Characterization and improved properties of Glutamine synthetase from *Providencia vermicola* by site-directed mutagenesis

Wu Zuo², Leitong Nie², Ram Baskaran², Ashok Kumar³ & Ziduo Liu¹,²

In this study, a novel gene for Glutamine synthetase was cloned and characterized for its activities and stabilities from a marine bacterium *Providencia vermicola* (*PveGS*). A mutant S54A was generated by site-directed mutagenesis, which showed significant increase in the activity and stabilities at a wide range of temperatures. The \( K_m \) values of *PveGS* against hydroxylamine, ADP-Na₂ and L-Glutamine were 15.7 ± 1.1, (25.2 ± 1.5) × 10⁻⁵ and 32.6 ± 1.7 mM, and the \( k_{cat} \) were 17.0 ± 0.6, 9.14 ± 0.12 and 30.5 ± 1.0 s⁻¹ respectively. *In-silico-* analysis revealed that the replacement of Ser at 54th position with Ala increased the catalytic activity of *PveGS*. Therefore, catalytic efficiency of mutant S54A had increased by 3.1, 0.89 and 2.9-folds towards hydroxylamine, ADP-Na₂ and L-Glutamine respectively as compared to wild type. The structure prediction data indicated that the negatively charged pocket becomes enlarged and hydrogen bonding in Ser54 steadily promotes the product release. Interestingly, the residual activity of S54A mutant was increased by 10.7, 3.8 and 3.8 folds at 0, 10 and 50 °C as compared to WT. Structural analysis showed that S54A located on the loop near to the active site improved its flexibility due to the breaking of hydrogen bonds between product and enzyme. This also facilitated the enzyme to increase its cold adaptability as indicated by higher residual activity shown at 0 °C. Thus, replacement of Ala to Ser54 played a pivotal role to enhance the activities and stabilities at a wide range of temperatures.

*Providencia vermicola*, is a marine bacterium isolated from juveniles of the entom pathogenic nematode *Steinernema thermophilumin*. Glutamine synthetase (GS, L-glutamate: ammonia ligase, ADP-forming, EC 6.3.1.2) is an important enzyme in nitrogen metabolism which catalyses the synthesis of glutamine using ammonia produced by nitrate reduction, amino acid degradation and photorespiration. It plays an important role in the metabolic pathways of marine bacteria found in oligotrophic oceans. Several researchers have studied the biological role, physico-chemical properties, and kinetic properties of GS from different sources. The phosphinothricin (PPT) and methionine sulfoximine (MetSox) are the inhibitors of GS activity, which tightly bound to its active site. Thus, GSI may play important role as bio-pesticide with potential usage in agricultural industry. Recently, the production of theanine using glutamine synthetase become more attractive and researchers are attempting to increase the total catalytic efficiency of GS by directed evolution, site directed mutagenesis for the synthesis of theanine in the industrial scale. In order to improve the catalytic activity and stability of industrially important enzymes directed evolution techniques and various immobilization strategies were used. The directed evolution is widely employed to generate mutants with increased catalytic activity and to investigate the role of particular amino acid residues in catalytic behaviour. The commonly used techniques in directed evolution to improve the enzyme activity were error-prone PCR, DNA shuffling and site-directed mutagenesis. The glutamine synthetase shows biosynthetic and \( \gamma \)-glutamyl transferase activities, which are regulated by the conversion of adenylylated and non-adenylylated forms. The biosynthetic activity can be

¹College of Food and Bio-science and Technology, Wuhan Institute of Design and Science, Wuhan, 430205, P.R. China. ²College of Life Science and Technology, State Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University, Wuhan, 430070, P.R. China. ³Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Waknaghat, Solan, 173234, Himachal Pradesh, India. Wu Zuo and Leitong Nie contributed equally. Correspondence and requests for materials should be addressed to Z.L. (email: lzd@mail.hzau.edu.cn)

Received: 21 November 2017
Accepted: 19 June 2018
Published online: 23 October 2018
significantly reduced by adenylylated form while the γ-glutamyl transfer activity exists in both forms 36. The bio-
synthetic activity of GS is catalyzes the reaction of glutamate and ammonia to form glutamine and the γ-
glutamyl transfer activity catalyzes the transfer of γ-glutamyl moieties to water or amino acids, or peptides6. GS are usually
categorized as GSII, GSIII, and GSI 37. GSI enzymes specially exist in prokaryotes, and their structures are dode-
cameric38,39 but recently GSI enzymes have also been identified in mammals and plants2,40. GSI enzymes are clas-
sified into two subdivisions 37, GSIα and GSIβ. GSIα genes are found in the thermophilic bacterium, Thermotoga
maritima and the Euryarchaeota. GSIβ type genes exist in other bacteria such as E. coli, Synechocystis PCC6803,
Aquifex aeolicus, Crenarcheon sp., and Sulfolobus acidocaldarius37.

In this study, a GSIβ glutamine synthetase protein structure from Salmonella typhimurium (PDB ID: 1F1H)
was adapted as a homology model to build up PveGS structure 41. This dodecameric structure was in complex with
two ammonium analogues, thallous ion (Tl+473, Tl+474), two manganese ion and a vital substrate ADP . Tl+
473 coincides with the ammonium substrate binding site reported by Liaw et al. 4, and the Tl+474 is the binding site
of the ammonium group of the substrate glutamate41. Eisenberg has reported that hydroxylamine in the transfer
reaction, ammonium ion in the biosynthetic reaction, and water in the glutamine hydrolysis reaction presumably
bind at the same site38.

Coupling the structural information with efficient site-directed mutagenesis, the microbial expression tech-
niques enabled successful engineering of a protein with the desired characteristics 21,23,42,43. The objective of
the present study was to obtain a mutant of glutamine synthetase obtained from a marine bacterium
P. vermicola. The gene has been successfully cloned, expressed and characterized to study its biochemical properties. A
mutant S54A with improved catalytic efficiency, cold adaptability and higher thermostability was obtained by
site-directed mutagenesis based on homology modelling of PveGS. The mutant with improved catalytic features

Results
Gene cloning and sequence analysis. The glutamine synthetase was cloned successfully from P. vermicola
genomic DNA with an ORF of 1410 base pairs encoding for 469 amino acids (primers presented in Table S1).
The amino acid sequence of PveGS was aligned with the five reported bacterial GS structures. Four highly con-
served amino acids located in the loop region (Asp51, Tyr179, Asn24, Tyr398) and one amino acid Glu328 was
located in the flap of active-site (Fig S1). Soluble PveGS protein was expressed and purified from E. coli BL21
(DE3) harbouring a recombinant plasmids pGEX-6p-1-PveGS. The molecular mass of the purified recombinant
GS was 52 kDa after the removal of GST-tag, while its molecular mass was observed larger than expected on
SDS-PAGE (Fig. 1A) due to the mino acids composition. The purified protein then loaded onto gel filtration

Figure 1. Expression and enzymatic analysis of PveGS and S54A. (A) SDS-PAGE analysis of the purified
glutamine synthetase produced in E. coli BL21. Marker lists the standard molecular weight and the lane of the
wild PveGS, mutant S54A and merged protein. (B) The optimum temperature of the wild type and S54A. The
activity was determined at a gradient temperature ranging from 0 to 60 °C and activity at 35 °C was defined as
100%; (C) The optimum pH of the WT and S54A. The activity assay was carried out at 35 °C for 30 min and the
specific activity under the optimum pH was defined as 100%. (D) and (E) Thermostability of WT and S54A was
measured under different temperatures for 2 h, and samples were taken every 20 min for 2 h. The specific activity
without incubation was defined as 100%. (F) pH stability of WT and S54A. The residual activity was measured
at 35 °C for 30 min and the highest activity was defined as 100%.
chromatography, and the elution volume revealed that the molecular weight of PveGS was more than 600 kDa (data not shown), which indicated its dodecameric structure.

**Homology modelling.** Homology modelling for PveGS was performed on the basis of target-template alignment, and its initial partial geometry. Heavy-atom coordinates were obtained from the conserved residues which were found between the template and PveGS. The backbone coordinates were collected when residue identity was variable. Backbone geometries were modelled from fragments of high-resolution chains from the PDB (Protein Data Bank) when there were no assigned backbone coordinates according to previous library44. Two appropriate structural templates were from PDB among a set of pre-alignment family, with an E value of 9.8 $\times$ 10$^{-231}$ and 7.3 $\times$ 10$^{-5}$. The best hit template 1F1H (PDB ID) is the crystal structure of glutamine synthetase from *S. typhimurium*, which was adapted as modelling template after removing the first amino acid methionine of PveGS. The model quality was evaluated using Ramachandran plot by MOE. The result of Ramachandran plot suggested that 462 residues were in the maximum allowable area of the plot excluding six residues (Phe81, Met98, Lys386, Lys395, Thr406 and Ser468), indicating that the model was stable in stereochemistry.

The structure modelling and molecular weight analysis indicated that it is a dodecameric structure consists of two stacked hexamers as the typical GSI enzymes. Hydrogen bonding and hydrophobic interactions hold the two GS rings together. Each subunit possesses a C-terminus and N-terminus, in which C-terminus stabilizes the GS structure by insertion into the hydrophobic region of the subunit across the other ring. The N-terminus located on the surface and exposed to the solution environment. In addition, the central channel is formed via six four-stranded $\beta$-sheets composed of anti-parallel loops from the twelve subunits 38,45. The PveGS monomer model (red chain) superimposed to a subunit (cyan chain) of 1F1H from *S. typhimurium* (green) were showed in Fig. 2A, as the black arrow pointing at, and different colours represented the different monomers. The superimposition between the model of PveGS and the crystal structure of 1F1H from *S. typhimurium* (green) was presented in Fig. 2B. The homology model constructed by MOE2009 and the structure of $\alpha$-helices and $\beta$-strands (yellow) was depicted in Fig. 2C.

**Ligand interaction analysis.** Every PveGS monomer possessed an active site named 'bifunnel' (Fig. 2D), which is the binding site of three distinct substrates: nucleotide, ammonium ion, and amino acid 4,46,47. The bifunnel top was the binding site for ATP, ADP as well as nucleic acids, and glutamine, glutamate, together with ammonium bind to the bottom region. As demonstrated in Fig. 2D, ADP located in the upside of bifunnel and two ammonium analogues, thallous ion, and ammonia group of glutamate binding sites, were in the lower end. Ligand interaction revealed that ADP molecules located in a pocket formed by Asp50' (from neighbour chain), Cys90, Ile92, Glu94, Lys170, Tyr180, Pro182, Glu221, Val222 and Glu293 (Fig. 2E). Tl‘473 interacts with Met49, Ser54, Tyr179’, His210, Glu212 and Val213 (Fig. 3A) in which Ser54, Asp 51, Glu212 and Tyr179’ (Fig. 3B) form

![Figure 2.](https://example.com/figure2.png)
a negatively charged ammonium pocket. The ammonium ion could occupy this pocket and donate hydrogen bonds, similar to that of the GS model from *S. typhimurium* as described by Liaw et al.\(^4\). Tl\(^{4+}\) 474 located in the negatively charged pocket for binding the amino group of the glutamate in the biosynthetic reaction, interacts with Glu132, Glu213, Gln219, Gly266, Ser267, Gly268 and His270 (Fig. 3C).

Based on the homology model, interaction analysis and sequence alignment, site-directed mutagenesis was performed on V15A, A36T, S54A, L126F, E213L, T224A, A249V and N265A. Among the generated mutants, S54A showed increased activity than wild type as well other mutants and thus selected for further investigation. The homology model was constructed for mutant GS S54A using the same template and the predicted E value was 2.2 \times 10^{-230}. From the homology model, it suggested that about 10 residues are out of the maximum allowable area, but it is still stable in stereochemistry. After site-directed mutagenesis in site Ser54, it was changed into Ala54 and the conformational analysis and ligand interaction of Tl\(^{4+}\) 473 enclosed by amino acids was presented in Fig. 3D-E.

**Effects of detergents.** To determine the potential industrial scale application of *PveGS*, its tolerance towards various detergents was analysed (Table S4). These results indicated that the WT enzyme has better tolerance than S54A, indicating S54A was more sensitive to the solution environment. The wild type enzyme noted the highest relative activity of 160 and 154% in 5 mM Triton X-100 and Tween 80, respectively, higher than that in the presence of non-ionic detergents such as 5 mM Tween 20 and 0.5% CHAPS as well as 5 mM cationic surfactant CTAB. However, the anionic surfactant SDS inhibited the activity at the concentration of 0.1 and 0.5% when compared to the control. Meanwhile, the mutant activity decreased to about 18 to 40% relative to that of the control and the native. The activity of native enzyme was also inhibited in the presence of 0.1 and 0.5% SDS.
mutant S54A. All the assays were carried out at the optimum pH and temperature for 30 min and data was given
as mean values ± S.D.

|        | ADP-Na2 | L-Glutamine | Hydroxylamine |
|--------|---------|-------------|---------------|
|        | WT      | S54A        | WT            | S54A         | WT      | S54A |
| \(K_a (\text{mM})\) | \((25.2 ± 1.5) \times 10^{-4}\) | \((10.2 ± 5.6) \times 10^{-2}\) | \(32.6 ± 1.7\) | \(23.8 ± 1.2\) | \(15.7 ± 1.1\) | \(4.6 ± 0.3\) |
| \(V_{\text{max}} (\text{mM/min})\) | \(0.20 ± 0.00\) | \(0.16 ± 0.00\) | \(0.66 ± 0.02\) | \(0.44 ± 0.01\) | \(0.37 ± 0.01\) | \(0.29 ± 0.01\) |
| \(k_\text{cat} (s^{-1})\) | \(9.14 ± 0.12\) | \(15.2 ± 0.2\) | \(30.5 ± 1.0\) | \(41.9 ± 1.1\) | \(17.0 ± 0.6\) | \(19.6 ± 0.5\) |
| \(k_\text{cat}/K_a (s^{-1} \cdot \text{mM}^{-1})\) | \((3.63 ± 0.17) \times 10^{3}\) | \((1.49 ± 0.80) \times 10^{3}\) | \(0.936 ± 0.02\) | \(1.76 ± 0.04\) | \(1.08 ± 0.04\) | \(4.27 ± 0.17\) |

Table 1. Kinetic parameters of the wild type PveGS and mutant S54A. Kinetic parameters of the wild type and mutant S54A. All the assays were carried out at the optimum pH and temperature for 30 min and data was given as mean values ± S.D.

**Kinetic measurements.** The enzyme kinetic parameters for the wild type and mutant enzymes were presented in Table 1. The kinetic analysis indicated that the \(K_a\) of PveGS against hydroxylamine, ADP-Na, and L-Glutamine were \((15.7 ± 1.1)\), \((25.2 ± 1.5) \times 10^{-5}\) and \((32.6 ± 1.7)\) mM, and the \(k_\text{cat}\) were \((17.0 ± 0.6)\), \((9.14 ± 0.12)\) and \((30.5 ± 1.0)\) s \(^{-1}\) respectively. The kinetic analysis of mutant S54A suggested that the \(K_a\) of PveGS against hydroxylamine, ADP-Na, and L-Glutamine were \((4.6 ± 0.3)\), \((10.2 ± 5.6) \times 10^{-4}\) and \((23.8 ± 1.2)\) mM, and the \(k_\text{cat}\) were \((19.6 ± 0.5)\), \((15.2 ± 0.2)\) and \((41.9 ± 1.1)\) s \(^{-1}\) respectively. The catalytic efficiency against hydroxylamine, ADP-Na, and L-Glutamine of the mutant S54A was increased by 3.1-, 0.89 and 2.9-folds respectively, as compared to wild type PveGS.

**Discussion**

In the present study, both the native and site-directed-mutagenesis-modified glutamine synthetases from marine *P. vermicola* were investigated for their catalytic properties under optimized conditions. The native (PveGS) and mutant (S54A) enzymes were characterized in terms of optimum temperature, pH, and thermal stability. The PveGS and S54A were found to be highly active at 40 °C for 2 h, which is similar to the previously reported GSI of *P. ruminicola*48. The mutant S54A showed a relatively high residual activity, about 64 and 35% at 10 °C and 0 °C respectively. These findings have been supported by a previous study similar to the wild esterase and lipase reported from *Psychrobacter sp.* Ant 300 and *Pseudomonas sp.*72.49.50. As reported previously, the low ratio of Arginine/Arginine + Lysine, low proportion of proline residues, small hydrophobic core, lesser salt bridges and aromatic–aromatic interactions are the common characteristics for a cold-adapted enzyme51–54. A smaller number of ion pairs and weak charge-dipole interactions in α-helices could contribute to the poor thermo-stability55.

In the present investigation, S54A was located on the loop of the active site, which leads to more plasticity and flexibility to the structure. Meanwhile, the hydrogen bonds between the product and enzyme was destroyed, which is facilitating the release of the substrate at a lower temperature. The cold-adapted enzyme is a promising source to the pharmaceutical industries, agriculture and chemical industries56, for cold conditions are required for biosynthesis of fragile pharmaceutical compounds to avoid adverse side-effects and conserve energy54.

Glutamine synthetase is a metallo-protein possessing a metal binding site in its active site to accommodate the cofactors (Mg\(^{2+}\) or Mn\(^{2+}\)) that are important for its activity. Cations binds to the metal binding site of the enzyme as previously reported57. A previous study revealed that there were mono-valent cation sites in GS so that these cations could stabilize the quaternary structure of GS and alkali ions could compete partially with NH\(_2\)OH in the γ-glutamyl transfer reaction. PveGS and S54A exhibited a relatively low residual activity, about 64 and 35% at 10 °C and 0 °C respectively. These findings have been supported by a previous study similar to the wild esterase and lipase reported from *Psychrobacter sp.* Ant 300 and *Pseudomonas sp.*72.49.50. As reported previously, the low ratio of Arginine/Arginine + Lysine, low proportion of proline residues, small hydrophobic core, lesser salt bridges and aromatic–aromatic interactions are the common characteristics for a cold-adapted enzyme51–54. A smaller number of ion pairs and weak charge-dipole interactions in α-helices could contribute to the poor thermo-stability55.

In the present investigation, S54A was located on the loop of the active site, which leads to more plasticity and flexibility to the structure. Meanwhile, the hydrogen bonds between the product and enzyme was destroyed, which is facilitating the release of the substrate at a lower temperature. The cold-adapted enzyme is a promising source to the pharmaceutical industries, agriculture and chemical industries56, for cold conditions are required for biosynthesis of fragile pharmaceutical compounds to avoid adverse side-effects and conserve energy54.

Glutamine synthetase is a metallo-protein possessing a metal binding site in its active site to accommodate the cofactors (Mg\(^{2+}\) or Mn\(^{2+}\)) that are important for its activity. Cations binds to the metal binding site of the enzyme as previously reported57. A previous study revealed that there were mono-valent cation sites in GS so that these cations could stabilize the quaternary structure of GS and alkali ions could compete partially with NH\(_2\)OH in the γ-glutamyl transfer reaction4. PveGS and S54A exhibited a relatively low residual activity (6.1 and 4.4%) at 10 °C and 0 °C respectively. These findings have been supported by a previous study similar to the wild esterase and lipase reported from *Psychrobacter sp.* Ant 300 and *Pseudomonas sp.*72.49.50. As reported previously, the low ratio of Arginine/Arginine + Lysine, low proportion of proline residues, small hydrophobic core, lesser salt bridges and aromatic–aromatic interactions are the common characteristics for a cold-adapted enzyme51–54. A smaller number of ion pairs and weak charge-dipole interactions in α-helices could contribute to the poor thermo-stability55.

**Material and Methods**

**Bacterial strains and plasmid.** The marine bacterium *P. vermicola* strain CGS6 was obtained from Marine Culture Collection of China (http://www.mccc.org.cn/). *P. vermicola* was grown in nutrient agar medium containing peptone (1%, w/v), yeast extract (0.5%, w/v), NaCl (2%, w/v), and agar (1.5%, w/v). Plasmid pGEX-6p-1 (GE Healthcare, USA) was used as a vector for protein expression. For cloning and expression, *E. coli* DH5α
Expression and purification. E. coli BL21 (DE3) cells harbouring pGEX-6P-1-PveGS were inoculated into LB broth with 100 μg/ml ampicillin. The culture was induced by adding 0.15 mM IPTG into when the OD600 was 0.6–0.8, then incubated at 18°C for 16 h under shaking (225 rpm/min). Finally, the induced cells were collected by centrifugation, resuspended in phosphate-buffered saline (PBS) buffer (NaCl 0.8%, KCl 0.02%, Na₂HPO₄ 0.14%, KH₂PO₄ 0.03%; pH 7.0) and homogenized using a high-pressure homogenizer (NS100IL 2K, Niro Soavi, Germany). PveGS recombinated with GST-tag were purified using a glutathione S-transferase (GST) Gene Fusion System (GE Healthcare, USA) and eluted from the GST tag by 3 C proteases (PreScission, Pharmacia). The protein was quantified using the Bradford reagent with bovine serum albumin (BSA) as a standard and the molecular weight was confirmed by sodium dodecyl sulfate-denatured polyacrylamide gel electrophoresis (SDS-PAGE) with 12% polyacrylamide gels. Then the tag-free protein sample was concentrated to load the gel filtration chromatography column “Superose 6, 10/300 GL” with regular buffer (25 mM Tris-HCl pH 7.5, 150 mM NaCl). Assay of glutamine synthetase activity. The GS activity assay contained 18 mM hydroxylamine-hydrochloride, 20 mM L-Glutamine, 1 mM MnCl₂, 25 mM potassium arsenate, 0.4 mM ADP-Na₂, and 135 mM imidazole hydrochloride. The reaction was terminated by adding 200 μL ‘stop mixture’ consisting of 55 g FeCl₃·6H₂O, 20 g trichloroacetic acid and concentrated HCl (21 mL/L). The absorbance was measured at 540 nm after centrifugation of reaction mixture. The L-Glutamic acid γ-monohydroxamate was added into the reaction mixture without enzyme to give a concentration from 0 to 6 mM for quantification by the absorbance at 540 nm. One unit of enzyme activity is defined as the amount of PveGS required convert 1 μmol L-Glutamic acid γ-monohydroxamate in one minute in one ml of reaction mixture under optimized conditions.

Effect of temperature and pH. The assay mixture and enzyme were incubated in the temperature gradient from 0 to 60°C for 5 min and the reaction was initiated by adding L-Glutamine and terminated after 30 min incubation using stop reagent described in previous sections. The thermostability assay was conducted in the temperature gradient from 30 to 70°C and the samples were taken after every 20 min for 2 h. The residual activity was determined under the optimized conditions. Phosphate-citrate (pH 4.0–8.0) and Glycine-NaOH (pH 8.0–11.0) buffer were used to analyze the effect of pH from 4.0 to 8.0 and 8.0 to 11.0, respectively. pH stability was determined by calculating the residual activity left after incubating the enzyme for 24 hours at 4°C.

Effect of metal ions and detergents on the native and mutant GS activity. The effects of various metal ions (K⁺, Mg²⁺, Mn²⁺, Zn²⁺, Cu²⁺, Ba²⁺, Ni²⁺, Fe³⁺, Co²⁺, Li⁺, NH₄⁺, Na⁺), reagents (EDTA, Urea, DTT, PMSF) and detergents (Tween 20, Tween 80, Triton X-100, CHAPS, CTAB, SDS) on the enzymatic activity were determined by using the different concentrations of reagents (1 and 5 mM) and detergents (1 and 5 mM, and 0.1 and 0.5%, v/v). The effects of metal ions K⁺, Mg²⁺, Mn²⁺, Zn²⁺, Cu²⁺, Ba²⁺, Ni²⁺, Fe³⁺, Co²⁺, Li⁺, NH₄⁺ and Na⁺ on the activity were tested by adding them into the reaction mixture without MnCl₂ at the concentration of 1 and 5 mM. The enzyme stability was tested with 2 mM MnCl₂ and 1 or 5 mM of various reagents. The relative activity was determined by comparing with the control.

The kinetic parameters Kₘ and kₘₐₓ. The substrates of the PveGS, L-Glutamine, hydroxylamine-hydrochloride and ADP-Na₂ were added into the mixture with the concentration of 2–40 mM, 0.05–20 mM and 0.00001–0.01 mM, respectively, to obtain the initial rate of reaction. Michaelis-Menten constant (Kₘ) was measured according to Lineweaver–Burkplot, and the value of Vₘₐₓ, the molecular mass and the concentration of the purified protein were used to calculate the catalytic constant (kₘₐₓ). Graphpad Prism software (Graphpad, San Diego, CA, USA) was also used to calculate these parameters for consistency and accuracy. Molecular docking. To search for the active site and investigate the interaction surface of the GS, molecular docking was performed. After obtaining the homologous sequences with a reasonable value, the structure containing the ligand was selected as the target substrate for docking. In present study, a GST3F glutamine synthetase protein structure from S. typhimurium (PDB ID: 1F1H) was adapted, which containing ligand like ADP, and two important ammonium analogues, Tl⁺, which were coincidence with ammonium substrate binding site and binding the ammonium group of the glutamate. Based on the homology modelling to WT and SS4A, molecular docking analysis was carried out on the instruction of docking tutorial in MOE 2009.
References

1. Somvanshi, V. S. et al. Providencia vermicola sp nov., isolated from infective juveniles of the entomopathogenic nematode Steinernema thermophilum. *Inf J Sys Evol Micr* 56, 629–633, https://doi.org/10.1099/ijs.0.63973-0 (2006).

2. Wyatt, K. et al. Lengsin is a survivor of an ancient family of class I glutamine synthetases re-engineered for evolution for a role in the vertebrate lens. *Structure* 14, 1823–1834, https://doi.org/10.1016/S0969-2126(06)00008 (2006).

3. Eisenberg, D. et al. Some evolutionary relationships of the primary biological catalysts glutamine synthetase and RhBiSCO. *Cold Spring Harbor Symp Quantitative Biol* 52, 483–490 (1987).

4. Liaw, S. H., Kuo, I. & Eisenberg, D. Discovery of the ammonium substrate site on glutamine synthetase, a third cation binding site. *Protein science: a publication of the Protein Society* 4, 2358–2365, https://doi.org/10.1023/A:10092507-0178-1 (1995).

5. Hirel, B., McNally, S. F., Gadal, P., Sumar, N. & Stewart, G. R. Cytosolic glutamine synthetase in higher plants. A comparative immunological study. *European journal of biochemistry/FEBS Letters* 138, 63–66 (1984).

6. Al-Gharawi, A. & Moore, D. Factors affecting the amount and the activity of the glutamate dehydrogenases of Coprinus cinereus. *Biochimica et biophysica acta* 496, 95–102 (1977).

7. Ebner, E., Wolf, D., Gancedo, C., Elsasser, S. & Holzer, H. ATP-glutamine synthetase adenyllyltransferase from Escherichia coli B. Purification and properties. *European journal of biochemistry/FEBS Letters* 14, 535–544 (1970).

8. El Alouai, S. et al. Glutamine synthetase from the marine cyanobacteria Prochlorococcus spp: characterization, phylogeny and response to nutrient limitation. *Environmental microbiology* 5, 412–423 (2003).

9. Yin, Z. M., Chen, Q. Y., Sima, J., Wu, Y. F. & Zhang, S. Q. The expression regulation and characterization of glutamine synthetase from the hyperthermophadophilic crenarcheon Sulfolobus acidocaldarius. *Proc Biochem* 30, 767–771 (2003).

10. Liaw, S. H. & Eisenberg, D. Structural model for the reaction mechanism of glutamine synthetase, based on five crystal structures of enzyme-substrate complexes. *Biochemistry* 33, 675–681 (1994).

11. Abell, L. M. & Villafranca, J. J. Investigation of the mechanism of phosphoinositol inactivation of Escherichia coli glutamine synthetase using rapid quench kinetic technique. *Biochemistry* 30, 6135–6144 (1991a).

12. Zhang, S. et al. Characterization of an L-phosphoinositol resistant glutamine synthetase from Exiguobacterium sp. and its improvement. *Applied microbiology and biotechnology* 1001–1009 (1977).

13. Wakisaka, S., Ohshima, Y., Ogawa, M., Tochikura, T. & Tachi, T. Characteristics and efficiency of glutamine production by coupling of a bacterial glutamine synthetase reaction with the alcoholic fermentation system of baker’s yeast. *Applied and environmental microbiology* 64, 2992–2997 (1998).

14. Yamamoto, S., Wakayama, M. & Tachi, T. Theanine production by coupled fermentation with energy transfer employing Pseudomonas taetrolens Y-30 glutamine synthetase and baker’s yeast cells. *Biosci, biotechnol, and biochem* 69, 784–789, https://doi.org/10.1271/bbb.69.784 (2005).

15. Yamamoto, S., Wakayama, M. & Tachi, T. Cloning and expression of Pseudomonas taetrolens Y-30 gene encoding glutamine synthetase: an enzyme available for theanine production by coupled fermentation with energy transfer. *Biosci, biotechnol, and biochem* 70, 500–507, https://doi.org/10.1271/bbb.70.500 (2006).

16. Yokogoshi, H., Kobayashi, M., Mochizuki, M. & Terashima, T. Effect of theanine, r-glutamylthylamide, on brain monoamines and striatal dopamine release in conscious rats. *Neurochemical research* 23, 667–673 (1998).

17. Sugiyama, T. & Sadzuka, Y. Theanine and glutamate transporter inhibitors enhance the antitumor efficacy of chemotherapeutic agents. *Biochimica et biophysica acta* 1633, 47–59 (2003).

18. Zhou, X. et al. Mn(2+) enhances theanine-forming activity of recombinant glutamine synthetase from Bacillus subtilis in Escherichia coli. *World J Microb Biot* 24, 1267–1272, https://doi.org/10.1127/wjmb/2007-007-9599-9 (2008).

19. Yokoyama, T. et al. Synthesis of L-theanine using enzyme/mesoporous silica conjugates under high pH conditions. *Materials letters* 65, 67–69 (2011).

20. Itoh, T. et al. Production of L-theanine using glutaminase encapsulated in carbon-coated mesoporous silica with high pH stability. *Biochemical engineering journal* 68, 207–214 (2012).

21. Zhang, S., Wu, G., Feng, S. & Liu, Z. Improved thermostability of esterase from Aspergillus fumigatus by site-directed mutagenesis. *Enzyme and microbial technology* 64–65, 11–16, https://doi.org/10.1016/j.enzmictec.2014.06.003 (2014).

22. Backer, M. S. & Liu, D. R. Methods for the directed evolution of proteins. *Nature Reviews Genetics* 16, 379 (2015).

23. Yao, P. et al. Improvement of glycine oxidase by DNA shuffling, and site-saturation mutagenesis of F247 residue. *International journal of biological macromolecules* 79, 965–970 (2015).

24. Chen, J., An, Y., Kumar, A. & Liu, Z. Improvement of chitinase Pachi with nematicidal activities by random mutagenesis. *International journal of biological macromolecules* 96, 171–176 (2017).

25. Dome, J. S. et al. High telomerase reverse transcriptase (hTERT) messenger RNA level correlates with tumor recurrence in patients with favorable histology Wilms’ tumor. *Cancer research* 59, 4301–4307 (1999).

26. Anwar, M. Z. et al. SnO2 hollow nanotubes: a novel and efficient matrix support for enzyme immobilization. *Scientific reports* 7, 15333, https://doi.org/10.1038/s41598-017-15550-y (2017).

27. Kumar, A. et al. Cellulose binding domain assisted immobilization of lipase (GSlip–CBD) onto cellulose nanogel: characterization and application in organic medium. *Colloids and Surfaces B: Biointerfaces* 136, 1042–1050 (2015).

28. Chen, L., Chen, J., Kumar, A. & Liu, Z. Effects of domains modification on the catalytic potential of chitinase from Pseudomonas aeruginosa. *International journal of biological macromolecules* 68, 262–272 (2015).

29. Kumar, A., Wu, G., Wu, Z. & Liu, Z. Improved catalytic properties of a serine hydroxymethyl transferase from Idiomarina loihiensis by site-directed mutagenesis. *Enzyme and microbial technology* 50, 26–30 (2016).

30. Kim, J., Kim, S., Yoon, S., Hong, E. & Ryu, Y. Improved enantioselectivity of thermotolerant esterase from Archaeoglobus fulgidus toward (S)-ketoprofen ethyl ester by directed evolution and characterization of mutant esterases. *Applied microbiology and biotechnology*, 1–9 (2015).

31. Chronopoulou, E. G. & Labrou, N. F. Site-saturation Mutagenesis: A Powerful Tool for Structure-Based Design of Combinatorial Mutation Libraries. Current Protocols in Protein Science. 26, 26.21–26.26.10 (2011).

32. Hennig, S. B., Anderson, W. B. & Ginsburg, A. Adenosine triphosphate: glutamine synthetase adenyllyltransferase of Escherichia coli: two active molecular forms. *Proceedings of the National Academy of Sciences of the United States of America* 67, 1761–1768 (1970).

33. Abell, L. M. & Villafranca, J. J. Effect of metal ions and adenyllylation state on the internal thermodynamics of phosphoryl transfer in the Escherichia coli glutamine synthetase reaction. *Biochemistry* 30, 1413–1418 (1991b).

34. Li, J., Gao, D., Xiao, Y., Li, Y., Liu, Z., Peng, J., Wang, Q. & Zhu, J. Mapping the structural properties of the glutamate synthase (GluATase) enzyme domain. *Biochemistry* 42, 767–771, https://doi.org/10.1021/bi0602050 (2007).

35. Bender, R. A. et al. Biochemical parameters of glutamine synthetase from Klebsiella aerogenes. *Journal of bacteriology* 129, 1001–1009 (1977).

36. Brown, J. R., Masuchi, Y., Robb, F. T. & Doolittle, W. F. Evolutionary relationships of bacterial and archaeal glutamine synthetase genes. *Molecular and evolutionary biology* 38, 566–576 (1994).

37. Eisenberg, D., Gill, H. S., Pluegg, G. M. & Rotstein, S. H. Structure-function relationships of glutamine synthetases. *Biochimica et biophysica acta* 1477, 122–145 (2000).
39. Almassy, R. J., Janson, C. A., Hamlin, R., Xuong, N. H. & Eisenberg, D. Novel subunit-subunit interactions in the structure of glutamine synthetase. Nature 323, 304–309, https://doi.org/10.1038/323304a0 (1986).
40. Mathis, R., Gamas, P., Meyer, Y. & Cullimore, J. V. The presence of GSI-like genes in higher plants: Support for the paralogous evolution of GSI and GSII genes. Journal of molecular evolution 50, 116–122, https://doi.org/10.1007/s002399910013 (2000).
41. Gill, H. S. & Eisenberg, D. The crystal structure of phosphinothricin in the active site of glutamine synthetase illuminates the mechanism of enzymatic inhibition. Biochemistry 40, 1903–1912 (2001).
42. Blundell, T. L., Sibanda, B. L., Sternberg, M. J. & Thornton, J. M. Knowledge-based prediction of protein structures and the design of novel molecules. Nature 326, 347–352, https://doi.org/10.1038/326347a0 (1987).
43. Sanchez, R. & Salo, A. Evaluation of comparative protein structure modeling by MODELLER 3. Proteins Suppl 1, 50–58 (1997).
44. Berman, H. M. et al. The Protein Data Bank. Nucleic Acids Res 28, 235–242, https://doi.org/10.1093/Nar.28.1.235 (2000).
45. Yamashita, M. M., Almassy, R. J., Janson, C. A., Cancio, D. & Eisenberg, D. Refined atomic model of glutamine synthetase at 3.5 A resolution. The journal of biological chemistry 264, 17681–17699 (1989).
46. Ginsburg, A., Yeh, J., Hennig, S. B. & Denton, M. D. Some effects of adenylylation on the biosynthetic properties of the glutamine synthetase from Escherichia coli. Biochemistry 9, 633–649 (1970).
47. Krajewski, W. W.
48. Kim, J. N., Cann, I. K. O. & Mackie, R. I. Purification, Characterization, and Regulation of Glutamine Synthetase from Prevotella ruminicola 23. Microb Ecol 57, 573–574 (2009).
49. Kulakova, L., Galkin, A., Nakayama, T., Nishino, T. & Esaki, N. Cold-active esterase from Psychrobacter sp. Ant300: gene cloning, characterization, and the effects of Glc–>Pro substitution near the active site on its catalytic activity and stability. Biochimica et biophysica acta 1696, 59–65 (2004).
50. Zhang, J. W. & Zeng, R. Y. Molecular cloning and expression of a cold-adapted lipase gene from an Antarctic deep sea psychrophilic bacterium Pseudomonas sp. 7323. Marine biotechnology 10, 612–621, https://doi.org/10.1007/s10126-008-9099-4 (2008).
51. Rahman, M. A. et al. A cold-adapted, solvent and salt tolerant esterase from marine bacterium Psychrobacter pacificensis. International journal of biological macromolecules 81, 180–187 (2015).
52. Rahman, M. A. et al. Characterization of a novel cold active and salt tolerant esterase from Zunongwangia profunda. Enzyme and microbial technology 85, 1–11, https://doi.org/10.1016/j.enzmictec.2015.12.013 (2016).
53. Rahman, M. A., Culsum, U., Kumar, A., Gao, H. & Hu, N. Immobilization of a novel cold active esterase onto Fe 3 O 4 nano-composite enhances catalytic properties. International journal of biological macromolecules 87, 488–497 (2016).
54. Joseph, B., Ramteke, P. W. & Thomas, G. Cold active microbial lipases: some hot issues and recent developments. Biotechnology advances 26, 457–470, https://doi.org/10.1016/j.biotechadv.2008.05.003 (2008).
55. Gomez-Baena, G., Dominguez-Martin, M. A., Donaldson, R. P., Garcia-Fernandez, J. M. & Diez, J. Glutamine Synthetase Sensitivity to Oxidative Modification during Nutrient Starvation in Prochlorococcus marinus PCC 9511. PloS one 10, e0135322, https://doi.org/10.1371/journal.pone.0135322 (2015).
56. Panda, T. & Gowrishankar, B. S. Production and applications of esterases. Applied microbiology and biotechnology 67, 160–169, https://doi.org/10.1007/s00253-004-1640-y (2005).
57. Gill, H. S. & Eisenberg, D. The crystal structure of phosphinothricin in the active site of glutamine synthetase illuminates the mechanism of enzymatic inhibition. Biochemistry 72, 248–254 (1976).
58. Shapiro, B. M. & Stadtman, E. R. The regulation of glutamine synthesis in microorganisms. Nature 323, 304–309, https://doi.org/10.1038/323304a0 (1986).
59. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical biochemistry 72, 248–254 (1976).
60. Shapiro, B. M. & Stadtman, E. R. Glutamine Synthetase Sensitivity to Oxidative Modification during Nutrient Starvation in Prochlorococcus marinus PCC 9511. PloS one 10, e0135322, https://doi.org/10.1371/journal.pone.0135322 (2015).
61. Kamnev, A. A. et al. Structural characterization of glutamine synthetase from Azospirillum brasilense. Biopolymers 74, 64–68, https://doi.org/10.1002/bip.20045 (2004).
62. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical biochemistry 72, 248–254 (1976).
63. Shapira, B. M. & Stadtman, E. R. The regulation of glutamine synthetase in microorganisms. Annual review of microbiology 24, 501–524, https://doi.org/10.1146/annurev.mi.24.100170.002441 (1970).
64. Rockmill, B. & Roeder, G. S. Telomere-mediated chromosome pairing during meiosis in budding yeast. Genes & development 12, 2574–2586 (1998).