



UNIVERSITI PUTRA MALAYSIA

***HETEROLOGOUS EXPRESSION AND CHARACTERIZATION OF
L-ASPARAGINASE FROM MARINE *Photobacterium* sp.
STRAIN J15***

MOHD ADILIN BIN YAACOB

FBSB 2014 21



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By

MOHD ADILIN BIN YAACOB

**Thesis submitted to the School of Graduate Studies, Universiti Putra Malaysia,
in Fulfillment of the requirements for the degree of Master of Science**

January 2014

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DEDICATION

I dedicate my dissertation work to my family and many friends. A special feeling of gratitude to my loving father and sisters, Yaacob, Rosliza, Roslina, Roslima, Rosliah, Amir Hakimi and Nur Farah Dilyani whose words of encouragement and push for tenacity ring in my ears. I also dedicate this dissertation to my many friends and supervisor who have supported me throughout the process. I will always appreciate all they have done, especially Zulhilmi for helping me develop my technology skills, and Dr. Adam Leow Thean Chor for the many hours of proofreading. I dedicate this work and give special thanks to my lovely wife Atiqah and my wonderful daughter Aamilah Hannani for being there for me throughout the entire master program. Both of you have been my best cheerleaders.



Abstract of thesis presented to the Senate of Universiti Putra Malaysia
In fulfilment of the requirement for the degree of Master of Science

**HETEROLOGOUS EXPRESSION AND CHARACTERIZATION OF
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STRAIN J15**

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MOHD ADILIN BIN YAACOB

January 2014

Chairman: Adam Leow Thean Chor, PhD

Faculty: Biotechnology and Biomolecular Sciences

L-asparaginase catalyzes the conversion of the L-asparagine to L-aspartate and ammonia. Although L-asparaginase is one of the main components used in chemotherapy, toxicity problem associated with glutaminase activity of commercial asparaginase products such as Elspar, oncaspar and Erwinaze derived from *Escherichia coli* and *Erwinia crysanthemi* becomes a major limitation in cancer treatment. The aim of the current study is to isolate L-asparaginase (J15 asparaginase) gene from *Photobacterium* sp. strain J15 and characterize J15 asparaginase biochemically by molecular expression and *in silico* modeling. Genome mining revealed an open reading frame of 1011 bp coding for J15 asparaginase gene from halo-tolerant *Photobacterium* sp. strain J15. The J15 asparaginase gene was isolated by using specific primer flanking the full length of gene overexpressed in pET-32b vector and transformed into *E. coli* strain Rosetta-gami B (DE3) pLysS. The J15 asparaginase was purified to homogeneity by using two-step chromatographic: Ni²⁺-Sephacel affinity chromatography and Q-Sepharose anion exchange chromatography. The final specific activity and yield of the enzyme achieved from these steps were 20 U/mg and 49.2 %, respectively.

The functional dimeric form of J15-asparaginase with molecular weight of ~70 kDa was characterized. The optimum temperature and pH was at 25 °C and pH 7 respectively. It was stable in the presence of 1 mM Ni²⁺ and Mg²⁺, but was inhibited by Mn²⁺, Fe³⁺ and Zn²⁺. J15 asparaginase actively hydrolyzed its natural substrate L-asparagine, but had low activity towards L-glutamine. The T_m for J15 asparaginase was about 51 °C, as revealed by denatured protein analysis of Circular Dichroism (CD) spectra. The K_m , K_{cat} , K_{cat}/K_m of J15 asparaginase was 0.76 mM, 3.2 S⁻¹, and 4.21 S⁻¹mM⁻¹, respectively. Structural analysis of J15 asparaginase was analysed by homology modeling and Molecular Dynamic (MD) simulations. The J15 asparaginase model was validated by using Ramachandran plot, and ERRAT 2.0. The monomer of J15 asparaginase comprises of 31 % α -helix and 18.3 % β -sheet. RMSD value of 0.436 with 76.85 % identities was recorded when the J15 asparaginase superimposed with template (pdb: 2OCD). MD simulation for 10 ns

was carried out to examine conformational changes of J15-asparaginase structure at different temperatures (25 °C, 45 °C, and 65 °C) and it was found that at 25 °C, J15 asparaginase was most stable hence able to hydrolyze the substrate at optimum rate. The function of mobile lid-loop at the active site was analyzed by using MD simulation at 10 ns. Tyr₂₄, His₂₂, Gly₂₃, Val₂₅, and Pro₂₆ residues might directly involve in 'open' and 'closed' of lid-loop conformation to facilitate the conversion of substrate during enzymatic reaction. Low glutaminase activity possessed by J15 asparaginase might serve as an ideal candidate to be used as enzyme-drug in cancer treatment.



Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
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**PENGEKSPRESAN SECARA HETEROLOGI DAN PENCIRIAN
L-ASPARAGINASE DARIPADA MARIN *Photobacteria* sp.
STRAIN J15**

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L-asparaginase memangkinkan penukaran L-asparagine kepada L-aspartate dan ammonia. Walaupun L-asparaginase adalah salah satu komponen utama yang digunakan dalam kemoterapi, masalah keracunan yang berkaitan dengan aktiviti glutaminase daripada asparaginase komersial seperti Elspar, oncaspar dan Erwinaze diperolehi daripada *Escherichia coli* dan *Erwinia caryophylli* menjadi had utama dalam rawatan kanser. Tujuan kajian semasa adalah untuk mengasingkan L-asparaginase (J15 asparaginase) gen daripada *Photobacterium* sp. J15 dan mencirikan J15 asparaginase secara biokimia dengan menggunakan kaedah biologi molekul dan model komputer. Perlombongan genom mendedahkan L-asparaginase jenis I (J15 asparaginase) dengan panjang gen 1011 bp dari spesies *Photobacteria* strain J15. Enzim ini telah dihasilkan dalam kuantiti yang banyak menggunakan vektor pET-32b dan *E. coli* Rosetta-gami B (DE3) pLysS sebagai hos serta dituliskan menggunakan dua langkah kromatografi: affiniti Nikel Sepharose dan Q-Sepharose. Aktiviti spesifik dan hasil yang dicapai daripada kaedah penulenan ini adalah masing-masing sebanyak 20 U/mg dan 49.2 %.

J15 asparaginase berbentuk dimerik dengan anggaran saiz sebesar ~70 kDa diperolehi dengan suhu optimum dan pH masing-masing pada 25 °C dan pH 7. J15 asparaginase stabil dengan kehadiran 1 mM Ni²⁺ dan Mg²⁺, tetapi direncatkan oleh Mn²⁺, Fe³⁺, dan Zn²⁺. J15 asparaginase aktif mengkatalisis substrat semulajadi L-asparagine tetapi mempunyai aktiviti yang rendah kepada L-glutamine. Analisis menggunakan CD spektrum menunjukkan T_m untuk J15 asparaginase adalah kira-kira 51 °C. Nilai K_m, K_{cat}, K_{cat} / K_m dari J15 asparaginase masing-masing adalah 0.76 mM, 3.2 S⁻¹, dan 4.21 S⁻¹mM⁻¹. Struktur J15 asparaginase telah dianalisis dengan model homologi dan simulasi Molekul Dinamik (MD). Model J15 asparaginase telah disahkan dengan menggunakan plot Ramachandran dan ERRAT 2.0. Monomer J15 asparaginase terdiri daripada 31% α-helik dan 18.3% β-sheets. Nilai RMSD sebanyak 0.436 dengan 76.85 persamaan identiti dicatatkan apabila J15 asparaginase dibanding

dengan struktur acuan (pdb: 2OCD). Simulasi MD selama 10 ns telah dijalankan untuk mengkaji perubahan struktur J15 asparaginase pada suhu yang berbeza (25 °C, 45 °C dan 65 °C) dan ia menunjukkan bahawa J15 asparaginase paling stabil pada 25 °C dan mampu menkatilisis substrat pada tahap optimum. Fungsi gelung mudah alih di tapak aktif juga dianalisis dengan menggunakan simulasi MD pada 10 ns. Tyr₂₄, His₂₂, Gly₂₃, Val₂₅, dan Pro₂₆ mungkin terlibat secara langsung dalam membuka dan menutup gelung mudah alih untuk memudahkan penukaran substrat semasa tindak balas enzim. J15 asparaginase yang menunjukkan glutaminase aktiviti yang rendah merupakan calon yang ideal untuk digunakan dalam rawatan kanser



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I certify that a Thesis Examination Committee has met on 16 January 2014 to conduct the final examination of Mohd Adilin bin Yaacob on his thesis entitled "Heterologous Expression and Characterization of L-Asparaginase from Marine *Photobacterium* sp. Strain J15" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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LIST OF ABBREVIATIONS

$\mu\text{g/mL}$	Microgram per millilitre
μM	Micromolar
\AA	Amstrong
\AA^2	Amstrong square
C	Celsius
cm	Centimeter
dH ₂ O	Distilled water
DNA	Deoxyribonucleic acid
g	Gram
g/L	Gram per Litre
h	Hour
IPTG	Isopropyl β -D Thiogalactoside
kbp	Kilobasepair
kDa	KiloDalton
MD	Molecular Dynamic
mg	Milligram
mL	Milliliter
mM	Millimolar
Nm	Nanometer
OD	Optical density
PCR	Polymerase chain reaction
pI	Isoelectrical point

RMSD	Root Mean Square Deviation
SASA	Solvent Accessible Surface Area
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
<i>sp.</i>	Species
U/mg	Unit per milligram
U/mL	Unit per millilitre
w/v	Weight per volume
YASARA	Yet Another Scientific Artificial Reality Application
µg	Microgram
µl	Microliter

CHAPTER 1

INTRODUCTION

Amino acid degrading enzyme L-asparaginase (EC 3.5.1.1) catalyzed the conversion of L-asparagine into aspartic acid and ammonia. The enzyme has been used for 40 years as chemotherapeutic agent to treat cancer cell especially Acute Lymphoblastic Leukemia (ALL) (Verma *et al.*, 2007) by degrading the L-asparagine in blood serum. Reduction of L-asparagine in circulatory system will selectively kill the tumor cell without affecting normal cells (Kotzia and Labrou, 2007). L-asparaginase has been used as diagnostic biosensor as the amount of ammonia produced during enzymatic reaction is directly correlates to the level of L-asparagine in a patient blood. Other than that, L-asparaginase also has successively been used in food industry. The pretreatment of carbohydrate rich food with L-asparaginase before baking has been proven to reduce the formation of acrylamide.

L-asparaginase is ubiquitous in environment but the enzymes from bacteria have obtained considerable interest because bacteria can be easily grown to high cell density thus economically viable. To date, L-asparaginase has been characterized from various bacterial genera. Despite of numerous studies on Gram positive bacteria such as *Pseudomonas fluorescense*, *Serratia marcescens*, *Mycobacterium sp.* and *Staphylococcus sp.*, their intrinsic glutaminase activity which could result serious toxicity restrict their clinical application. However, L-asparaginase from Gram negative bacteria such as *Erwinia caryophylli* (ErA) and *Escherichia coli* (ECII) were found to be the most effective, owing to less immunorelated toxicity (Vidya *et al.*, 2011). Their application however has limitation that restricted their application as effective cancer treatment. Because ECII is a bacterial protein, hypersensitivity ranging from mild allergic reaction to death is often observed during treatment (Moola *et al.*, 1990). The development of neutralizing antibodies after a few series of treatment also reduces the efficacy of ECII. In such cases, ErA is often used as substitute since it has different pharmacokinetic and immunogenic properties. However, ErA has shorter half-life than ECII and need to administer frequently. Other than that, the used of ErA also associated with neurotoxicity which partially attributed to its glutaminase activity (Narta *et al.*, 2007)

Asparaginases from different environment have different biochemical and kinetic properties hence exploring a new source of L-asparaginase with different serological properties but same therapeutic effect is crucial to produce safer enzyme-drug based in cancer treatment (Eden *et al.*, 1990). The enzyme from marine microbe is an interesting subject to be further investigated because they have diverse range of enzymatic activity and capable of catalyzing various biochemical reaction. Marine enzyme biotechnology can offer novel biocatalysts with properties like high salt tolerance, hyperthermostability, barophilicity, cold adaptivity, and ease in large-scale cultivation (Debahish *et al.*, 2005). Halophilic bacteria possess many hydrolytic enzymes and capable of functioning under condition that lead to precipitation and denaturation of most protein. In addition of that, human body contain the element of salt such as sodium, potassium and calcium in almost the same proportion of ocean and this could provide microbial product such as enzyme that could be safer, more

effective with no or less toxicity when used for therapeutic application in human being.

In the present study, the genome from marine Gram negative *Photobacterium sp.* strain J15 was isolated, partially sequenced and annotated. The gene sequence coded for L-asparaginase was isolated and characterized. In order to have better understanding on this new isolated gene, the following strategic were implemented:

- 1) To isolate J15 asparaginase gene from *Photobacterium sp.* strains J15 and express in *E.coli* expression system.
- 2) To purify and characterize J15 asparaginase biochemically
- 3) To predict J15 asparaginase structure by using Homology modelling and Molecular Dynamic simulation.



REFERENCES

- Adrio, J.L., and Demain, A.L. (2010). Recombinant organism for production of industrial products. *Bioeng Bugs*. 1 (2) : 116-131.
- Amann, E., Brosius, J., and Ptashne, M. (1983). Vectors bearing a hybrid trplac promoter useful for regulated expression of cloned genes in *Escherichia coli*. *Gene*. 25 : 167-178.
- Amena, S., Vishalakshi, N., Prabhakar, M., Dayanand, A., and Lingappa, K. (2010). Production, purification and characterization of L-asparaginase from *Streptomyces gulbargensis*. *Braz. J. Microbiol.* 41 : 173-178.
- Araujo, A.P., Oliva, G., Henrique-silva, F., Garret, R.C., Caceres, O., and Beltramini, L.M. (2000). Influence of the histidine tail on the structure and activity of recombinant chlorocatechol 1,2-dioxygenase, *Biochem. Biophys. Res. Commun.* 272 : 480-484.
- Aung, H.P., Bocola, M., Schleper, S. and Rohm, K.H. (2000). Dynamics of a mobile loop at the active site of *Escherichia coli* asparaginase. *Biochim. Biophys. Acta.* 1481 (2) : 349-359.
- Baldauf, S.L. (2003). The deep roots of eukaryotes. *Science* 300 : 1703 1706.
- Baneyx, F. (1999). Recombinant protein expression in *Escherichia coli*. *Curr. Opin. Biotechnol.* 10 : 411-421.
- Bansal, S., Srivastava, A., Mukherjee, G., Pandey, R., Verma, A. K., Mishra, P. and Kundu, B. (2012). Hyperthermophilic asparaginase mutants with enhanced substrate affinity and antineoplastic activity: structural insights on their mechanism of action. *FASEB J.* 26 : 1-11.
- Bansal, S., Gnaneswari, D., Mishra P., and Kundu, B. (2010). Structural Stability and Functional Analysis of L-Asparaginase from *Pyrococcus furiosus*. *Biochemistry* 75 (3) : 375-381.
- Basha, N.S., Rekha, R., Komala, M., and ruby, S. (2009). Production of extracellular anti-leukaemic enzyme L-asparaginase from marine actinomycetes by solid state and submerged fermentation: purification and characterization, *Trop. J. Pharm. Res.* 8 (4) : 353-360.
- Becalski, A., Lau, B. P., Lewis, D., and Seaman, S.W., (2003). Acrylamide in foods: occurrence, sources, and modelling. *J. Agric. Food Chem.* 51: 802 808.
- Belasco, J.G., and Higgins, C.F. (1988). Mechanisms of mRNA decay in bacteria: A perspective. *Gene* 72:15-23.

- Bessette, P.H., Åslund, F., Beckwith, J., and Georgiou, G., (1999). Efficient folding of proteins with multiple disulfide bonds in the *Escherichia coli* cytoplasm. *Proc. Natl. Acad. Sci. USA*. 96 : 13703-13708
- Brondyx, W.H. (2009). Selecting an Appropriate method for expressing a recombinant protein. *Methods enzymol.* 463 : 131-147.
- Broome, J.D. (1961). Evidence that the L-asparaginase activity of guinea pig serum is responsible for its anti lymphoma effects. *Nature* 191 : 1114-1115.
- Brosius, J., Erfle, M., and Storella, J. (1985). Spacing of the -10 and -35 regions in the *tac* promoter. Effect on its *in vivo* activity. *J. Biol. Chem.* 260 : 3539-3541.
- Cadel, S., Gouzy-Darmon, C., Petres, S., Piesse, C., Pham, V.L., Beinfeld, M.C., Cohen, P., and Foulon, T. (2004). Expression and purification of rat recombinant aminopeptidase B secreted from baculovirus infected insect cells. *Protein Expr. Purif.* 36 : 19-30.
- Cambillau, C., Claverie, J.M. (2000). Structural and genomic correlates of hyperthermostability. *J. Biol. Chem.* 275 (42) : 32383-32386.
- Capener, C.E., Shrivastava, I.H., Ranatung, K.M., Forres, L.R., Smith, G.R. and Sansom, M.S. (2000). Homology modelling and molecular dynamics simulation studies of an inward rectifier potassium channel. *Biophys. J.* 78 : 2929 - 2942.
- Cappelletti, D., Chiarelli, L. R., Paschetto, M. Stivala, V.S., Valentini, G., and Scotti, C. (2008). *Helicobacter pylori* L-asparaginase: A promising chemotherapeutic agent. *Biochem. Biophys. Res. Commun.* 377 : 1222-1226.
- Carrington, J.C., and Dougherty, W.G. (1988). A viral cleavage site cassette: identification of amino acid sequences required for tobacco etch virus polyprotein processing. *Proc. Natl. Acad. Sci. USA* 85: 3391-3395
- Caspeta, L., Flores, N., Pérez, N.O., Bolívar, F. and Ramírez, O.T. (2009). The effect of heating rate on *Escherichia coli* metabolism, physiological stress, transcriptional response, and production of temperature induced recombinant protein: a scale-down study. *Biotechnol. Bio.* 102: 468-482.
- Ceulemans, H., and Russell, R.B. (2004). Fast fitting of atomic structures to low resolution electron density maps by surface overlap maximization, *J. Mol. Biol.* 338 : 783-793.
- Chant, A., Kraemer-Pecore, C.M., Watkin, R., and Kneale, G.G. (2005). Attachment of a histidine tag to the minimal zinc finger protein of the *Aspergillus nidulans* gene regulatory protein AreA causes a conformational change at the DNA binding site. *Protein Expr. Purif.* 39 : 152-159.

- Chen, D., and Texada D.E. (2006). Low-usage codons and rare codons of *E.Coli*. *Gene Ther. Mol. Biol.* 10 : 1-12.
- Chen, H., Xu, Z., Xu, N., and Cen, P. (2005). Efficient production of a soluble fusion protein containing human beta-defensin-2 in *E. coli* cell-free system. *J. Biotechnol.* 115 : 307–315.
- Chen, H., Bjercknes, M., Kumar, R. and Jay, E. (1994). Determination of the optimal aligned spacing between Shine-Dalgarno sequence and the translation initiation codon of *Escherichia coli* mRNAs. *Nucleic Acids Res.* 22 (23) : 4953-4957.
- Chohan, S. M., and Rashid, N., (2013). TK1656, a thermostable L asparaginase from *Thermococcus kodakaraensis* exhibiting highest ever reported enzyme activity. *J. Biosci. Bioeng.* 116(4) : 438-443.
- Choi, S.I., Song, H.W., Moon, J.W., Seong, B.L. (2001). Recombinant enterokinase light chain with aYnity tag: expression from *Saccharomyces cerevisiae* and its utilities in fusion protein technology. *Biotechnol. Bioeng.* 75 : 718–724.
- Chou, C.P. (2007). Engineering cell physiology to enhance recombinant protein production in *Escherichia coli*. *Appl. Microbiol. Biotechnol.* 76: 521–53.
- Ciesarova, Z., Kiss, E., and Boegl, P. (2006). Impact of L-asparaginase on acrylamide content in potato products. *J. Food Nutr. Res.* 45 : 141 146.
- Cragg, G.M., and Newman, D.J. (1999). Discovery and development of antineoplastic agents from natural sources. *Cancer Invest.* 17 : 153 163.
- D'incalci M., and Galmarini C.M.(2010) A review of trabectedin(ET-743): a unique mechanism of action. *Mol Cancer Ther.* 9(8);2157-63
- De Rienzo, F., Fanelli, F., Menziani, M.C., and De Benedetti P.G. (2000). Theoretical investigation of substrate specificity for cytochromes P450 IA2, P450 IID6 and P450 IIIA4. *J. Comput-Aided Mol. De.* 14 : 93 116.
- De Vries, E.G.E., de Hooge, M.N., Gietema, J.A., and de Jong, S. (2003). Target of Cancer Therapy. *Clin.. Cancer Res.* 9 : 912
- Demetri, G., Seiden, M., and Garcia, C. R., (2000). Ecteinascidin (ET-743) shows Promising activity in distinct populations of sarcoma patients: *Proceeding of ASCO.* 19 : 553-563
- Derewenda, Z.S. (2004). The use of recombinant methods and molecular engineering in protein crystallization. *Methods* 34 : 354–363.
- Dian, C., Eshaghi, S., Urbig, T., McSweeney, S., Heijbel, A., Salbert, G., and Birse, D. (2002). Strategies for the purification and on-column cleavage of

glutathione S-transferase fusion target proteins. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* 769: 133–144.

Dong, H., Nilsson, L., and Kurland, C.G. (1996). Co-variation of tRNA abundance and codon usage in *Escherichia coli* at different growth rate. *J. Mol. Biol.* 260 : 649-663.

Donovan, R.S., Robinson, C.W., and Glick, B.R. (1996). Review: optimising inducer and culture conditions for expression of foreign proteins under the control of the *lac* promoter. *Journal Ind. Microbiol.* 16 : 145–154.

Dougherty, W.G., Parks, T.D., Cary, S.M., Bazan, J.F., and Fletterick, R.J., (1989). Characterizations of the catalytic residues of the tobacco etch virus 49-kDa proteinase. *Virology* 172:302–310.

Duval, M., Suciu, S., Ferster, A., Rialland, X., Nelken, B., Lutz, P., Benoit, Y., Robert, A., Manel, A.M., Vilmer, E., Otten, J., and Philippe, N. (2002). Comparison of *Escherichia coli*–asparaginase with *Erwinia* asparaginase in the treatment of childhood lymphoid malignancies. *Blood* 99 : 2734–2739.

Dyxhoorn, D.M., Pierre, R.S. and Linn, T. (1996). A set of compatible *tac* promoter expression vectors. *Gene* 177 : 133-136.

Ebrahiminezhad, A., Rasoul-Amini, S., and Ghasemi, Y. (2011). L asparaginase Production by Moderate halophilic bacteria isolated from Maharloo Salt Lake. *Indian J. Microbiol.* 51(3): 307-311

Eden, O.B., Shaw, M.P., Lilleyman, J.S., and Richards S. (1990). Non randomized study comparing toxicity of *Escherichia Coli* and *Erwinia* asparaginase in children with leukaemia. *Med. Pediat. Oncol.*, 18: 497-502.

El-Bessoumy, A.A., Sarhan, M., and Mansour, J. (2004). Production, isolation a purification of L-asparaginase from *Pseudomonas aeruginosa* 50071 using solid-state fermentation. *J. Biochem. Molecular Biol.* 37: 3873932.

Elshafei, A.M., Hassan, M.M., Abouzeid, M.A.E., Mahmoud, D.A., and Elghonemy, D.H., (2012). Purification, Characterization and Antitumor Activity of L asparaginase from *Penicillium brevicompactum* NRC 829. *British Microbiology Research Journal* 2(3): 158-174.

Elvin, C.M., Thompson, P.R., Argall, M.E., Hendry, P., Stamford, N.P., Lilley, P.E., Dixon, N.E., (1990). Modified bacteriophage lambda promoter for overproduction of proteins in *Escherichia coli*. *Gene* 87: 123–126.

Fenchel, T., (2001). Ecology– marine bugs and carbon flow. *Science* 292: 2444-2445

- Floudas, C.A., Fung, H.K., McAllister S.R., Mönnigmann M., and Rajgaria R. (2006). Advances in protein structure prediction and de novo protein design: A review. *Chem. Eng. Sci.* 61 : 966 - 988.
- Fonda, I., Kenig, M., Gaberc-Porekar, V., Pristovaek, P., and Menart, V. (2002). Attachment of histidine tags to recombinant tumor necrosis factor α drastically changes its properties, *Sci. World J.* 15: 1312–1325.
- Francoijs, C.J., Klomp, J.P., and Knegtel, R.M., (2000). Sequence annotation of nuclear receptor ligand-binding domains by automated homology modeling. *Protein Eng.* 13:391-4.
- Fu, J., Togna, A.P., Shuler, M.L. and Wilson, D.B., (1992). *E. coli* host cell modifications in continuous culture affecting heterologous protein overexpression: a population dynamics study. *Biotechnol. Progr.* 8 : 340-346.
- Furth, O., and Friedmann, M. (1910). Über die Verbreitung asparagins paltender Organ fermente. *Biochem Z.* 26: 435-440.
- Goel, A., Colcher, D., Koo, J.S., Booth, B.J., Pavlinkova, G., and Batra, S.K., (2000). Relative position of the hexahistidine tag effects binding properties of a tumor-associated single-chain Fv construct, *Biochem. Biophys. Acta* 1523: 13–20.
- Gold, L., (1990). Expression of heterologous proteins in *Escherichia coli*. *Methods Enzymol.* 185: 11-14.
- Goldman, E., Rosenberg, A.H., Zubay, G., and Studier, F.W. (1995). Consecutive low usage leucine codons block translation only when near the 5' end of a message in *Escherichia coli*. *J. Mol. Biol.* 245: 467–473.
- Gronenborn, (1976). Overproduction of phage lambda repressor under control of the *lac* promoter of *Escherichia coli*. *Mol. Gen. Genet.* 148: 243–250
- Guanasekaran, S., McDonald, L., Manavathu, M., Manavathu, E., and Gunasekaran M., (1995). Effect of culture media on growth and L asparaginase production in *Nocardia asteroides*. *Biomed. Lett.* 52(207):197-203.
- Gurvich, O.L., Baranov, P.V., Gesteland, R.F., and Atkins J.F., (2005). Expression levels influence ribosomal frame shifting at the tandem rare arginine codons AGG_AGG and AGA_AGA in *Escherichia coli*. *J. Bacteriol.* 187:4023- 4032.
- Guzman, L.M., Belin, D., Carson, M.J., Beckwith, J., (1995). Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter. *J. Bacteriol.* 177: 4121–4130.

- Haldimann, A., Daniels, L., Wanner, B. (1998). Use of new methods for construction of tightly regulated arabinose and rhamnose promoter fusions in studies of the *Escherichia coli* phosphate regulon. *J. Bacteriol.* 180:1277–1286.
- Harley, C.B. and Reynolds, R.P. (1987). Analysis of *E. coli* promoter sequences. *Nucleic Acids Res.* 15 : 2243-2361.
- Haun, R.S., and Moss, J. (1992). Ligation-independent cloning of glutathione fusion genes for expression in *Escherichia coli*. *Gene* 112:37–43.
- Hernandez-Espinosa, D., Minano, A., Martinez, C., Perez-Ceballos, E., Heras, I., Fuster, J.L., Vicente, V., and Corral, J.(2006) L asparaginase –induced antithrombin type I deficiency: Implication for conformational disease. *Am J. Pathol.* 169:142-153
- Hoof, R.W., Sander, C., Vriend, G., and Abola, E. (1996). Errors in protein structures. *Nature* 381:272.
- Hosfield, T., and Lu, Q. (1999). Influence of the amino acid residue downstream of (Asp)₄Lys on enterokinase cleavage of a fusion protein. *Anal. Biochem.* 269 : 10–16.
- Huang, H.C., Sherman, M.Y., Kandror, O., Goldberg, A.L. (2001). The molecular chaperone DnaJ is required for the degradation of a soluble abnormal protein in *Escherichia coli*. *J. Biol. Chem.* 276:3920–3928.
- Ikemura, T. (1981). Correlation between the abundance of *Escherichia coli* transfer RNAs and the occurrence of their respective codons in its protein genes: a proposal for asynonymous codon choice that is optimal for the *E. coli* translational system. *J. Mol. Biol.* 151 : 389-409.
- Jones, S., and Thornton, J.M., (1996). Principle of protein-protein interactions. *Proc. Natl. Acad. Sci. USA.* 93:13-20
- Jordan M.A., Kamath K., manna T. *et. al.*,(2005) The primary antimiotic mechanism of action of the synthetic halichondrin E7389 is suppression of microtubule growth. *Mol Cancer Ther.* 4:1086-1095
- Jonasson, P., Liljeqvist, S., Nygren, P.A., and Stahl, S. (2002). Genetic design for facilitated production and recovery of proteins in *Escherichia coli*. *Biotechnol. Appl. Biochem.* 35 : 91-105.
- Jonasson, P., Liljeqvist, S., Nygren, P.A. and Stahl, S.(2002). Genetic design for facilitated production and recovery of recombinant proteins in *Escherichia coli*. *Biotechnol. Appl. Biochem.* 35 : 91-105.
- Kane, J.F. (1995). Effects of rare codon clusters on high-level expression of heterologous proteins in *Escherichia coli*. *Curr. Opin. Biotechnol.* 6:494-500.

- Karl, D.M. (2002). Hidden in a sea of microbes. *Nature* 415: 590–591.
- Karplus, M. and McCammon, J.A.(2002). Molecular dynamics simulations of biomolecules, *Nat. Struct Biol.* 9 (9) : 646–652.
- Kelly, S.M. and Price, N.C. (2000). The use of circular dichroism in the investigation of protein structure and function. *Curr. Protein Pept. Sci.* 1(4):349-384.
- Kidd, J.G. (1953). Regression of transplanted lymphomas induced *in vivo* by means of normal guinea pig serum.Course of transplanted cancers of various kinds in mice and rats given guinea pigserum, horse serum, or rabbit serum. *J. Exp. Med.* 98(6) : 565–582
- Klepeis, J.L. Lindorff-Larsen,K., Dror, R.O., andShaw, D.E. (2009).Long timescale molecular dynamics simulations of protein structure and function, *Curr. Opin. Struc. Biol.* 19 120–127.
- Ko, Y.H., Thomas, P.J., Delannoy, M.R., Pedersen, P.L. (1993). The cystic fibrosis transmembrane conductance regulator : Overexpression, purification, and characterization of wild type and delta F508 mutant forms of the first nucleotide binding fold in fusion with the maltose-binding protein. *J. Biol.Chem.* 268 : 24330–24338.
- Kotzia, G.A., and Labrou, N.E. (2007). L-asparaginase from *Erwinia Chrysanthemi* 3937: Cloning, expression and characterization. *Biotechnol.* 127(4) :657-669.
- Kou, G., Shi, S. Wang, H., Tan, M., Xue, J., Zhang, D., Hou, S., Qian W., Wang S., Dai, J., Li, B. and Guo, Y. (2007). Preparation and characterization of recombinant protein ScFv (CD11c) -TRP2 for tumor therapy from inclusion bodies in *Escherichia coli*. *Protein Expr. Purif.* 52:131-138.
- Krasotkina J., Borisova A.A., Gervaziev Y.V., Sokolov N.N. (2004).One-step purification and kinetic properties of the recombinant L-asparaginase from *Erwinia carotovora*. *Biotechnol. Appl. Biochem.*, 39: 215-221.
- Kozak, M., Borek, D., Janowski, R., and Jaskolski, M. (2002). Crystallization and preliminary crystallographic studies of five crystal forms of *Escherichia coli* L-asparaginase II (Asp90Glu mutant). *Acta Crystallography. D. Biol. Crystallogr.* 58 (1) : 130–132.
- Krieger, E, Koraimann, G., Vriend, G. (2002). Increasing the precision of comparative models with YASARA NOVA—a self-parameterizing forcefield. *Proteins* 47 (3): 393–402.
- Kuilman, M., and Wilms, L. (2007). Safety of the enzyme asparaginase, a means of reduction of acrylamide in food. *Toxicol. Lett.* 172: 196 S197.

- Kumar, S., Dasu, V.V., and Pakshirajan, K. (2010). Localization and production of novel L-asparaginase from *Pectobacterium carotovorum* MTCC 1428. *Process Biochem.* 45 : 223–229.
- Kurland, C. and Gallant, J. (1996). Errors of heterologous protein expression. *Curr. Opin. Biotechnol.* 7:489–493.
- Kumar, S. Tsai, C.J., and Nussinov, R. (2000). Factors enhancing thermostability, *Protein Eng.* 13(3): 179-191.
- Laatsch, H. (2008). Anti-Base, A Data Base for Rapid Structural Determination of Microbial Natural products, and annual updates, Wiley VCH: Weinheim, Germany
- Lang, S. (1904). Uber desamidierung im Tierkorper. *Beitr. Chem. Physiol. Pathol.* 5: 321-345.
- Law, A.S., and Wriston, J.C. (1971). Purification and properties of *Bacillus coagulans* L-asparaginase. *Arch. Biochem. Biophys.* 147(2) : 4744 4752.
- Mahajan, R.V, Saran, S., Kameswaran, K., Kumar, V., and Saxena, R.K. (2012). Efficient production of L-asparaginase from *Bacillus licheniformis* with low glutaminase activity: optimization, scale up and acrylamide degradation studies. *Bioresour. Technol.* 125: 11-16
- Makrides, S.C. (1996). Strategies for achieving high-level expression of genes in *Escherichia coli*. *Microbiol. Rev.* 60 (3) : 512-538.
- Manna, S., Sinha, A., Sadhukhan, R. and Chakrabarty, S.L. (1995). Purification, characterization and antitumor activity of L-asparaginase isolated from *Pseudomonas stutzeri*. MB-405. *Curr. Microbiol.* 30: 291-298.
- Mannan. S., Sinha, A., Sadhukhan R., and Chakrabarty, S. L., (1995). Purification, characterization and antitumor activity of Lasparaginase isolated from *Pseudomonas stutzeri* MB-405. *Curr Microbiol.*, 30, 291 298
- Mergulhao, F.J., Monteiro, G.A. (2004). Secretion capacity limitations of the sec pathway in *Escherichia coli*. *J. Microb. Biotechnol.* 14 : 128–133.
- Mesas, J.M., Gil, J.A., and Mart, J.F. (1990). Characterization and partial purification of L-asparaginase from *Corynebacterium glutamicum*, *J. Gen. Microbiol.* 136 (51) : 515-519.
- Michalska, K. and Jaskolski, M. (2006). Structural aspects of L asparaginases, their friends and relations, *Acta Biochimica Polonica* 53 : 627-640.

- Moola, Z.B., Scawen, M.D., Atkinson, T., Nicholls, D.J. (1994). *Erwinia chrysanthemi* L-asparaginase: epitope mapping and production of antigenically modified enzymes. *Biochem J.* 302 : 921–927.
- Moorthy, V., Ramalingam, A., Sumantha, A., and Shankaranaya, R.T. (2010). Production, purification and characterisation of extracellular L asparaginase from a soil isolate of *Bacillus sp.* *J. Microbiol. Res.* 4:1862-1867
- Moreno-Enriquez, Z., Evangelista-Martinez, E.G., Gonzalez-Mondragon, A., Calderon-Flores, R., Arreguin, E., Perez-Rueda, A., and Saquera, H. (2012). Biochemical characterization of recombinant L-asparaginase (AnsA) from *Rhizobium etli*, a member of an increasing rhizobial-type family of L-asparaginases, *J Microbiol Biotechnol.* 22(3): 292-300
- Mottram, D. S. and Wedzicha, B. L. (2002). Acrylamide is formed in the maillard reaction. *Nature* 419 : 448–449.
- Mukharjee, J., Majumdar, S., and Scheper, T. (2000). Studies on nutritional and oxygen requirements for production of L-asparaginase by *Enterobacter aerogenes*. *Appl. Microbiol. Biotechnol.* 53(2) : 180–184.
- Muller, H.J. and Boos, J. (1998). Use of L-asparaginase in childhood ALL. *Crit. Rev. Oncol. Hematol.* 28: 97–113
- Nagai, K., and Thogersen, H.C. (1984). Generation of b-globin by sequence specific proteolysis of a hybrid protein produced in *Escherichia coli*. *Nature* 309 : 810-812.
- Narta, U.K., Kanwar, S.S., and Azmi, W. (2007). Pharmacological and clinical evaluation of L-asparaginase in the treatment of leukemia. *Crit. Rev. Oncol. Hematol.* 61(3) : 208–221
- Narayana, K. J.P., Kumar, K.G. and Vijayalakshmi, M. (2008). L asparaginase production by *Streptomyces albidoflavus*. *Indian Journal of Microbiology* 48: 331-336.
- Nordström, K., and Uhlin, B.E. (1992). Runaway-replication plasmids as tools to produce large quantities of proteins from cloned genes in bacteria. *Bio/Technology* 10 : 661-666.
- Offman, M.N., Krol, M., Patel, N., Krishnan, S., Liu, J. Z., Saha, V. and Paul, A. (2011). Activity for cancer cell toxicity rational engineering of L asparaginase reveals importance of dual activity for cancer cell toxicity , *Blood* 117: 1614-1621
- Pattnaik, S., Kabi, R., Janaki, R.K., and Bhanot, K.K. (2000). L-asparaginase activity in *Aeromonas sp.* isolated from freshwater mussel. *Indian J. Exp. Biol.* 38 (11) : 1143-1146.

- Petsch, D., and Anspach, F.B., (2000). Endotoxin removal from protein solutions. *J. Biotechnol.* 21 76 (2-3) : 97-119.
- Phillips, T.A., Van Bogelen R.A., Neidhardt F.C. (1984). Ion gene product of *Escherichia coli* is a heat-shock protein. *J. Bacteriol.* 159: 283–287.
- Pichuantes, S. and Nguyen, A.T. and Franzusoff, A. (1996). Expression of heterologous gene products in yeast. In *Protein Engineering: Principles and Practice*, eds Jeffrey L. Cleland and Charles S. Craik p.p 518.. New York :John wiley and son Ltd.
- Pokrovskaya, M.V., Aleksandrova, S.S., Pokrovsky, V.S., Omeljanjuk, N.M. Borisova, A.A. Anisimova, N.Y. and Sokolov, N.N. (2012). Cloning, expression and characterization of the recombinant *Yersinia pseudotuberculosis* L-asparaginase. *Protein Expres. Purif.* 82 : 150-154.
- Prakasham, R.S., Rao, S., Lakshmi G.S., and Sarma, P.N. (2007). L-Asparaginase production by isolated *Staphylococcus sp.*– 6A: design of experiment considering interaction effect for process parameter optimization *J. Appl. Microbiol.* 102 :1382–1391
- Pritsa, A.A, and Kyriakidis, D.A. (2001). L-asparaginase of *Thermus thermophilus*: Purification, properties and identification of essential amino acids for its catalytic activity. *Mil. cell. Biochem.* 216 : 93-101
- Prokop, A., Bajpai, R.K. and Ho, C. (1991). Recombinant DNA Technology and Applications. New York: McGraw-Hill, Inc.
- Rahman, H. (2008). Unusual Sesquiterpenes: Gorgonenes and Further Bioactive Secondary Metabolites Derived from Marine and Terrestrial Bacteria. PhD Thesis, Universität Göttingen, Germany p. 158.
- Rinehart, K.L. (2000). Antitumor compounds from tunicates. *Med Res Rev.* 20: 1–27.
- Ritz, D., Lim, J., Reynolds, C.M., Poole, L.B., Beckwith, J. (2001). Conversion of a peroxiredoxin into a disulfide reductase by a triplet repeat expansion. *Science* 294:158–160.
- Robinson, M., Lilley, R., Little, S., Emtage, J.S., Yarranton, G., Stephens, P., Millican, A., Eaton, M., and Humphreys, G. (1984). Codon usage can affect efficiency of translation of genes in *Escherichia coli*. *Nucl. Acids Res.* 12 : 6663-6671.
- Rosen, J., and Hellena's, K. E. (2002). Analysis of acrylamide in cooked foods by liquid chromatography tandem mass spectrometry. *Analyst.* 127: 880–882.
- Rosenberg, M. and Court, D. (1979). Regulatory sequences involved in the promotion and termination of RNA transcription. *Annu. Rev. Genet.* 13:319-353.

- Russell, R.J., Ferguson, J.M., Hough, D.W., Danson, M.J., Taylor, G.L. (1997). The crystal structure of citrate synthase from the hyperthermophilic Archeon *Pyrococcus furiosus* at 1.9Å resolution. *Biochemistry*. 36 (33) : 9983-9984.
- Sahu, M.K., Poorani, E., Sivakumar, K., Thangaradjou, T., and Kannan, L., (2007). Partial purification and anti-leukemic activity of L-asparaginase enzyme of the actinomycete strain LA-29 isolated from the estuarine fish, *Mugil cephalus* (Linn.). *J Environ Biol*. 28(3) : 645-650.
- Salminen, T., Teplyakov, A., Kankare, J., Cooperman, B.S., Lahti, R., and Goldman, (1996). An unusual route to thermostability disclosed by the comparison Of *Thermus thermophilus* and *Escherichia coli* inorganic pyrophosphatases. *Protein Sci*. 5(6): 1014-25.
- Sambrook, J., and Russell, D.W., (2001), Molecular cloning: A laboratory manual, 3rd edition, Cold Spring Harbor Laboratory Press, Cold spring Harbor, New York. 1; 105- 118
- Sanches, M., Krauchenco K., and Polikarpov, I. (2007). Structure, substrate complexation and reaction. Mechanism of bacterial asparaginases, *Curr. Chem. Biol*. 175–86.
- Sanchez-Ruiz, J.M. and Makhatadze, G.I. (2001). To charge or not to charge? *Trends Biotechnol*. 19(4) : 132 - 135.
- Sarquis, M.I., Oliveira, E.M., Santos, A.S., Costa, G.L., (2004). Production of L asparaginase by Filamentous Fungi. *Mem Inst Oswaldo Cruz*, 99(5): 489-492
- Saxena, R.K., Sheoran, A., Giri, Bhoopander, Davidson, W.S., (2003). Purification strategies for microbial lipases. *J. Microbiol. Methods* 52:118.
- Schröder, O., Tippner, D., Wagner, R. (2001). Towards the three-dimensional structure of the *Escherichia coli* DNA-binding protein H-NS: a CD and fluorescence study. *Biochem. Biophys. Res. Commun*, 282 : 219-227.
- Schwartzmann, G., (2000). Marine organisms and other novel natural sources of new cancer drugs. *Ann Oncol*. 11: 235–243.
- Shine, J. and Dalgarno, L. (1974). The 3'-terminal sequence of *Escherichia coli* 16S ribosomal RNA: Complementary to nonsense triplets and ribosome binding sites. *Proc. Natl. Acad. Sci. USA* 71(4): 1342-1346.
- Shokri, A., Sande'n, A.M., and Larsson, G. (2003). Cell and process design for targeting of recombinant protein into the culture medium of *Escherichia coli*. *Appl. Microbiol. Biotechnol*. 60:654–664.

- Skerra, A. (1994) Use of the tetracycline promoter for the tightly regulated production of a murine antibody fragment in *Escherichia coli*. *Gene* 151: 131-135.
- Smyth, D.R., Mrozkiewicz, M.K., Mcgrath, W.J., Listwan, P. and Kobe, B.(2003).Crystal structures of fusion proteins with large-affinity tags. *Protein Sci.* 12:1313-1322.
- Sorensen, H.P. and Mortensen, K.K. (2005). Advanced genetic strategies for recombinant protein expression in *Escherichia coli*. *J. Biotechnol.* 115: 113-128.
- Sreerama, N. Venyaminov, S.Y., Woody, R.W. (2000). Estimation of protein secondary structure from circular dichroism spectra: Inclusion of denatured proteins with native proteins in the analysis. *Anal. Biochem.* 287: 243-251.
- Stecher, A.L., de-Deus, P.M., Polikarpov, I., and Abrahao-Neto, J. (1999). Stability of L-asparaginase: an enzyme used in leukemia treatment. *Pharm. Acta Helv.* 74: 1–9
- Studier, F.W., and Moffatt, B.A. (1986). Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol.* 189:113-130.
- Sun, Q.M., Chen, L.L., Cao, L., Fang, L., Chen, C., and Hua, Z.C., (2005). An improved strategy for high-level production of human vasostatin 120 180, *Biotechnol. Prog.* 21:1048–1052.
- Suresh, J.V., and Raju K.J. (2012). Studies on the Production of L Asparaginase by *Aspergillus terreus* MTCC 1782 using Agro Residues under Mixed Substrate Solid State Fermentation. *JCBPS* 3(1);314-325.
- Swartz, J.R., (2001). Advances in *Escherichia coli* production of therapeutic proteins. *Curr. Opin. Biotechnol.* 12: 195-201.
- Talukdar, A.S. and Wilson, D.L. (1999). Modeling and optimization of rotational C arm stereoscopic X-ray angiography. *IEEE Trans Med Imaging*,18:604-16.
- Tang , W., Sun Z.Y., panel, R., Gurewich, V., and Liu, J.N. (1997). An efficient system for production of recombinant urokinase type plasminogen activator, *Protein Expr. Purif.* 11: 279-283.
- Terpe, K. (2006). Overview of bacterial expression systems for heterologous protein production: from molecular and biochemical fundamentals to commercial systems. *Appl. Microbiol. Biotechnol.* 72: 211–222.
- Terpe, K. 2003. Overview of tag protein fusions: from molecular and biochemical fundamentals to commercial systems. *Appl. Microbiol. Biotechnol.* 60: 523 533.

- Tetin, S.Y., Prendergast, F.G. and Venyaminov, S.Y. (2003). Accuracy of protein secondary structure determination from circular dichroism spectra based on immunoglobulin examples. *Anal. Biochem.* 321:183-187.
- Theantana, T., Hyde, T., and Lymyong, S., (2009). Asparaginase production by endophytic fungi from Thai medicinal plants: cytotoxicity properties. *Int. J. Integrative Biol.* 7: 1-8.
- Vang Hendriksen, H., Stringer, M. A., Ernst, S., Held-Hansen, P., Schafermayer, R., and Corrigan, P. (2006). Novozymes A/S. Procter & Gamble Inc., Patent No. WOO6053563
- Verdino, P. and Keller, W. (2004). Circular dichroism analysis of allergens. *Methods* 32:241-248.
- Verma N., Kumar K., Kaur G., Anand S.,(2007). L-asparaginase: A promising chemotherapeutic agent. *Crit Rev Biotechnol.* 27: 45-62.
- Vidya, J., Vasudevan, U.M., Socco, C.R., and Pandey, A.(2011). Cloning, functional expression and characterization of L-Asparaginase II from *E. coli* MTCC 739, *Food Technol. Biotechnol* 49 (3) : 286–290.
- Vind, J., Sorensen, M. A., Rasmussen, M. D., and Pedersen, S. (1993). Synthesis of proteins in *Escherichia coli* is limited by the concentration of free ribosomes. *J. Mol. Biol.* 231, 678-688.
- Vitkup, D., Melamud, E., Moul,t J., and Sander, C. (2001). Completeness in structural genomics. *Nat Struct Biol* 8 : 559-66.
- Wang, X Campoli, M. Ko, E., Luo, W., and Ferrone, S. (2004). Enhancement of scFv fragment reactivity with target antigens in binding assays following mixing with anti-tag monoclonal antibodies, *J. Immunol. Methods* 294 : 23–35.
- Whitman, W.B, Coleman D.C., and Wiebes W.V. (1998). Prokaryotes: the unseen majority. Diversity of bacterio plankton. *Trends Ecol.* 95: 6578 6583
- Wilkinson, D.L., and Harison, R.G. (1991). Predicting the solubility of recombinant proteins in *Escherichia coli* .*Biotechnology* 9, 443-448.
- Willis, R.C. and Woolfolk, C.A, (1974). Asparagine utilization in *Escherichia coli* *J. Bacteriol* , 118, 231–241.
- Wong, W.C., Stroh, S.M., and Eisenhaber, F. (2011). Not all transmembrane helices are born equal: Towards the extension of the sequence homology concept to membrane proteins. *Biol Direct* 6:1-30.
- Wriston, J.C, and Yellin, T.O.(1973). L-asparaginase: a review. *Adv Enzymol Relat Areas Mol Biol* 39:185–248.

- Yano, S., Minato R., Thongsanit, J., Tachiki, T., and Wakayama, M., (2008). Overexpression of type I L-asparaginase of *Bacillus subtilis* in *E. coli*, rapid purification and characterization of recombinant type I L asparaginase. *Annals of Microbiology* 58 (4) : 711-716.
- Yao, M., Yasutake, Y., Morita H. and Tanaka, I., (2005) Structure of the type I L asparaginase from hyperthermophilic archaeon *Pyrococcus horikoshii* at 2.16 angstroms resolution, *Acta Crystallogr D*. 61, 294–301.
- Yellin, T.O., and Wriston J.C., (1966). Antagonism of purified asparaginase from guinea pig serum towards lymphoma. *Science* 151(713) : 998–999.
- Yu, L., Deng K.P., Yu C.A. (1995). Cloning, gene sequencing, and expression of the small molecular mass ubiquinone-binding protein of mitochondrial ubiquinol cytochrom c reductase. *J. Biol Chem* 270 : 25634–25638.
- Yun, M.K., Nourse, A., White, S.W., Rock, C.O., and Heath, R.J., (2007). Crystal structure and allosteric regulation of the cytoplasmic *Escherichia coli* L-asparaginase I, *J.Mol Biol.* 369 :794-811
- Zhou, Y., and Johnson, M.E., (1999). Comparative molecular modelling analysis of-5 amidinoindole and benzamidine binding to thrombin and trypsin: specific H bond formation contributes to high 5 amidinoindole potency and selectivity for thrombin and factor Xa. *J. Mol Recognit* 12 : 235-41.
- Wang Y., Qian S., Meng G., and Zhan S., (2001) cloning and expression of L asparaginase gene in *Escherichia coli* . *Adv. Biochem. Eng. Biotechnol* 95 ; 94-99