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Molecular Characterization of a Thermostable L-Asparaginase from *Geobacillus* SBS-4S

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ABSTRACT

L-Asparaginase enzyme is known for its utilization in the treatment of acute lymphoblastic leukemia (ALL) and in the food industry as it depletes acrylamide which is a carcinogen often produced in baked foods. L-Asparaginase enzyme hydrolyzes L-asparagine into L-aspartate. This enzyme also bears a glutaminase activity which is a side effect of this enzyme when used for ALL treatment. In this study, molecular characterization of unique L-asparaginase from *Geobacillus* strain SBS-4S (geoAsnase) has been explored which involves *in silico* analysis of its 3D structure followed by its characterization. Visualization of structure-based docking of L-asparaginase with L-asparagine showed Phe176 and Glu270 residues to be involved in forming hydrogen bonds and Tyr161, Ala175, Tyr298 and Asn299 forming hydrophobic interactions with the substrate. L-asparaginase gene was cloned and expressed in *E. coli*. Purified enzyme (~35kDa) was used to check its asparaginase as well as glutaminase activity. This enzyme showed asparaginase specific activity of 39.8 U/mg at 65 ˚C and pH 6.9 whereas its glutaminase activity was found to be almost negligible i.e. 0.0066 U/mg. This specific regioselectivity of geoAsnase towards asparagine, makes it much more appropriate asparaginase treatment rather than other enzymes, for its application in the treatment of ALL, as it will not exert cytotoxic effect due to circulating glutamine in patient's plasma. The thermostability of the enzyme at 65 ˚C also makes it suitable for its utilization in food industry. Based on the present study geoASNase has a potential for its application in medicinal and food industries.

INTRODUCTION

L-Asparaginase (L-asparagine amidohydrolase, E.C.3.5.1.1) hydrolyzes L-asparagine into L-aspartic acid and ammonia (Sallan *et al*., 1983). This holds extreme significance in medicinal world (Schwartz *et al*., 1966; Beacham and Jennings, 1990). Antitumor activity of this enzyme cannot go unnoticed and is currently being used to cure acute lymphoblastic leukemia (ALL) (Appel *et al*., 2007; Jennings and Beacham, 1990; Avramis and Tiwari, 2006; Kumar *et al*., 2014; Pui and Evans., 2006; Verma *et al*., 2007). All cells in human body can make their own L-asparagine using L-asparagine synthetase whereas tumor cells lack this ability due to low level of synthetase enzyme in them (Haskell and Canellos, 1969). Deficiency of asparagine in neoplastic cells leads to decrease in protein and glycoprotein production and ultimately death of leukemia cells by apoptosis (Bussolati *et al*., 1995). Cancer cells depend on circulatory blood L-asparagine to fulfill their needs (Rogers., 1989). Introducing exogenous L-asparaginase enzyme depletes L-asparagine in blood hence leading to lack of nutrition for cancer cells. This enzyme has proven to be an excellent choice for ALL treatment (Crowther, 1971; McCreadie *et al*., 1973; Wriston and Yellin, 1973; Yoshimoto *et al*., 1986) but its long term use elicits allergic reactions on skin (Sarquis *et al*., 2004) and anaphylaxis (Verma *et al*., 2007). Use of prokaryotic L-asparaginase leads towards hypersensitivity and hence immune inactivation because of secondary L-glutaminase activity of enzyme (Lopes *et al*., 2017) and hence that of eukaryotes has proven to be better for medicinal purposes (Narta *et al*., 2007). It is generally anticipated that by overcoming these problems can hopefully open ways to

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cure blood cancer completely (Schrappe *et al*., 2000). Asparaginase from different sources like *Escherichia coli* and *Erwinia chrysanthemi* is currently available in market both in native and PEGylated forms (Tong *et al*., 2014).

Other uses of asparaginase include food industry where fried starchy food develops acrylamide due to the reaction between L-asparagine and reducing sugars present in food (Millard reaction) (Tareke *et al*., 2002). Possible hazards related to acrylamide include carcinogenicity, genotoxicity, neurotoxicity and reproductive toxicity (Lyon, 1994; Vinci *et al*., 2012; Boegl, 2006; Friedman, 2003; Dybing *et al*., 2005). Pretreatment of carbohydrates with L-asparaginase ceases the acrylamide synthesis (Dhanam and Kannan, 2013; Kumar et al., 2014). Another beneficial use of L-asparaginase is as biosensor to measure the amount of L-asparagine present in the food or tumor cells. The measure is based on the extent of increasing of pH due to depletion of asparagine, resulting in the release of ammonia by the action of enzyme.

In this study, L-asparaginase from *Geobacillus* strain SBS-4 is recombinantly produced in *E. coli*. *Geobacillus* SBS-4S is a thermostable microorganism isolated from Gilgit, Northern areas of Pakistan and is known to produce industrially important thermostable enzyme such as lipase, protease and amylase (Tayyab *et al*., 2011a, b). L-Asparaginase from this source contains 323 amino acids with a theoretical molecular weight of 35.2 kD (see Supplemantary Fig. 1 for amino acid sequence), and isoelectric point (pI) of 6.47 (https://web.expasy.org/protparam/). Structure of L-asparaginase is mostly homo-tetrameric (Epp *et al*., 1971) when isolated from *E. coli* but can be hexameric as well when isolated from other microbial sources. AlphaFold based prediction showed the 3D structure of the monomeric protein. Binding affinities of L-asparagine, L-glutamine and D-asparagine with geoAsnase enzyme were determined using structure-based molecular docking. Molecular dynamic (MD) simulations predicted better L-asparaginase activity of this enzyme as compared to glutaminase activity. This study also manifest the amino acid residues involved in glutaminase activity which can be directed through mutagenesis for improving other L-asparaginases to get rid of their glutaminase activity.

MATERIALS AND METHODS

Bioinformatic analysis

L-asparaginase 3D structure prediction was done using AlphaFold software. Binding affinities of L-asparagine (L-ASN), L-glutamine (L-GLN) and D-asparagine (D-ASN) with L-asparaginase were determined using CB dock2 software and Vina score values were used as a predictor of the best docking model. For the molecular dynamics simulation of geoAsnase enzyme with L-ASN and L-GLN, we employed GROMACS. The structures of L-ASN and L-GLN were modeled and energy minimized using Avogadro. Topologies for the enzyme and ligands were generated using the GROMACS pdb2gmx tool with the CHARMM36 force field. The protein-ligand complex was solvated in a cubic water box using the SPC water model. Ions were added to neutralize the system by replacing water molecules with sodium or chloride ions. An energy minimization step was conducted using the steepest descent algorithm, with a convergence criterion set to a maximum force of 1000 kJ/mol/nm. The system was equilibrated in two phases: initially in the NVT ensemble (constant number of particles, volume, and temperature) for 500 ps at 300 K using a V-rescale thermostat, followed by the NPT ensemble (constant number of particles, pressure, and temperature) for an additional 500 ps at 300 K and 1 bar pressure. Finally, a 100 ns production run was performed to analyze the dynamics of the enzyme-ligand interactions. The results were analyzed using GROmancer (https://bioinfoxpert.de/gromancer-the-md-wizard/). Root mean square deviations (RMSD), root mean square fluctuations (RMSF) and hydrogen bonds formations over time between the binding pocket of geoAsnase and ligands (L-ASN and L-GLN) were determined.

Bacterial strains, culture conditions and plasmids

Geobacillus SBS-4S strain was provided by Naeem Rashid from School of Biological Sciences, University of the Punjab, and was used in this study as a source of L-asparaginase gene. *Escherichia coli* DH5α was used for gene cloning and *E. coli* BL21-CodonPlus (DE3)-RIL (Stratagene, USA) was used for gene expression. LB medium (1% tryptone, 0.5% yeast extract and 0.5% NaCl) was used to grow all the three above mentioned strains, at 37 ˚C, and glycerol stocks were stored at -80 ˚C. pCR-TOPO cloning vector (Thermo fisher Scientific, USA) was used for general DNA cloning, pET-21a (Stratagene) was used for the expression of the gene.

PCR, cloning and expression of geoAsnase

Forward (5’-CATATGACGAAACGGAAAGTG-GTGCTCCTG-3’) and reverse (5’-CTAATAAGCGAA-CAAGCCATCAACGGCGG-3’) primers were designed using Primer 3 software against L-asparaginase gene of *G. cillus* SBS-4S, DNA sequence information available in the NCBI database (Accession number AYSF01000054). Primers were confirmed by using BLAST and Oligocalc tool.

L-Asparaginase gene was amplified from the whole genome of *Geobacillus* SBS-4S by PCR using forward and reverse primers. PCR amplified DNA was

inserted in pCR-TOPO vector (TOPO PCR cloning, Thermofischer Scientific). The *Nde*I-*Eco*RI restriction fragment of L-asparaginase gene was purified by using GF-1 nucleic acid extraction kit (Vivantis) and ligated in pET-21a(+) expression vector at the corresponding sites and transformed in DH5-α cells. The resulting plasmid named pET-asp was used to transform *E. coli* strain BL21-CodonPlus(DE3)-RIL. *E. coli* host cells with the pET-asp plasmid were grown overnight at 37 °C in LB medium containing ampicillin (50 µg/ml). The culture was diluted (1%) into fresh LB medium containing ampicillin (50 µg/ml) and then cultivation was continued until A_{660} reached 0.5. Heterologous expression of the geoAsnase gene was induced by the addition of 0.07 mM (final concentration) isopropyl-β-D-thiogalactopyranoside (IPTG), and incubation was continued for 4 h at 37 °C or overnight at 18 °C.

Enzyme activity assay and its characterization

Enzyme activity assay was based on the principle that geoAsnase hydrolyzes L-asparagine to L-aspartic acid and ammonia, this released ammonia reacts with Nessler's reagent to produce an orange-colored product. Enzyme activity assays were performed by measuring the amount of ammonia liberated from asparagine or glutamine (Campbell and Mashburn, 1969). Routine assay mixture, 200 µL, consisted of 100 mmol/L citrate-phosphate buffer (pH 6.5), 10 mmol/L substrate, and 5 µg of geoAsnase. The reaction was allowed to proceed for 10 min, stopped by adding 50 µL of 15% trichloroacetic acid (TCA), and centrifuged at 12000 rpm for 5 min and 100 µL of clear supernatant was taken into a fresh tube. Nessler's reagent (100 µL) and water (800 µL) were added and optical density was measured at 425 nm. The experiments were performed in triplicates and repeated twice. The absorbance concentration of the ammonia liberated One unit of geoAsnase activity was defined as the amount of enzyme liberating 1 µmol of NH_3 in 1 min at 65 °C.

Biochemical characterization

geoAsnase enzyme activity was monitored at different temperatures from 35 °C- 95 °C and pH values of 6.0-8.0. geoAsnase enzyme was purified by dialysis and gel filtration chromatography. Purified geoAsnase enzyme was used for further analysis.

RESULTS AND DISCUSSION

3D structure prediction and structure-based ligand docking

The monomeric structure of geoAsnase using AlphaFold software was predicted (Fig. 1). Binding affinities of L-asparagine and L-glutamine with geoAsnase enzyme were determined using CB dock2 software and Vina score values (Table I). Vina score of a lower value is considered to indicate better ligand binding affinities. The docked complex visualization with L-ASN (Fig. 2) revealed the involvement of Phe176 and Glu270 residues of geoAsnase in hydrogen bond formation with Asn molecule. Other amino acid residues Tyr161, Ala175, Tyr298 and Asn299 were observed to be involved in hydrophobic interactions in ligand binding. Structure-based docking done with L-GLN molecule (Fig. 3), another substrate for L-asparaginase enzyme, shows the involvement of five residues Tyr99, Ala146, Lys160, Arg197 and Glu199 at the binding site of enzyme in hydrogen bonding with the substrate. Other residues are involved in making hydrophobic interactions in stabilizing the substrate. D-asparagine which is stereoisomer of L-asparagine was also used for enzyme docking. D-asparagine binding with geoAsnase was stabilized by four amino acid residues Ala146, Lys160, Arg197 and Glu199 involved in hydrogen bonding (Supplementary Fig. 2). Docking energies of the D-asparagine binding with L-asparaginase were also predicted (Supplementary Table I). Theoretically predicted docking results show more favorable binding with L-GLN as compared to L-ASN. However, these results are based on monomeric geoAsnase while this enzyme possesses a homo-tetrameric structure which might affect the binding affinities of the enzyme with its substrate when present in cellular environment.

Table I. Docking energies of L-asparagine with geoAsnase enzyme.

Vina score	Cavity size	Center			Size		
		X	Y	Z	X	Y	Z
-5	642	4	-5	8	16	16	25
-4.6	709	1	7	10	23	16	16
-4.3	131	8	-3	-6	16	16	16
-4	218	6	17	-5	16	16	16
-3.7	250	17	3	15	24	16	16

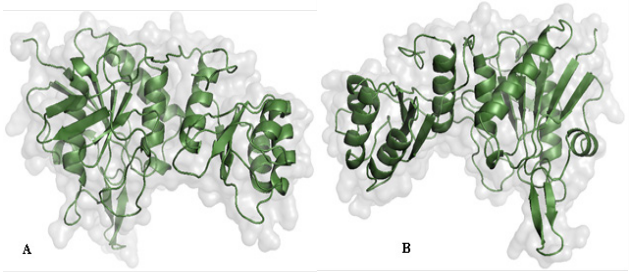

Fig. 1. 3D structure prediction of monomeric geoAsnase (front and back view).

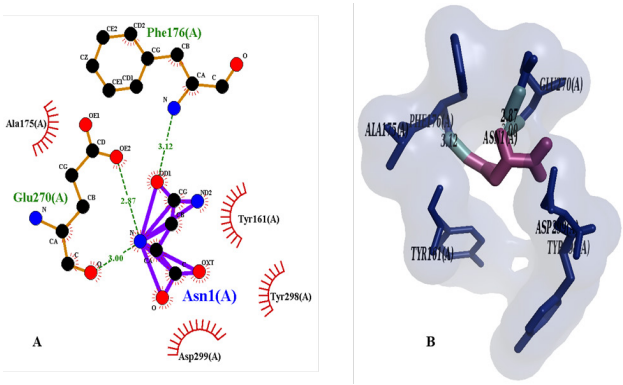

Fig. 2. Structure-based docking of geoAsnase with substrate L-asparagine. (A) 2D image and (B) 3D image of the docking complex showing Asn1 in the center forming hydrogen bonds with Phe (176) and Glu (270) residues of the enzyme. Hydrophobic interactions indicated by (Ala 175, Tyr 161, Tyr 298 and Asp299) in the periphery (A) are involved in stabilizing the complex.

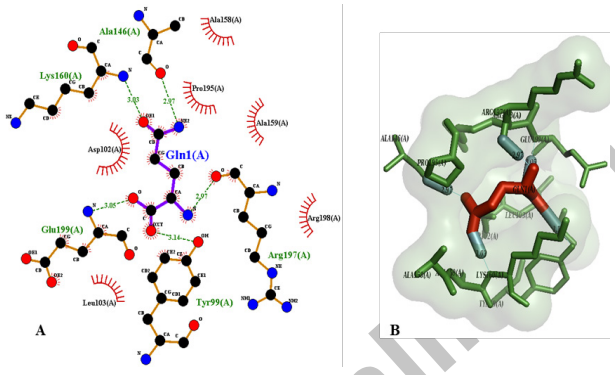

Fig. 3. Structure-based docking of geoAsnase with substrate L-glutamine. (A) 2D image and (B) 3D image of the docking complex showing Gln1 in the center forming hydrogen bonds with Tyr99, Ala146, Lys160, Arg197 and Glu199 residues of the enzyme. Hydrophobic interactions indicated by (Asp102, Leu103, Ala158, Ala159, Pro195, Arg198) in the periphery (A) are involved in stabilizing the complex.

Mechanism of substrate binding interactions by MD simulation

To further evaluate the binding affinities of L-ASN and L-GLN with geoAsnase molecular dynamic simulations were done. RMSD plot (Fig. 4A) showed less deviations in the binding affinities of geoAsnase with L-ASN as compared to L-GLN over time, as L-GLN shows larger peaks spread out over a large area showing high deviations which makes this enzyme-substrate complex less stable. RMSF plot (Fig. 4B) showed the difference in the amino acid residues of geoAsnase involved in forming the complex with L-ASN and L-GLN. Residues number 50-55 are involved in the difference in the binding of the two substrates with the geoAsnase enzyme. It will be important to explore these residues for future studies, also in other L-asparaginases, in order to improve their asparaginase activity by lowering glutaminase activity via site directed mutagenesis. Number of the hydrogen bonds formed over time (Fig. 4C) between geoAsnase and L-ASN and L-GLN depicted more favorable binding interactions between geoAsnase and L-ASN as compared to L-GLN which predicts that this enzyme will have low glutaminase activity.

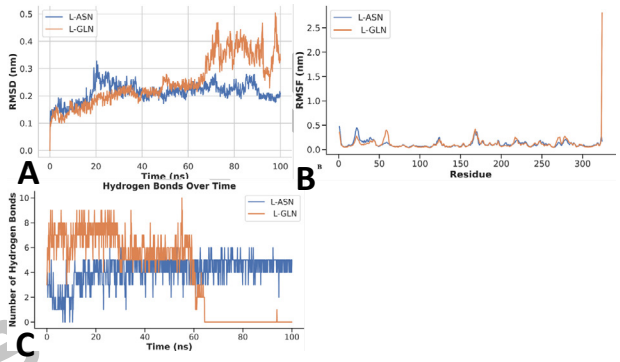

Fig. 4. Molecular dynamics simulations of geoAsnase with L-asparagine (L-ASN, blue lines) and L-glutamine (L-GLN, orange line). (A) RMSD plot showing deviations in the ligand binding with geoAsnase enzyme. L-ASN shows better binding with geoAsnase enzyme over time as compared to L-GLN which shows more peaks spread out as time increased indicating increased deviations. (B) RMSF plot showing the frequency of geoAsnase residues interacting with ligands L-ASN and L-GLN. (C) Hydrogen bond formation over time between geoAsnase and L-ASN and L-GLN.

Expression of recombinant geoAsnase

L-Asparaginase gene from *Geobacillus* SBS-4S was amplified (~1 kb gene, Fig. 5A) and successively cloned in TOPO cloning vector and for expression it was cloned in pET expression vector and transformed in *E. coli* strain BL21(DE3) Codon plus. The expression of enzyme at 0.07mM IPTG concentration after 4 h induction was clearly visible via SDS-PAGE analysis (Fig. 5B). It was observed that protein was expressed in the form of inclusion bodies at high IPTG concentrations (0.1 mM) (unpublished data). However, lowering the IPTG concentration to 0.07mM, protein was also expressed in soluble form (Fig. 5B). The highest enzyme activity of purified heat treated (65 °C) (Fig. 5C) geoAsnase at pH 6.9 was measured to be 39.8 U/mg while glutaminase activity of the recombinant geoAsnase was calculated to

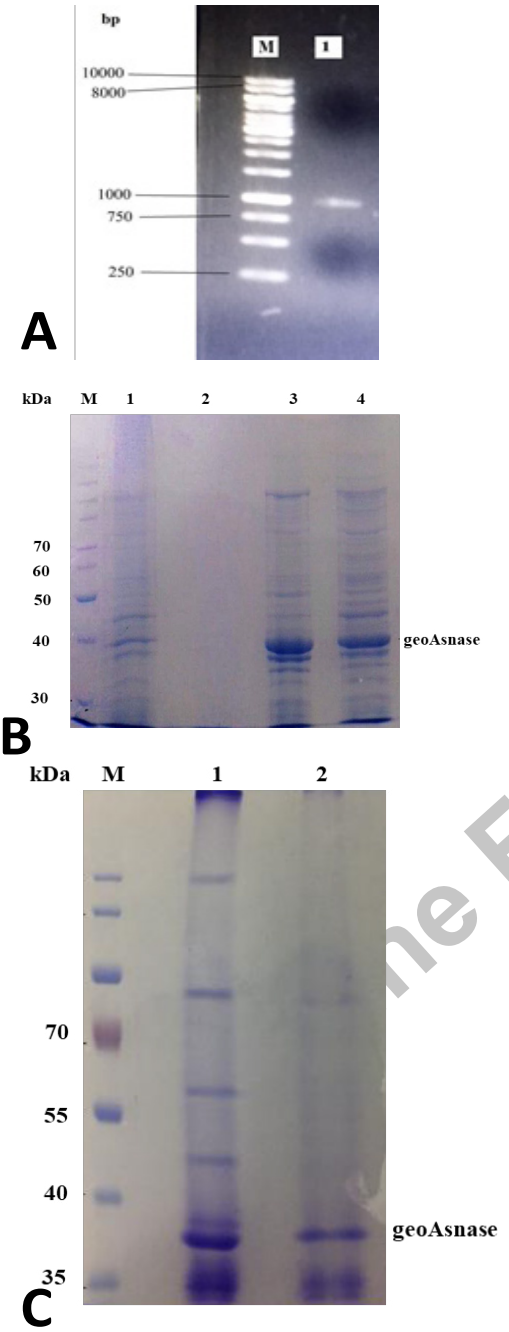

Fig. 5. **A**, PCR product (~1 kb) of geoAsnase gene in 1% agarose gel (Lane I), Lane M shows *1kb* gene marker. **B**, analysis showing geoAsnase expressed with 0.07mM IPTG with 4 h induction at 37 °C in 12% SDS-PAGE. Lane M: Unstained protein marker (PageRulerTM #26614, Lane 1: Uninduced pellet, Lane 2: Uninduced supernatant, Lane 3: Induced pellet, Lane 4: Induced supernatant. **C**, 12% SDS-PAGE showing prestained protein ladder in Lane L; geoAsnase after dialysis in Lane 1, and geoAsnase after gel filtration chromatography in Lane 2.

be 0.0066 U/mg which was almost negligible. This makes geoAsnase a potential enzyme for antitumor therapy of ALL due to its low glutaminase activity because this is a major side effect of many asparaginase enzymes (Ollenschläger, *et al*., 1988; Hussain *et al*., 2016).

Effect of temperature and pH

Enzyme activity of recombinant geoASNase was examined at various temperature ranging from 37-95 °C, and found to increase till 65 °C (Supplementary Fig. 3A), after which the decrease in activity was observed. This can be attributed to the optimum temperature required for the growth of *Geobacillus* strain SBS-4S (Tayyab *et al*., 2011). Similarly, effect of pH was observed by increasing pH 6.6-7.9 and found to be optimally maximum at pH 6.9 (Supplementary Fig. 3B). GeoAsnase enzyme shows better thermostability and pH sensitivity as compared to other L-asparaginases (Chohan *et al*., 2019; Chi *et al*., 2022).

CONCLUSION

The present study demonstrates molecular characterization of geoAsnase enzyme from *Geobacillus* SBS-4S in *E. coli*. Structure-based docking studies indicate the involvement of Phe176 and Glu270 residues in hydrogen bond formations with the substrate as well as several hydrophobic residues are involved in stabilizing the enzyme-substrate complex. MD simulation for substrate interactions revealed more favorable binding interactions between L-ASn as compared to L-GLN. This was confirmed experimentally where glutaminase specific activity of geoAsnase was found to be almost negligible. Our study shows that geoAsnase will be beneficial for therapeutic use in ALL treatment as well as for industrial purposes. Further studies on the enzyme at large scale in food industry an *in vivo* studies in cancer cell lines will delineate its predicted effects in the cellular environment.

DECLARATIONS

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Data availability

Data used in the study will be available when required.

Supplementary material

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.pjz/20240531082915

Statement of conflict of interest

The authors have declared no conflict of interest.

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Supplementary Material

Molecular Characterization of a Thermostable L-Aparaginase from *Geobacillus* Strain SBS-4S

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Protein sequence

MTKRKVVLLTTGGTIASKRNERSGRLRA-GALSGEELAAMCHLPKEIEIIVESVFQLPS-MH LTFSHWLELKKRIEAHFTDPSVDGIVVTH-GTDALEETAYFLDLTIDDPRTIVVTGSQRAP SDLGSDVYINIRHAIYAACAESLRGAGAVVVF-NERIFAAKYVKKEHASNIQGFNAFGFGY LGIIDN-DEVYVYQKPIRREYYKLVRPLPPVDIVKCYLEADG-KFIKAARESGVAGIVLEGV GRGQVAPKMVDEIVKAIEAGIKVVVTTSAEEG-AVYTTYDYPGSAYDLHKKGVILGSDYDA KKA-RIKLAVALAADAAVDGLFAY

Supplementary Fig. 1. Amino acid sequence of L-asparaginase

Supplementary Table I. Docking energies of L-asparagine with geoAsnase enzyme.

Vina score	Cavity size	Center			Size		
		X	Y	Z	X	Y	Z
-5	642	4	-5	8	16	16	25
-4.7	709	1	7	10	23	16	16
-4.1	131	8	-3	-6	16	16	16
-3.7	250	17	3	15	24	16	16
-3.6	218	6	17	-5	16	16	16

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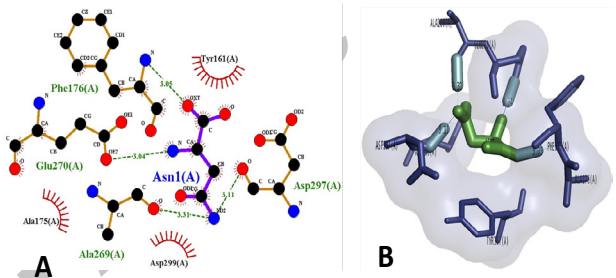
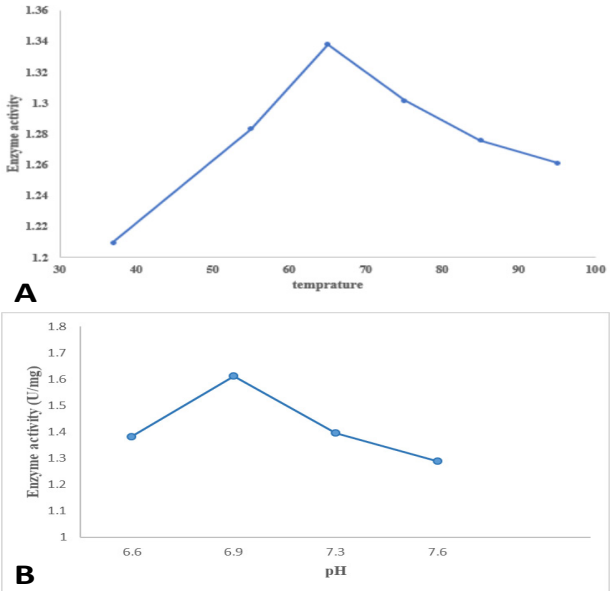
0030-9923/2025/0001-0001 $ 9.00/0



Supplementary Fig. 2. Structure-based docking of geoAsnase with substrate D-asparagine. (A) 2D image and (B) 3D image of the docking complex showing Asn1 in the center forming hydrogen bonds with Phe176, Ala269, Glu270 and Asp297 residues of the enzyme. Hydrophobic interactions indicated by (Tyr161, Ala175 and Asp299) in the periphery (A) are involved in stabilizing the complex.



Supplementary Fig. 3. Effect of temperature (A) and pH (B) on geoAsnase on enzyme activity.