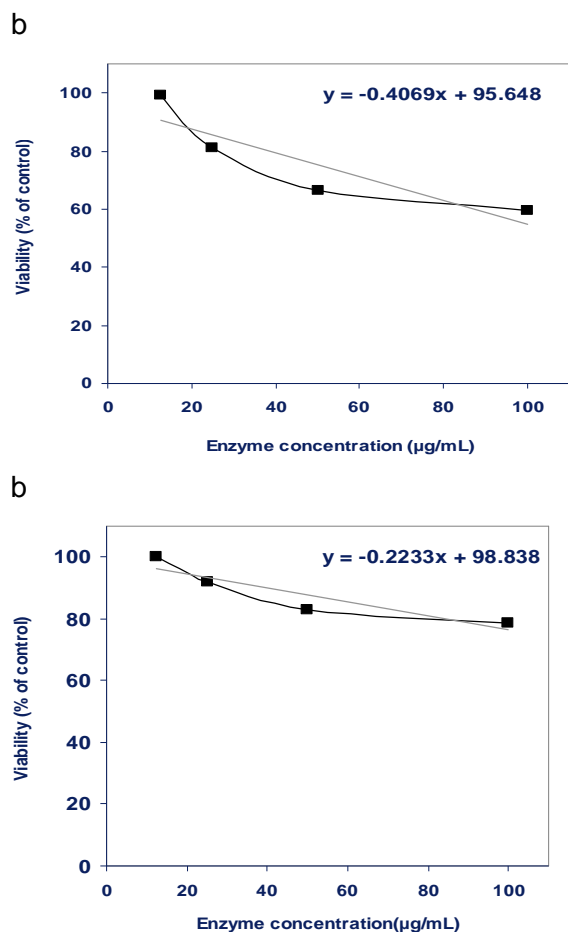
b

$y = -0.2233x + 98.838$

Fig (5): Anti- tumor activity against a. hepatocellular carcinoma cells (Hep-G2) and b. colon carcinoma cells (HCT-116). All data are average value of triplicate measurements.

Corresponding author:
Maysa Moharam,
Microbial Chemistry Department, Genetic Engineering and Biotechnology Division, National Research Center, Dokki, Giza, Egypt
email: maysa12000@hotmail.com.
Phone: 002 02 33 37 13 62 – 1523, Fax: 202 3370931

4. References:

1. Ettinger, L. J., Ettinger, A.G., Avramis V.I. and Gaynon P.S. Acute lymphoblastic leukaemia: a guide to asparaginase and pegaspargase therapy. Bio drugs 1997; 7: 30-9.

2. Athale UH, Chan KCA. Thrombosis in children with acute lymphoblastic leukemia part II. Pathogensis of thrombosis in children with acute lymphoblastic leukemia: Effects of the disease and therapy.Thromb Res 2003;111:199-212.

3. Narta UK, Kanwar SS, Azmi W. Pharmacological and clinical evaluation of L-asparaginse in the treatment of leukemia. Crit Rev Oncol Hematol 2007; 61: 208- 21.

4. Reitzer L.J. Sources of nitrogen and their utilization. In: Neidhardt FC, Curtiss R III, Ingraham J.L., Lin ECC, Low KB, Magasanik B, Reznikoff WS, Riley M,: Schaechter M, Umbarger H. E, editors , *Escherichia coli* and *Salmonella typhimurium*. Cellular and molecular biology Vol. 1. Washington, D.C.: American Society for Microbiology, 1996. P. 380-90.

5. Stecher A.L., de Deus P.M. Polikarpov I. and Abrahäo- Neto J. Stability of L-asparaginase: an enzyme used in leukemia treatment. Pharm. Acta Helv. 1999 74 :1-9.

6. Hüser A., Klöppner U. and Rohm K.H. Cloning, sequence analysis and expression of ans B from *pseudomonas fluorescens,* encoding periplasmic glutaminase/ asparaginase. FEMS Microbiol lett. 1999;178 : 327-35.

7. Fisher SH, Wray Jr LV. *Bacillus subtilis*168 contains two differentially regulated gene encoding L-asparaginase. J Bacteriol.2002;184:2148-54.

8. Geckil H., Ates, B., Gencer S., Uckun M. and Yilmaz I. Membrane permeabilization of gram negative bacteria with a potassium phosphate/ hexane aqueous phase system for the release of L-asparaginase an enzyme used in cancer therapy. Process Biochem. 2005; 40: 573-9.

9. Broome J.D. L-asparaginase discovery and development as a tumor- inhibitory agent. Cancer treat. Rep 1981,65: 111-14.

10. Abdel –Fattah Y. R. and Olama Z.A. L-asparaginase production by *pseudomonas aeruginosa* in solid state culture: evaluation and optimization of culture conditions using factorial designs. Process Biochem. 2002 ; 38: 115-22.

11. Bullock C. Immobilized enzymes. Sci prog. 1995; 78: 119- 34.

12. Klibanov A.M. Immobilized enzymes and cells as potential catalysis. Science 1983:219-27.

13. Wriston JC. Jr., Asparaginase. Method Enzymol. 1970 XVII: 732-42.

14. Abdel Naby A.M. Immobilization of *Aspergillus niger* NRC-107 xylanase and -xylosidase and

properties of immobilized enzyme. Appl. Biochem. Biotech. 1993, 38: 69-81.

15. Hansen MB, Nielsen SE and Berg K. Re-examination and further development of a precise and rapid dye method for measuring cell growth / cell kill. J. immunol Methods. 1989, 119:203-10.

16. Van Amsterdam, F.T., Roveri A., Maiorino M., Ratti E., and Ursini F. Lacidipine : a dihydro-pyridine calcium antagonist with antioxidant activity. Free rad. Biol Med. 1992, 12: 183-7.

17. Triantafillou D.J.,Georgatsos J. G., Kyriakidis D. A. Purification and properties of a membrane-bound L- asparaginase of *Tetrahymena pyriformis.* Mol Cell Biochem. 1988; 81 : 43- 51.

18. Barnes W R, Dorn C L, and Vela CR. **:** Effect of culture conditions on synthesis of L-asparaginase by *Escherichia coli* A-1. Appl Environ Microbiol 1997; 33 ;257-61.

19. Kafkewitz D. and Goodman D. L-asparaginase production by the rumen anaerobe *Vibrio succinogenes*. Appl Microbiol 1974; 27:206 – 9.

20. Peterson R. E. and Ciegler A. L asparaginase production by *Erwinia aroideae.* Appl Microbiol 1969; 18: 64-7.

21. Heinemann B. and Howard A. Production of tumor inhibitory L-asparaginase by submerged growth of *Serratia marscences*. Appl Microbiol 1969; 18: 550- 4.

22. Mukherjee J. Majumdar S. and Scheper T. Studies on nutritional and oxygen requirements for production of L-asparaginase by *Enterobacter aerogenes*. Appl Microbiol Biotechnol 2000; 53: 180- 4**.**

23. Ceckil H. Cencer S. and Uckun M. *Vitreoscilla* hemoglobin expressing *Enterobacter aerogenes* and *Pseudomonas aeruginosa* respond differently to carbon catabolite and oxygen repression for production of L- asparaginase, an enzyme used in cancer therapy. Enzyme Microbiol Technol 2004;35:182-9.

24. Kumar S., Dasu V.V., and Pakshirjan K. **:** Localization and production of novel L-asparaginase from *Pectobacterium carotovorum* MTCC 1428. Process Biochem. 2009, Article in press.

25. Prakasham R.S., Hymavathi M., Subba Rao., Arepalli S.K., Venkateswara Rao., Kavin Kennady P., Nasaruddin K., Vijayakumar J.B.and Sarma P.N. Evaluation of antineoplastic

activity of extracellular asparaginase produced by isolated *Bacillus circulans.* Appl. Biochem Biotechnol 2009 :8679 – 88.

26. Prakasham RS, Rao ChS., Rao RS., Lakshmi Gs., Sarma PN. L-asparaginase production by isolated *Staphylococcus* sp.-6A :design of experiment considering interaction effect for process parameter optimization. J Appl Microbiol 2007, 102 (5):1382-91.

27. Jones CE., Genetics and physiological relationships between L-asparaginase I and L-asparaginase II in *Saccharomyces cervisiae.* J Bacteriol 1977; 130: 128 -30.

28. Kumar S. Pakshirajan K. Venkata Dasu V.**:** Development of medium for enhanced production of glutaminase free L- asparaginase from *Pectobacterium carotovorum* MTCC 1428. Appl. Microbiol. Biotechnol. 2009;84(3):477-86.

29. Hymavathi M. Sathish T. Subba Rao Ch. Prakasham R.S.: Enhancement of L-asparaginase production by isolated *Bacillus circulans* (MTCC8574) using response surface methodology. Appl. Biochem. Biotechnol. 2009; 159 (1): 191- 8.

30. Anon: USPDI: Drug Information for the Health Care Professional, 17th ed US Ph convention Inc, Rockville, MD 1997.

31. Zhang, Y. Q. Tao M. L., Shen W.D., Zhou, Y.Z., Ding Y., Ma Y. and Zhou W.L. Immobilization of L-asparaginase on the micro particles of the natural silk sericin protein and its characters. Biomaterials 2004, 25: 3751-59.

32. [32] Vina. I., Karsa Kevich, A. and Bekers, M. Stabilization of anti-leukemic enzyme L-asparaginase by immobilization on polysaccharide levan. J. Mol. Cat B 2001, 11: 551-558.

33. Balcäo, V. M., Maleoc, C., Fernan dez-lafuentec, R., Malcata F. X. and Guisan J.M. Coimmobilization of L-asparaginase and glutamate dehydrogenase onto highly activated supports. Enzyme Microbiol Technol. 2001,28: 696-704.

34. Kotzia G.A., Labrou N.E. **:** L-asparaginase from *Erwinia chrysantheni* 3937: Cloning expression and characterization. J Biotechnol. 2007 (127): 657-69.

35. Tabandeh M.R. Aminlari M. Synthesis, physicochemical and immunological properties of oxidized inulin –L- asparaginase bioconjugate. J Biotechnol. 2009 (141) : 189-95.

36. Amiri.S. Ramezani R. Aminlari M. **:** Antibacterial activity of dextran- conjugated lysozyme against *Escherichia coli* and *Staphylococcus aureus* in cheese curd.J Food Prot.2008, 71 :411-15.

37. Scaman C., Nakia S., Amirlari M. : Effect of pH, temperature and sodium bisulfate or cysteine on the level of Maillard –based conjugation lysozyme with dextran, galactomannan and mannan. Food Chem. 2006 (99) : 368-80.

38. Cappelletti D., Chiarelli L.R., Pasquetto M. V. Stivala C.V. and Scotti C. *Helicobacter pylori* L-asparaginase : A promising chemotherapeutic agent. Biochem. Biophys. Research Communications. 2008; 377 : 1222 -6.

4/20/2010