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Molecular Cloning, Heterologous Expression And Structural Modelling Of L-Asparaginase From Pseudopedobacter Saltans In E. Coli

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Abstract

L-Asparaginase aminohydrolase (EC 3.5.1) or L-asparaginase (L-ASP) is an enzyme capable of hydrolyzing L-Asn into aspartic acid and ammonia, which is used as a treatment for acute lymphoid leukemia. Cloning of L-asparaginase gene from A-novel Pseudopedobacter sltans DM12145 in E. coli. with accession number NC_015177.1. full-length of P. saltans L- asparaginase is 1019pb, protein encoding 339 amino acids; and molecular weight evaluated to be 37.8kDa, with theoretical (pI) is 6.13 kDa. It was cloned on the expression vector pET-28α-His (+) by EZ Clone method synonymously called ligation independent cloning (LIC) with protein ID WP_013634621.1". by Genscript Co., USA. Respectively. The recombinant of L-asparaginase I gene of P. saltans was expressed in pET28-αHis and transformed in E. coli BL21 (DE3); as a (6 his-tag fusion protein).and induced by one mM of (IPTG) for 18 hours at 30˚C, and purify by IMAC Chromatography, then analyzed by SDS-PAGE to assess the solubility and molecular weight of recombinant protein band was exactly as expected at 36.0 kDa. P. saltans L-asparaginase I enzyme maintained its enzymatic activity at a pH8.5, temperature 60˚C, with variable of kinetics Km value equal to 3 mM and a Vmax of 168.2 µmol/min/mg, finding of this study revel It is quite similar to L-asparaginases I of E. coli which is distinctly specific for L-Asparagine and act as homodimer cytosolic protein. Finally, the cloning and expression of A Novel-bacterial of P. saltans L-asparaginase enzyme in soluble and active stats, was successfully achieved.

Keywords: Pseudopedobacter saltans, L-asparaginase, IPTG I sopropyl β-D-1- thiogalactopyranoside, homodimer, Cloning, expression

Introduction

L-asparaginase (L-ASP), or l-Asparagine aminohydrolase (EC 3.5.1), is an enzyme capable of hydrolyzing L-Asn into aspartic acid and ammonia (Kumar, 2012), It is used in the treatment of Acute Lymphoid Leukemia, which

promotes the cleavage of the amino acid asparagine through the use of water and cleaves non-peptide carbon-nitrogen bonds (Erva, 2016). Two types of L-ASPs are observed: type I found in the cytosol and type II in the bacterial periplasm. L-asparaginase from gram-negative bacteria (EcA) is widely used in the treatment of ALL but its administration requires a crucial control for the maintenance of the patient's well-being. L-asparaginase from E. coli is thought to be the most potent tumor-killing enzyme in the world. Broome (1968) postulated that this high tumor inhibition capacity was due to the main factors, including high affinity of the enzyme for L- asparagine, translated into a low Michaelis-Menten (KM) constant considering that its glutaminase activity is identified as the responsible for the side effects.

These include allergic reactions (difficulty breathing, rash, fever, pain, redness, swelling in the injection area), symptoms of liver problems (darkness of urine, nausea, loss of appetite, pancreatitis) and neurological seizure (Erva, 2016). It was postulated that a low cross-glutaminolytic activity is important to avoid excessive side effects of the enzyme treatment. Therefore, several researches have been developed aiming the isolation of microbial strains that produce this important enzyme, such as Pseudomonas fluorescens, Serratia marcescens, Escherichia coli, Erwinia carotovora, Proteus vulgaris, Saccharomyces cerevisiae, Karnatakensis Streptomyces, Streptomyces venezuelae and several genera of fungi such as Aspergillus, Penicillium and Fusarium (Gallagher, 2010).Pseudomonas fluorescens, Mycobacterium phlei, Staphylococcus bacteria, Tetrahymena pyriformis and Thermus aquaticus, a thermophilic bacterium, were identified as producers (Verma et al., 2007). So, in this present study we will be describing the cloning, expression, purification of recombinant l-asparaginase from a novel bacterium Pseudopedobacter saltans into E. coli BL21 DE3.

Materials and Methods

Vectors and Bacterial strains

The pET-28a (+) vector was used as an expression vector, E. coli (BL21) Rosetta strain from (Promega, USA) that was used for expression hosts for recombinant proteins. (Laura Bertani media, Difco Laboratories/India), (Modified ezapelc's dox broth, interon/Korea), (Wizard genomic DNA purification, Promega/USA), (Wizard genomic DNA purification, Thermo Fisher/USA), (PCR master mix, Healthcare/USA), PMSF; Triton X100(Amresco/USA).

Cloning of asparaginase I encoding gene from Pseudopedobacter saltans

The open reading frame (ORF) encoding the asparaginase type I gene from the novel bacteria Pseudopedobacter sltans DM12145, and all its genetic information that related to determination its possession of the l-asparaginase gene was obtained from the NCBI website (http://www.ncbi.nlm.nih.gov). The L-asparaginase gene type I of Pseudopedobacter sltans DSM 12145 with the accession number NC_015177.1. associated with protein ID WP_013634621.1". It was cloned on the expression vector pET-28a (+) by EZ Clone method synonymously called ligation independent cloning (LIC) by Genscript Co., USA.

transformation and Heterologous expression of pET-28α-His (+)/asp_pseudopedo construct in bacterial system

The E. coli BL21(DE3) chemo-competent strain was transformed with the vectors pET-28a (+) and incubated on LB agar plates with kanamycin (34 µg/mL), at 37 °C for Overnight under 200 rpm agitation Expression induction was performed using 1 mM of (IPTG), incubating the culture medium at 30°C for 18 hours, under 200 rpm rotation.

Screening of the transformants E. coli BL21 (DE3) Rosetta cells harboring pET-28α-His (+)/asp_pseudopedo construct

The pET-28a (+)/asp_pseudopedo construct, assuming to carry the asparaginase type I gene, was isolated from five single colony and subjected to restriction digestion with both restriction enzyme XhoI and MluI, figure (1). then, subjected to PCR reaction using the universal primer set of the pET-28a (+) vector T7 promoter/T7 terminator. The sequence of the universal primer set T7 promoter/T7 terminator was as follow: T7 promoter (5'-TAATACGACTCACTATAG-3') and T7 terminator (5'- TAGTTATTGCTCAGCGGTGG -3'). The run PCR condition (Initial denaturation (1 cycle): 95℃, 5 min; Amplification (16 cycles): Each segment has: Denaturation: 98℃, 20s; Annealing: 69℃, 30s; Extension: 72℃, 30 s; Then amplification for 6 cycles Each segment has: Denaturation: 94℃, 20s; Annealing: 58℃, 30s; Extension: 72℃, 30 s; Final extension (1 cycle): 72oC, 10 min. finally the PCR products was subjected to Electrophoresis and SDS-PAGE.

In silico sequence and Phylogenetic analysis

The sequence of P. saltans L- asparaginase amino acid, was obtained by translating the nucleotide sequence using the translation tool at the ExPASy server (http://web.expasy.org/translate/). The prediction of secondary structure was performed according to the SAS online program (sequence annotated by structure) (https://www.ebi.ac.uk/thornton-srv/databases/sas/). Then, the dimensional structure expectation was done by submitting the sequence of the protein to the Swiss model server to obtain the data and the Three-dimensional structural prediction, were analyzed.

Results and Discussion

Pattern of PCR amplification of the insert using pET-28a (+)/asp_pseudopedo construct as a template and T7 promoter and T7 terminator primer set

To detect the transformation of the pET-28a (+)/asp_pseudopedo construct, by using the universal primer vector set T7 promoter and T7 terminator (Figure2). The amplified genomic the expected size of the PCR product is 1500 bp including the L-asparaginase type I gene (1019 bp) from P. saltans, and the remaining extra nucleotide were from the vector itself, after genomic sequencing, the correct nucleotide sequence was confirmed.

SDS-PAGE analysis of Heterologous expression in prokaryotic system and purification of pET-28a (+)/asp_pseudopedo

After the cell lysis the protein extract (soluble fraction) of the recombinant protein were purify with IMAC Chromatography, then was analyzed by SDS-PAGE to assess the solubility of the protein as well as to determine (M.W) of recombinant protein band was exactly as expected at 36.0 kDa. as show in figure 3. the result indicates that the P. saltans L-asparaginase is quite similar to type I- asparaginase. which is distinctly specific for L-asparagine located into cytosol and act in solution as homodimer. This homodimer is quite necessary for acting site formulation and in turn, it is essential for catalysis.

Determination of recombinant strain for L-asparaginase production

Modified ezapelc's dox broth way used for enrichment of bacteria producing L-asparaginase enzyme. The (MED) media was supplement asparagine as substrate and phenol red, as an indicator /when L-asparaginase act on L-asparagine, ammonia is liberated leading to conversion of yellow color in phenol red to pink, in alkaline condition. pH 6.8 at 25oC phenol red 0.0094. and sterilized by autoclaving at 121C for 20minute. the result as shown in figure 4, indicated that all the tested colonies by (MED) media, were an able to produce L-asparaginase enzyme with different levels.

Effect of pH and temperature on enzyme activity

The evaluation of the influence caused by the pH variation on the activity of the pET-28a (+)/L-ASP enzyme, was carried out based on the colorimetric method of Nesslerization, using different buffers, which ranged from pH 3 to 11. As can be seen in Figure 5, asp_pseudopedo, showed maximum catalytic activity at pH 8.5, as well as the assay that determines the optimum temperature for the enzymatic activity of pET-28a (+)/L-ASP, with the application of a temperature elevation ramp ranging from 20 °C to 90 °C. Regarding the influence of temperature on the activity of asp_pseudopedo, an optimal temperature of 60°C was obtained as shown in figure (6).

Enzyme Kinetics

To assesses the kinetic mechanism of the pET-28a (+)/L-ASP enzyme, where gradual concentrations of L-asparagine, whose variation range ranged from 62.5 µM to 9.91 mM, The kinetic curve assay aims to evaluate the hydrolytic capacity of the enzyme against different substrate concentrations up to the complete saturation point of the catalytic sites, under optimum pH and fixed temperature, As shown in Figure 7, asp_pseudopedo reaches the plateau at 8 mM L-asparagine, Thus, the kinetic calculations were calculated using the SigrafW software and indicated a Km value equal to 3 mM and a Vmax of 168.2 µmol/min/mg, approaching that shown by Willis & Woolfolk (1974).

Phylogenetic tree analysis Of Pseudopedobacter saltans

by sing the neighbor rejoining approach to obtain the genetic algorithms based on evolutionary distances was estimated from nucleotides and amino acid sequences of P. saltans L-asparaginase I, the phylogenetic relationship of P. saltans L. asparaginase I with other Pseudopedobacter species may be seen in the tree at the nucleotide and amino acid levels. Bacterial species shifted to separate clusters for the L. asparaginase gene at both nucleotide and amino acid levels (Fig 8), indicating that the organisms had diverged. (Saeed et al., 2018).

Annotation of the Pseudopedobacter saltans structure and 3D structure prediction

The secondary structure of asparaginase type I gene was predicted by SAS online program, The output of SAS program revealed that the asparaginase type I from P. saltans showed similarity sequence identity of 41.8% with the crystal structure of PDB (Protein Database Bank): 2OCD_A: asparaginase type I from Vibrio cholerae O1 biovar eltor str. N16961. The primary structure of P. saltans L-asparaginase I and protein module's secondary structure annotation expectations revealed some maintained distinctive lineaments, characteristic lineaments see Figure (9 and 10); P. saltans L. asparaginase I Classified by unique signature of common conserved for microbial L. asparaginase that characterized by the invariant amino acid residues; Thr12, Ala23, Lys25, Ser88, Asp89, Lys165, Leu291. that included in catalysis. Also, the P. saltans L-asparaginase I primary 2D structure involve a conserved catalytic residue to L. glutaminase I, the amino acid residues; Thr12, Ala23, Ser88, Glu286, Leu291 (fig 9 and 11). the expectation of the 2D structure was performed by using PSIPRED program Based on the amino acid composition, the predicted calculated (pI) for P. saltans L. asparaginase was found to be 6.13 (Saeed et al., 2018).

CONCLUSION AND RECOMMENDATIONS

1. Conclusively, this study did successfully produce the L-asparaginase from A-novel bacteria Pseudopedobacter saltans cloning and heterologous expression of the L-asparaginase in E. coli for the first time, it is become ready to study its therapeutic properties against cancer in the future.
2. and the finding of this study revel It is quite similar to type L-asparaginases I of E. coli which is distinctly specific for L-Asparagine that located into cytosol and act in solution as homodimer.
3. Finally, the cloning and expression of A Novel-bacterial of P. saltans L-asparaginase enzyme in soluble and active stats, was successfully achieved.

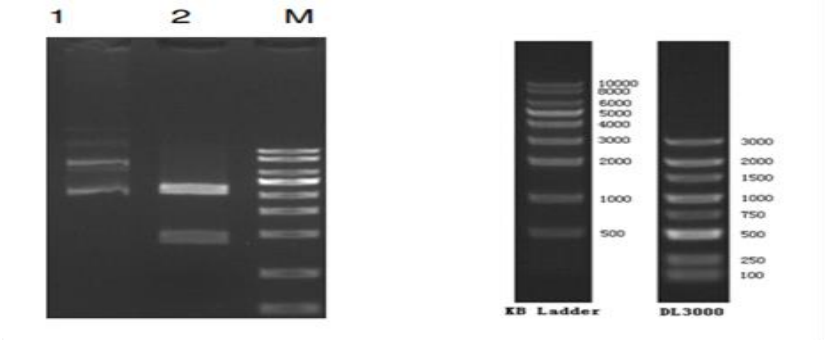


Figure 1: Agarose gel electrophoresis (1%) showing pattern of restriction digestion of pET-28a (+)/asp_pseudopedo with MluI and XhoI. The upper band: 4200 bp of the plasmid vector. The lower band: 1999 bp: including the insert L-asparaginase type I gene. M: 1kbp DNA ladder.

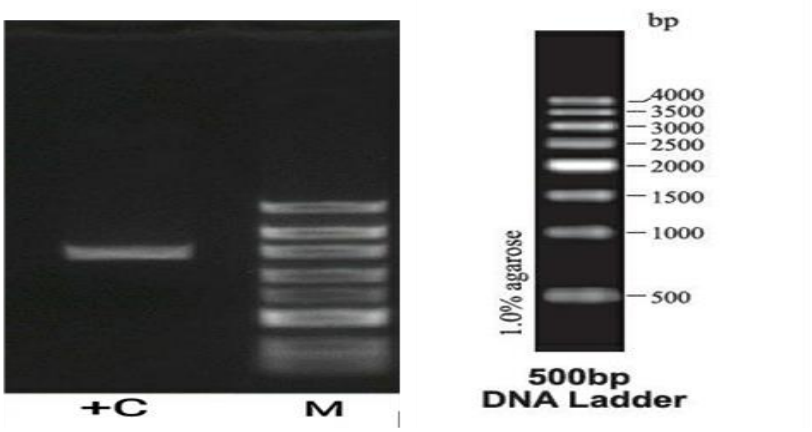


Figure 2: 1% agarose gel electrophoresis showing the PCR product resulting from using the universal primer vector set T7 promoter and T7 terminator and the pET-28a (+)/asp_pseudopedo construct as a template. [1% agarose gel proving positive colony for insertion of recombinant plasmid pET-28a (+)/asp_pseudopedo; MM: molecular marker (500bp DNA ladder Promega®), and +C; positive control of pET-28a (+)/asp_pseudopedo]

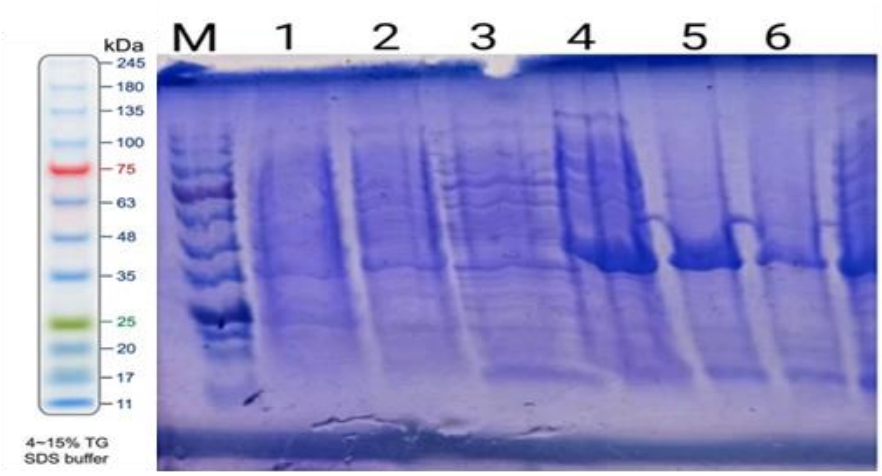


Figure 3: SDS-PAGE (10%) showing M: protein ladder, the lanes (4;5;6) is the expression of recombinant L-ASP soluble fraction proteins of induced E. coli BL21 (DE3) Rosetta harbouring asp_pseudopedo, using 1 mM IPTG. and the lanes (1;2;3) is represented the insoluble fraction proteins of uninduced E. coli BL21 (DE3) Rosetta / asp_pseudopedo.

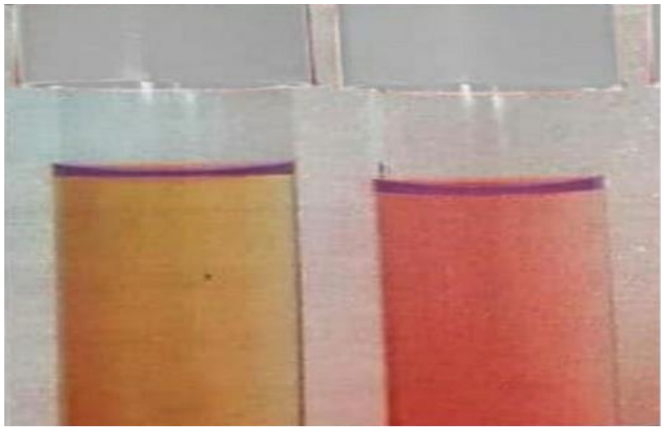


Figure 4: screening of L-asparaginase show pink color modified ezapelc's dox media supplemented with phenol Red; A/ yellow media devoid of L-asparaginase. pink color of phenol red in basic medium due to ammonium formation.

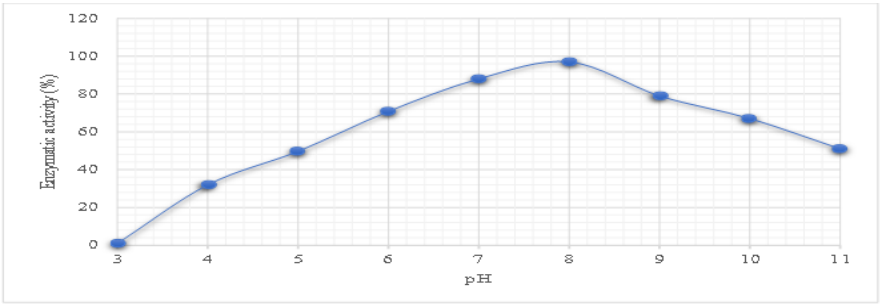


Figure 5: pET-28a (+)/L-ASP enzymatic activity at different pH [asp_pseudopedo, enzymatic activity profile (in percentage) for l-Asn consumption under different pH. N of samples of each protein = 3, Mean ± standard deviation (SD)]

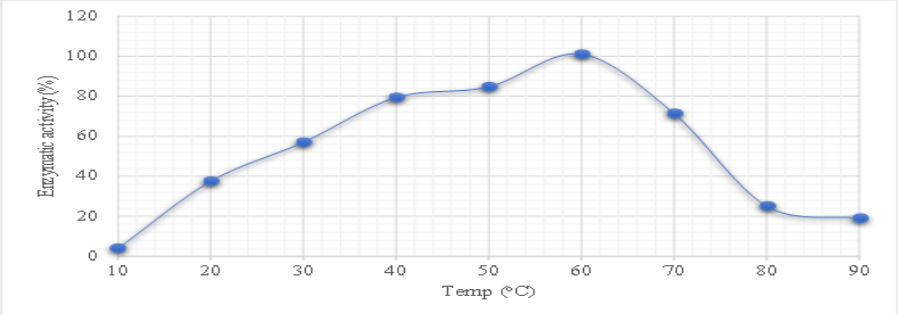


Figure 6: asp_pseudopedo, enzymatic activity at different temperatures

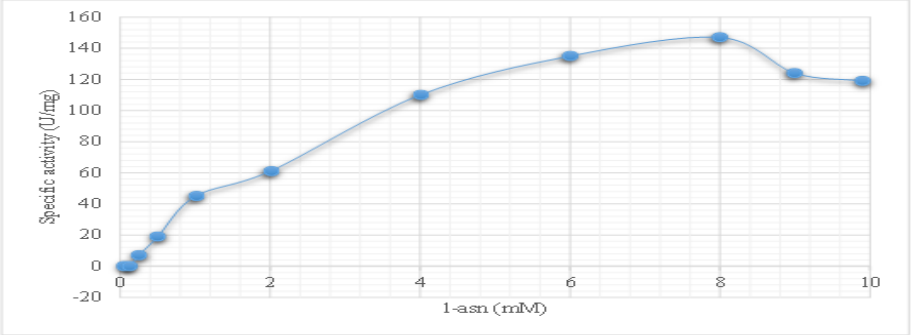


Figure 7: Kinetic curve of asp_pseudopedo, enzyme

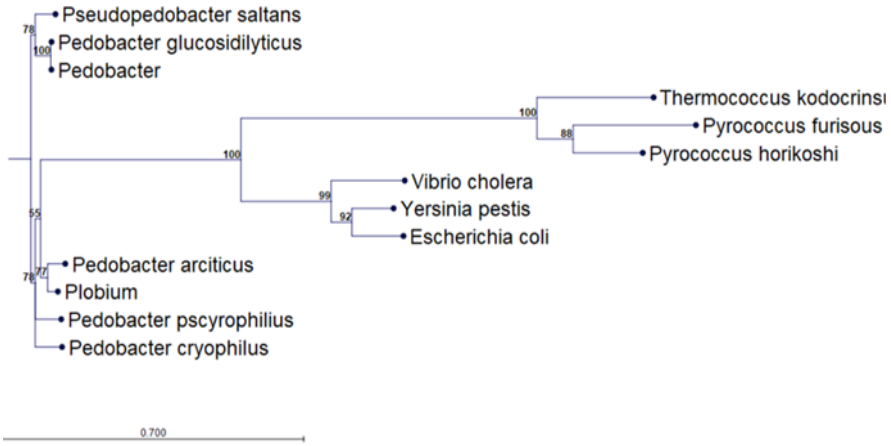


Figure 8: The Phylogenetic Tree of *Pseudopedobacter saltans*

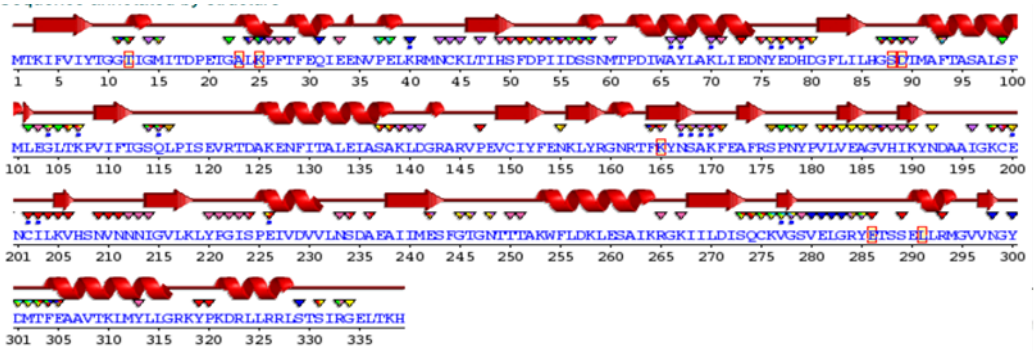


Figure 9: Predicted Secondary structure of asparaginase type I from *Pseudopedobacter saltans* by SAS online program based on the template 2OCD_A: asparaginase type I from *Vibrio cholerae* O1 biovar eltor str. N16961. It consisted of 17 β-sheets and 15 α-helices. Amino acid residues highlighted in red rectangles refer to amino acid residues involved in the catalytic residues: Thre12, Ala23, Pro26, Ser88, Asp 89, Lys 165, Phe164, Glu286, and Leu291.

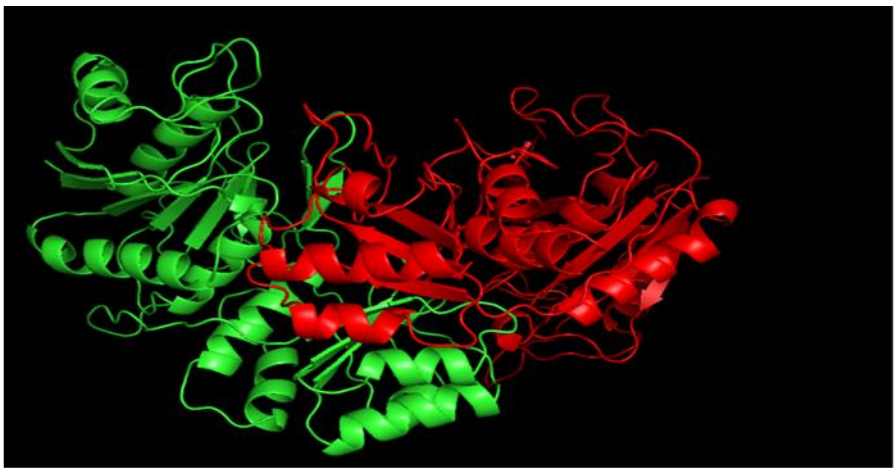
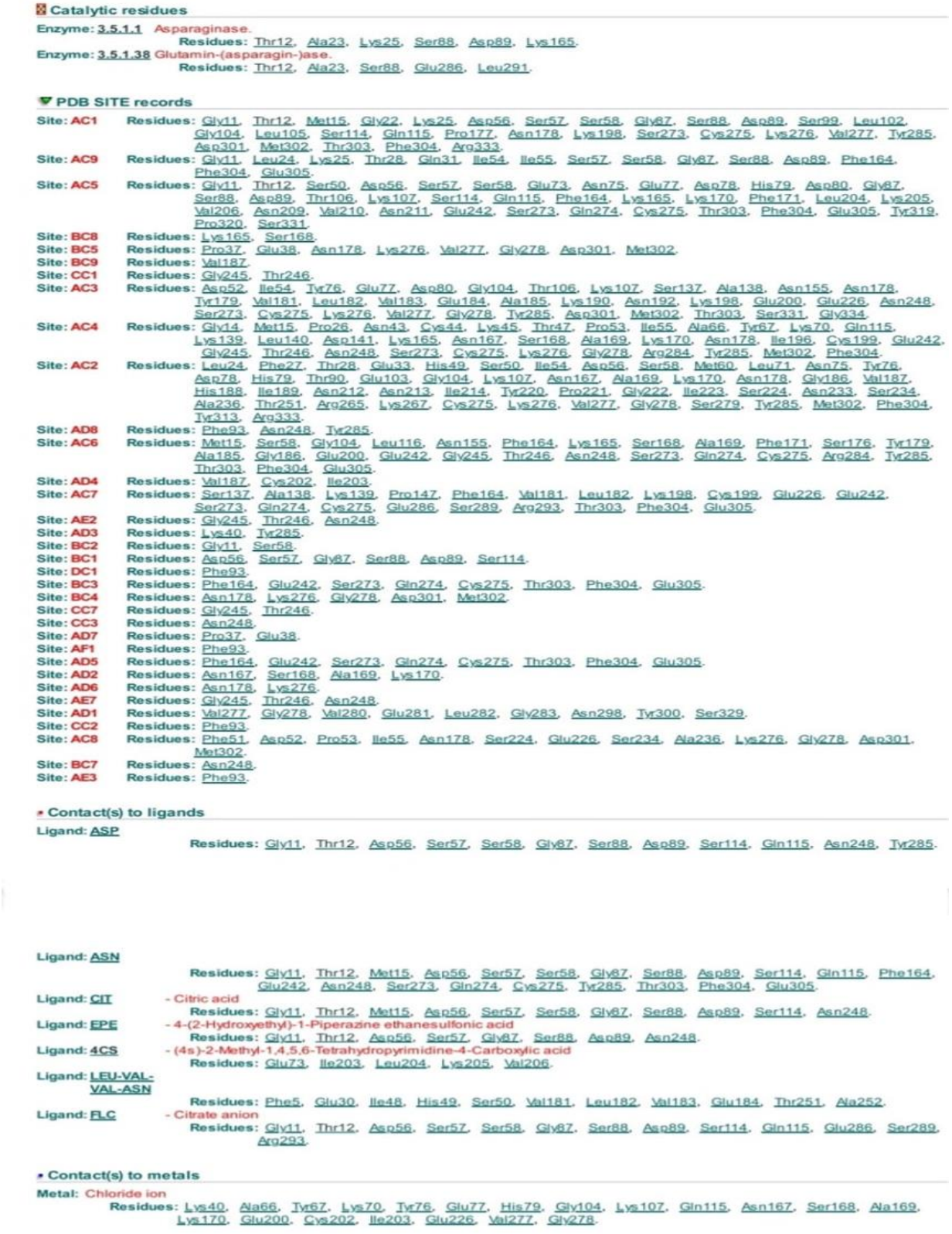


Figure 10: Protein homodimer structure view of *P. saltans* L-asparaginase I. the monomer in green color represents Methionine; and the monomer in red color represented the Lysin.

Figure 11: preserved the P. saltans L. asparaginase I of amino acid residues that is essential in different ligands and metal ions binding



Catalytic residues

Enzyme: 3.5.1.1 Asparaginase.
Residues: Thr12, Ala23, Lys25, Ser88, Asp89, Lys165.
Enzyme: 3.5.1.38 Glutamin-(asparagin-)ase.
Residues: Thr12, Ala23, Ser88, Glu286, Leu291.

PDB SITE records

Site: AC1 Residues: Gly11, Thr12, Met15, Gly22, Lys25, Asp56, Ser57, Ser58, Gly87, Ser88, Asp89, Ser99, Leu102, Gly104, Leu105, Ser114, Gln115, Pro177, Asn178, Lys198, Ser273, Cys275, Lys276, Val277, Tyr285, Asp301, Met302, Thr303, Phe304, Arg333.
Site: AC9 Residues: Gly11, Leu24, Lys25, Thr28, Gln31, Ile54, Ile55, Ser57, Ser58, Gly87, Ser88, Asp89, Phe164, Phe304, Glu305.
Site: AC5 Residues: Gly11, Thr12, Ser50, Asp56, Ser57, Ser58, Glu73, Asn75, Glu77, Asp78, His79, Asp80, Gly87, Ser88, Asp89, Thr106, Lys107, Ser114, Gln115, Phe164, Lys165, Lys170, Phe171, Leu204, Lys205, Val206, Asn209, Val210, Asn211, Glu242, Ser273, Gln274, Cys275, Thr303, Phe304, Glu305, Tyr319, Pro320, Ser331.
Site: BC8 Residues: Lys165, Ser168.
Site: BC5 Residues: Pro37, Glu38, Asn178, Lys276, Val277, Gly278, Asp301, Met302.
Site: BC9 Residues: Val187.
Site: CC1 Residues: Gly245, Thr246.
Site: AC3 Residues: Asp52, Ile54, Tyr76, Glu77, Asp80, Gly104, Thr106, Lys107, Ser137, Ala138, Asn155, Asn178, Tyr179, Val181, Leu182, Val183, Glu184, Ala185, Lys190, Asn192, Lys198, Glu200, Glu226, Asn248, Ser273, Cys275, Lys276, Val277, Gly278, Tyr285, Asp301, Met302, Thr303, Ser331, Gly334.
Site: AC4 Residues: Gly14, Met15, Pro26, Asn43, Cys44, Lys45, Thr47, Pro53, Ile55, Ala66, Tyr67, Lys70, Gln115, Lys139, Leu140, Asp141, Lys165, Asn167, Ser168, Ala169, Lys170, Asn178, Ile196, Cys199, Glu242, Gly245, Thr246, Asn248, Ser273, Cys275, Lys276, Gly278, Arg284, Tyr285, Met302, Phe304.
Site: AC2 Residues: Leu24, Phe27, Thr28, Glu33, His49, Ser50, Ile54, Asp56, Ser58, Met60, Leu71, Asn75, Tyr76, Asp78, His79, Thr90, Glu103, Gly104, Lys107, Asn167, Ala169, Lys170, Asn178, Gly186, Val187, His188, Ile189, Asn212, Asn213, Ile214, Tyr220, Pro221, Gly222, Ile223, Ser224, Asn233, Ser234, Ala236, Thr251, Arg265, Lys267, Cys275, Lys276, Val277, Gly278, Ser279, Tyr285, Met302, Phe304, Tyr313, Arg333.
Site: AD8 Residues: Phe93, Asn248, Tyr285.
Site: AC6 Residues: Met15, Ser58, Gly104, Leu116, Asn155, Phe164, Lys165, Ser168, Ala169, Phe171, Ser176, Tyr179, Ala185, Gly186, Glu200, Glu242, Gly245, Thr246, Asn248, Ser273, Gln274, Cys275, Arg284, Tyr285, Thr303, Phe304, Glu305.
Site: AD4 Residues: Val187, Cys202, Ile203.
Site: AC7 Residues: Ser137, Ala138, Lys139, Pro147, Phe164, Val181, Leu182, Lys198, Cys199, Glu226, Glu242, Ser273, Gln274, Cys275, Glu286, Ser289, Arg293, Thr303, Phe304, Glu305.
Site: AE2 Residues: Gly245, Thr246, Asn248.
Site: AD3 Residues: Lys40, Tyr285.
Site: BC2 Residues: Gly11, Ser58.
Site: BC1 Residues: Asp56, Ser57, Gly87, Ser88, Asp89, Ser114.
Site: DC1 Residues: Phe93.
Site: BC3 Residues: Phe164, Glu242, Ser273, Gln274, Cys275, Thr303, Phe304, Glu305.
Site: BC4 Residues: Asn178, Lys276, Gly278, Asp301, Met302.
Site: CC7 Residues: Gly245, Thr246.
Site: CC3 Residues: Asn248.
Site: AD7 Residues: Pro37, Glu38.
Site: AF1 Residues: Phe93.
Site: AD5 Residues: Phe164, Glu242, Ser273, Gln274, Cys275, Thr303, Phe304, Glu305.
Site: AD2 Residues: Asn167, Ser168, Ala169, Lys170.
Site: AD6 Residues: Asn178, Lys276.
Site: AE7 Residues: Gly245, Thr246, Asn248.
Site: AD1 Residues: Val277, Gly278, Val280, Glu281, Leu282, Gly283, Asn298, Tyr300, Ser329.
Site: CC2 Residues: Phe93.
Site: AC8 Residues: Phe51, Asp52, Pro53, Ile55, Asn178, Ser224, Glu226, Ser234, Ala236, Lys276, Gly278, Asp301, Met302.
Site: BC7 Residues: Asn248.
Site: AE3 Residues: Phe93.

Contact(s) to ligands

Ligand: ASP
Residues: Gly11, Thr12, Asp56, Ser57, Ser58, Gly87, Ser88, Asp89, Ser114, Gln115, Asn248, Tyr285.

Ligand: ASN
Residues: Gly11, Thr12, Met15, Asp56, Ser57, Ser58, Gly87, Ser88, Asp89, Ser114, Gln115, Phe164, Glu242, Asn248, Ser273, Gln274, Cys275, Tyr285, Thr303, Phe304, Glu305.
Ligand: CIT - Citric acid
Residues: Gly11, Thr12, Met15, Asp56, Ser57, Ser58, Gly87, Ser88, Asp89, Ser114, Asn248.
Ligand: EPE - 4-(2-Hydroxyethyl)-1-Piperazine ethanesulfonic acid
Residues: Gly11, Thr12, Asp56, Ser57, Gly87, Ser88, Asp89, Asn248.
Ligand: 4CS - (4s)-2-Methyl-1,4,5,6-Tetrahydropyrimidine-4-Carboxylic acid
Residues: Glu73, Ile203, Leu204, Lys205, Val206.
Ligand: LEU-VAL-VAL-ASN
Residues: Phe5, Glu30, Ile48, His49, Ser50, Val181, Leu182, Val183, Glu184, Thr251, Ala252.
Ligand: FLC - Citrate anion
Residues: Gly11, Thr12, Asp56, Ser57, Ser58, Gly87, Ser88, Asp89, Ser114, Gln115, Glu286, Ser289, Arg293.

Contact(s) to metals

Metal: Chloride ion
Residues: Lys40, Ala66, Tyr67, Lys70, Tyr76, Glu77, His79, Gly104, Lys107, Gln115, Asn167, Ser168, Ala169, Lys170, Glu200, Cys202, Ile203, Glu226, Val277, Gly278.

References

1. Abraham, M. J. et al. Gromacs: High performance molecular simulations through multi-level parallelism from laptops to supercomputers. Software X, v. 1–2, p. 19–25, 2015.

2. Acharya, K. R.; Lloyd, M. D. The advantages and limitations of protein crystal structures. Trends in Pharmacological Sciences, [S.l.], v. 26, n. 1, p. 10–14, 2005.

3. Allen, M. P.; Tildesley, D. J. Computer Simulation of Liquids. 2. ed. [s.l.] Oxford University Press, 2017.

4. Anandakrishnan, R.; Aguilar, B.; Onufriev, A. V. H++ 3.0: Automating pK prediction and the preparation of biomolecular structures for atomistic molecular modeling and simulations. Nucleic Acids Research, v. 40, n. 1, p. W537–W541, 2012.

5. Bejger, M. et al. Na+/K+exchange switches the catalytic apparatus ofpotassium- dependent plant L-asparaginase. Acta Crystallographica, [S.l.], v. 70, p. 1854 - 1872, 2014.

6. Berendsen, H. J. C. et al. Interaction models for water in relation to protein hydration. Intermolecular Forces, p. 331, 1981.

7. Berendsen, H. J. C. et al. Molecular dynamics with coupling to an external bath. The Journal of Chemical Physics, v. 81, n. 8, p. 3684–3690, 1984.

8. Bonvin, A. M. et al. Building macromolecular assemblies by information-driven docking: introducing the HADDOCK multibody docking server. Molecular and Cellular Proteomics, [S.l.], p. 1784-1794, 2010.

9. Broome, J. D. Antilymphoma activity of L-asparaginase in vivo: clearance rate of enzyme preparations from guinea pig serum and yeast in relation to their effects on tumor growth. Journal of the National Cancer Institute, [S.l.], v. 35, p. 967–74, 1965.

10. Brown, T.L.; Lemay, H.E., Brusten, B.E., Burdge, J.R. Chemistry: The Central Science, 9th ed., Pearson Education: São Paulo, 2005.

11. Caetano, L. F. Expression and biochemical characterization of a L-asparaginase II from Bacillus subtilis expressed in Escherichia coli: preliminary study of a potential biopharmaceutical, Course Conclusion Paper, Federal University of Ceará, Fortaleza, 63 f. 2017.

12. Carvalho, I.; Pupo, M.T.; Borges, A.D.L.; Bernardes, L. S. C. Introduction to molecular modeling of drugs in the experimental course of Pharmaceutical Chemistry. Química Nova, São Paulo, v. 26, p. 428-438, 2003.

13. Liolios, K., Sikorski, J., & Lu, M. (2011). Complete genome sequence of the gliding, heparinolytic Pedobacter saltans type strain. Standards in Genomic Sciences, 5 (1).

14. Steyn, P. L., Segers, P., Vancanneyt, M., Sandra, P., Kersters, K., & Joubert, J. J. (1998). Classification of heparinolytic bacteria into a new genus, Pedobacter, comprising four species: Pedobacter heparinus comb. nov., Pedobacter piscium comb. nov., Pedobacter africanus sp. nov. and Pedobacter saltans sp. nov. Proposal of the family Sphingobacteria ceae fam. nov. International Journal of Systematic and Evolutionary Microbiology, 48(1), 165-177.

15. Clementi, A. Enzymatic desemidation of asparagine in different animal species and the physiological significance of its presence in the organism. Arch. Int. Physiol. 19 (1922) 369–76.

16. Deehan, M.; Garces, S.; Kramer, D.; Baker, M. P.; Rat, D.; Roettger, Y.; Kromminga, A. Managing unwanted immunogenicity of biologicals. Autoimmunity Reviews, Elsevier, p. 1–6, 2015.

17. Deschavanne, P. et al. Bact Pep DB: a database of predicted peptides from an exhaustive survey of complete prokaryote genomes. The Journal of Biological Databases and Curation, [S.l.], v.106, p. 1–9, 2014.

18. E. Albanese and D. Kafkewitz, Effect of Medium Composition on the Growth and Asparajinase Production of Vibrio succinogenes, Appl. Environ. Microbiol., 36(1978) 25-30.

19. Erenler, S. Gencer, H. Geckil, B.C. Stark and D.A. Webster, Cloning and expression of the Vitreoscilla hemoglobin gene in Enterobacter aerogenes: effect on cell growth an oxygen uptake. Appl. Biochem. Microbiol., 40(2004) 241-248.

20. Erva, R. R. et al. Molecular dynamic simulations of Escherichia coli l-asparaginase to illuminate its role in deamination of asparagine and glutamine residues. 3 Biotech, [S.l.], v. 6, n. 1, 2016, 2 p.

21. Essmann, U. et al. A smooth particle mesh Ewald method. The Journal of Chemical Physics, v. 103, n. 19, p. 8577–8593, 1995.

22. Francisco Assis Leone, SigrafW: An easy-to-use program for fitting enzyme kinetic data, Biochemistry and Molecular Biology Education, Volume33, Issue6, November 2005, Pages 399-403

23. Gallagher E. R., Urayama K. Y., Buffler P. A., Ayoob J. M., Ma X. A meta-analysis of the association between day-care attendance and childhood acute lymphoblastic leukaemia. Int. J. Epidemiol., v. 39, n. 3, p. 718–732, nov. 2010.

24. Geckil H., Salihgencer; 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 and Microbial Technology, v. 35, n. 2–3, p. 182-189, ago. 2004.

25. Gomes, D. C. Biochemical and kinetic characterization of bacterial L-asparaginases and evaluation of their cytotoxic potential, Course Conclusion Paper, Federal University of Ceará, Fortaleza, 31 f. 2019.

26. Greenfield, N. J. Using circular dichroism spectra to estimate protein secondary structure. Nat Protoc., v. 1 (6) p. 2876–2890, 2006.

27. H.J. Müller and J. Boos Use of L-asparajinase in childhood ALL. Crit. Rev. Oncol. Hemat., 28(1998) 97-113.

28. Harrison C. J. Blood Spotlight on iAMP21 acute lymphoblastic leukemia (ALL), a high-risk pediatric disease. Blood, v. 125, p. 1383–1386, 2015.

29. Hess, B. P-Lincs: A parallel linear constraint solver for molecular simulation. Journal of Chemical Theory and Computation, v. 4, n. 1, p. 116–122, 2008.

30. J. Broome, Evidence that the L-asparajinase activity of guinea pig serum is responsible for its antilymphoma effects, Nature, 191(1961)1114–1115.

31. J. Mukherjee, S. Majumdar and T. Scheper, Studies on nutritional and oxygen requirements for production of L-asparajinase by Enterobacter aerogenes, Appl. Microbiol. Biotechnol., 53 (2000) 180-184.

32. J.C. Jr. Wriston and T.O.Yellin, L-asparajinase: a review. Adv. Enzymol. Relat. Areas. Mol. Biol. 39(1973)185-248.

33. Jawa, V.; Cousens, L. P.; Awwad, M.; Wakshull, E.; Kropshofer, H.;Groot, A. S. D. T-cell dependent immunogenicity of protein therapeutics: Preclinical assessment and mitigation. Clinical Immunology, Elsevier, v. 149, p. 534–555, 2013.

34. Khushoo, A; Pal, Y.; Singh, B. N.; Mukherjee, K. J. Extracellular expression and single step purification of recombinant Escherichia coli L asparaginase II. Protein Expression and Purification, v. 3838, p. 29 36, 2004.

35. Krupka, M., Masek, J., Barkocziova, L., Turanek Knotigova, P., Kulich, P., Plockova, J., Lukac, R., Bartheldyova, E., Koudelka, S., Chaloupkova, R., Sebela, M., Kyka, D., Droz, L., Effenberg, R., Ledvina, M., Miller, A. D., Turanek, J., Raska, M. The Position of His-Tag in Recombinant OspC and Application of Various Adjuvants Affects the Intensity and Quality of Specific Antibody Response after Immunization of Experimental Mice. PLOS ONE, v. 11(2), e. 0148497, 2016.

36. Kumar, K., Verma, N., The various sources and application of L-asparaginase. Asian Journal of Biochemical and Pharmaceutical Research, v. 2, n. 3, p. 197–205, 2012.

37. Lehninger, A.L.; Nelson, D.L.; Cox, M.M. Principles of Biochemistry. 6th ed. São Paulo: Artmed, p.200-212, 2014.

38. Lengauer, T.; Rarey, M. Computational methods for biomolecular docking. Current Opinion in Structural Biology, [S.l.], v. 6, p. 402-406, 1996.

39. LI, Y. F. Commonly used tag combinations for tandem affinity purification. Biotechnol. Appl. Biochem. v. 55, p. 73–83, 2010.

40. Liu, H.; Naismith, J. H. An efficient one-step site-directed deletion, insertion, single and multiple-site plasmid mutagenesis protocol. BMC biotechnology, v. 8, p. 91, 2008.

41. Lopes A. M., Oliveira-Nascimento L., De Ribeiro A., et al. Therapeutic l-asparaginase: upstream, downstream and beyond. Crit Rev Biotechnol, v. 8551, p. 1- 18, dec. 2015.

42. M.S. Nawaz, A.A. Khan, J.E. Seng, J.E. Leakey, P.H. Siitonen and C.E. Cerniglia, Purifcation and characterization of an amidase from an acrylamide-degrading Rhodococcus sp., Appl. Environ. Microbiol. 60(1994) 3343-3348.

43. Mashburn, L. T.; Wriston, J. C. Tumor inhibitory effect of L-asparaginase from Escherichia coli. Archives of Biochemistry and Biophysics, [S.l.], v. 105, p. 451–452, 1964.

44. Mccoy, T. A., Maxwell, M., Neuman, R. E. The amino acid requirements of the Walker Prakcarcinosarcoma 256 in vitro. Cancer Research, v. 16, p. 979–984, 1956.

45. Michalska, K.; Jaskólski, M. Structural aspects of l -sparaginases, their friends and relations. Acta Biochimica Polonica, [S.l.], v. 53, n. 4, p. 627–640, 2006.

46. Nelson, D.L.; COX, M.M. Lehninger's Principles of Biochemistry. 5. ed. Porto Alegre: Artmed, 2011.

47. Noura El Ahmady, E. N., Sara, M. E. E., Nancy, M. E. S. Microbial asparaginase as a potential therapeutic agent for the treatment of acute lymphoblastic leukemia: the pros and cons. International Journal of Pharmaceutics v. 10, p. 182 199, 2014.

48. OECD (Organisation for Economic Co-Operationd Development). Centralised and Decentralised Public Procurement. Sigma Working Papers 108. Paris: OECD Publishing. 2000.

49. Pacheco, D.L.A. Deimmunization of L-asparaginase from Escherichia coli by Genetic Algorithm. Course Completion Paper, Federal University of Ceará, Fortaleza, 86 f. 2019.

50. Porath, J., Carlsson, J., Olsson, I., & Belfrage, G. Metal chelate affinity chromatography, a new approach to protein fractionation. Nature, v. 258(5536), p. 598– 599, 1975.

51. S. Iuchi and E.C.C Lin, Adaptation of Escherichia coli to respiratory conditions: regulation of gene expression. Cell, 66(1991) 5-7

52. Salzer, W. L., Asselin, B. L., Plourde, P. V., Corn, T., Hunger SP. Development of asparaginase Erwinia chrysanthemifor the treatment of acute lymphoblastic leukemia. Annals of the New York Academy of Sciences, v. 1329, p. 81–92, 2014.

53. Sanches M., Barbosa J. A., De Oliveira R. T., Abrahao Neto J., Polikarpov I. Structural comparison of Escherichia coli L-asparaginase in two monoclinic space groups. Acta Crystallogr D Biol Crystallogr, v. 59, n. 3, p. 416–422, nov. 2003.

54. Sánchez, M. A. O., Ortega, M. L, O., Barrientos, J. V. R.; Acute lymphoblastic leukemia, Internal Medicine of Mexico, v.23, n.1, 2007.

55. Segel, I.H. Biochemistry: Theory and Problems. Translated Grassiano, DM Rio de Janeiro, Technical and scientific books s, 1979.

56. Shen, Y.; Maupetit, J.; Derreumaux, P.; Tuffery, P. Improved PEP-FOLD Approach for Peptide and Miniprotein Structure Prediction. Journal of Chemical Theory and Computation, [S.l.], v. 10, p. 4745–4758, 2014.

57. Sockolosky, J. T. Szoka, F. C. Periplasmic production via the pET expression. system of soluble, bioactive human growth hormone. Protein Expression and Purification, v. 87, p. 129-135, 2013.

58. Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). Molecular cloning: a laboratory manual (No. Ed. 2). Cold spring harbor laboratory press.

59. Swain, A. Crystal structure of Escherichia coli L-asparaginase, an enzyme used in cancer therapy. Proceedings of the National Academy of Sciences, [S.l.], v. 90, p. 1474–1478, 1993.

60. T. Tosa, R. Sano, K. Yamamoto, M. Nakamura, K. Ando and I. Chibata, L- Asparajinase from Proteus vulgaris, Appl. Microbiol., 22 (1971) 387-392.

61. Verma N., Kumar K., Kaur G., Anand S. L-asparaginase: a promising chemotherapeutic agent. Crit Rev Biotechnol, v. 27, n. 1, p. 45-62, out. 2007. Disponível em: http://dx.doi.org/10.1080/07388550601173926

62. Vidya, J., Vasudevan, U. M., Soccol, C. R., Pandey, A. L Asparaginase II from E. coli MTCC 739. Food Technol. Biotechnol, v. 49, n. 3, p. 286 2 90, 2011.

63. Warrel, R. P. et al. Clinical evaluation of succinylated Acinetobacter glutaminase- asparaginase in adult leukemia. Cancer Treatment Reviews, [S.l.], v. 66, n. 7, p.1479-1485, 1982.

64. Weeb, E. C. Enzyme nomenclature 1992. Recommendations of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology on the Nomenclature and Classification of Enzymes, 6. ed. [S.l.], 1992.

65. Willis, R. C., Woolfolk, C. A. Asparagine utilization in Escherichia coli, Journal of Bacteriology, v. 118, n. 1, p. 231–241, 1974.

66. Yan Y, Steinherz P, Klingemann HG, Dennig D, Childs BH, McGuirk J, O'Reilly RJ. Antileukemia activity of a natural killer cell line against human leukemias. Clin Cancer Res. 1998 Nov;4(11):2859-68. PMID: 9829753.

67. Yang, J. et al. The I-TASSER Suite: protein structure and function prediction. Nature Methods, [S.l.], p. 7-8, 2015.

68. Yao, H., Wynendaele, E., De Spiegeleer, B. Thermal sensitivity as a quality control attribute for biotherapeutics: The L-asparaginase case. Drug Test Anal; 1–11. 2019.

69. Yazdi, Mehdi & Kolahi, Maryam & Foroghmand, A. & Tabandeh, Mohammad reza. (2020). In silico assessment of plant L-asparaginase and estimating its allergenicity in comparison to bacteria asparaginase. Pediatric Hematology/Oncology and Immunopathology. 19. 35-46. 10.24287/1726-1708-2020-19-1-35-46.

70. Young, D. C. Computational chemistry: a practical guide for applying techniques to real- world problems. Wiley-Interscience, [S.l.], 2001, 369 p.

71. Zhang, Y. I-TASSER server for protein 3D structure prediction. BMC Bioinformatics, [S.l.], 2008.

72. Zhao, H.; Verma, D.; Li, W.; Choi, Y.; Ndong, C.; Fiering, S. N.; Bailey-Kellogg, C.; Griswold, K. E. Depletion of T cell epitopes in lysostaphin mitigates anti-drug antibody response and enhances antibacterial efficacy in vivo. Chemistry and Biology, Elsevier, v. 22, p. 629–639, 2015.

73. Saeed, H., Ali, H., Soudan, H., Embaby, A., El-Sharkawy, A., Farag, A., ... & Ataya, F. (2018). Molecular cloning, structural modeling and production of recombinant Aspergillus terreus L. asparaginase in Escherichia coli. International journal of biological macromolecules, 106, 1041-1051.

74. Swain, A. L., Jaskólski, M., Housset, D., Rao, J. K., & Wlodawer, A. (1993). Crystal structure of Escherichia coli L-asparaginase, an enzyme used in cancer therapy. Proceedings of the National Academy of Sciences, 90(4), 1474-1478.

75. Yun, M. K., Nourse, A., White, S. W., Rock, C. O., & Heath, R. J. (2007). Crystal structure and allosteric regulation of the cytoplasmic Escherichia coli L-asparaginase I. Journal of molecular biology, 369(3), 794-811.

76. El-Naggar, N. E., El-Ewasy, S. M., & El-Shweihy, N. M. (2014). Microbial L-asparaginase as a potential therapeutic agent for the treatment of acute lymphoblastic leukemia: the pros and cons. Int J Pharmacol, 10(4), 182-199.

77. Nagarethinam, S., Naik, A. N., Udupa, N., Rao, V. J., & Vanathi, M. B. (2012). Microbial L-Asparaginase and its future prospects. Asian Journal of Medical Research, 1(4), 159-168.

78. Abdel-Fattah, M. K. (1995). Studies on the asparaginolytic enzymes of Streptomycetes. 1.-culture conditions for the production of L. as paraginase enzyme from Streptomyces longs porusflavus (F-15) strain. Egyptian Journal of Microbiology (Egypt).

79. Gurunathan, B., & Sahadevan, R. (2012). Optimization of Culture Conditions and Bench-Scale Production of $ _L $-Asparaginase by Submerged Fermentation of Aspergillus terreus MTCC 1782. Journal of microbiology and biotechnology, 22(7), 923-929.

80. WEHNER, A., HARMS, E., JENNINGS, M. P., BEACHAM, I. R., DERST, C., BAST, P., & RÖHM, K. H. (1992). Site-specific mutagenesis of Escherichia coli asparaginase II: None of the three histidine residues is required for catalysis. European journal of biochemistry, 208(2), 475-480.

81. Kotzia, G. A., & Labrou, N. E. (2005). Cloning, expression and characterisation of Erwinia carotovora L-asparaginase. Journal of Biotechnology, 119(4), 309-323.

82. Makrides, S. C. (1996). Strategies for achieving high-level expression of genes in Escherichia coli. Microbiological reviews, 60(3), 512-538.

83. Baneyx, F. (1999). Recombinant protein expression in Escherichia coli. Current opinion in biotechnology, 10(5), 411-421.

84. Radha, R., Arumugam, N., & Gummadi, S. N. (2018). Glutaminase free L asparaginase from Vibrio cholerae: heterologous expression, purification and biochemical characterization. International journal of biological macromolecules, 111, 129-138.

85. Pokrovskaya, M. V., Aleksandrova, S. S., Pokrovsky, V. S., Omeljanjuk, N. M., Borisova, A. A., Anisimova, N. Y., & Sokolov, N. N. (2012). Cloning, expression and characterization of the recombinant Yersinia pseudotuberculosis L-asparaginase. Protein expression and purification, 82(1), 150-154.

86. Ho, P. P., Milikin, E. B., Bobbitt, J. L., Grinnan, E. L., Burck, P. J., Frank, B. H., ... & Squires, R. W. (1970). Crystalline l-asparaginase from Escherichia coli B: I. purification and chemical characterization. Journal of Biological Chemistry, 245(14), 3708-3715.

87. Jalil, A. T., & Karevskiy, A. (2020). The Cervical Cancer (CC) Epidemiology and Human Papillomavirus (HPV) in the Middle East. International Journal of Environment, Engineering & Education, 2(2), 7-12. https://doi.org/10.5281/zenodo.3972634

88. Turki Jalil, A., Hussain Dilfy, S., Oudah Meza, S., Aravindhan, S., M Kadhim, M., & M Aljeboree, A. (2021). CuO/ZrO2 nanocomposites: facile synthesis, characterization and photocatalytic degradation of tetracycline antibiotic. Journal of Nanostructures.

89. Jalil, A. T. (2020). COVID-19 most affected age groups and lethality in Europe, Glob. J. Public Health Med, 2, 179-184. https://doi.org/10.37557/gjphm.v2iSP1.51

99. Mezal, E. H., Yousif, A. F., Hanan, Z. K., Hanan, A. K., & Jalil, A. (2020). Isolation, Assessment of Antimicrobial Sensitivity of Bacterial Pathogens from Post-Cesarean section Infection of patients in Thi-Qar Province. European Journal of Molecular & Clinical Medicine, 7(3), 958-964.

100. Mubark, N. N., Jalil, A. T., & Dilfi, S. H. (2020). DESCRIPTIVE STUDY OF HYDATIDIFORM MOLE ACCORDING TO TYPE AND AGE AMONG PATIENTS IN WASIT PROVINCE, IRAQ. Global Journal of Public Health Medicine, 2(1), 118-124. https://doi.org/10.37557/gjphm.v2i1.30

101. Turki Jalil, A. ., Dilfi, S. H. ., & Karevskiy, A. . (2019). SURVEY OF BREAST CANCER IN WASIT PROVINCE , IRAQ. Global Journal of Public Health Medicine, 1(2), 33–38. https://doi.org/10.37557/gjphm.v1i2.7

102. Jaleel, A. T. (2018). SURVEY THE PREVALENCE OF VIRAL HEPATITIS A, B, C INFECTION IN DHI-QAR PROVINCE (IRAQ). ББК 20.1 А43 Редакционная коллегия: ИБ Заводник (отв. ред.), АЕ Каревский, ОВ Янчуревич, ОВ Павлова, 95.

103. Jalil, A. A. T. EPIDEMIOLOGY OF CERVICAL CANCER AND HIGH RISK OF HUMAN PAPILLOMA VIRUS IN PATIENT. ББК 28.6 3, 85(7).