



OPEN Tapping the untapped: type II L-asparaginase from marine crustacean-derived *Chryseomicrobium amylolyticum* as a potential bio better for blood cancer therapy

V. P. Lailaja¹, T. G. Sumithra¹ , S. R. Krupesha Sharma¹, M. Ashif Mohammed², Unnikrishnan Sivan³ & V. N. Anusree¹

L-asparaginase is a vital antineoplastic drug. The side effects of the current formulations intrigued researchers to explore novel candidates. The study evaluated a novel type-II L-asparaginase from *Chryseomicrobium amylolyticum* from marine crab, *Scylla serrata*. Statistical optimization enhanced production to 281.6 from 115.69 IU/ml. The purified enzyme had 173.69 IU/mg protein specific activity. The *K_m*, *V_{max}*, and *K_{cat}* were 6.364 μ M, 909.09 μ M/min, and 222.82 /sec. The enzyme didn't react with L-glutamine, D-glutamine, L-glutamic acid, and urea, a clinically desirable feature. In vitro trypsin and serum half-life were ~ 39 h and ~ 27 min. The study enlisted ions, protein-modifying agents, and surfactants influencing its activity. The detailed in silico analyses of the functional gene predicted its physicochemical and structural features. The low sequence identity with the commercial enzymes and the predicted nonallergenic and nontoxic features suggested its clinical suitability. Molecular docking revealed a higher binding affinity for L-asparagine than L-glutamine and urea. The IC₅₀ against MCF7, MOLT4 and THP-1 cell lines were 0.043, 0.041, and 0.05 IU/ml, respectively. Phase contrast microscopy confirmed its cytotoxicity. Flow cytometry demonstrated 81.4% apoptotic cells in MOLT-4 cells at IC₅₀, signifying potent anticancer activity. The enzyme did not affect normal human fibroblast cells and was non-haemolytic. The results underscore the therapeutic potential of *C. amylolyticum* type-II L-asparaginase and support further in vivo studies to advance L-asparaginase-based cancer therapies.

Keywords Statistical optimization, Antineoplastic enzyme, Molecular docking, In vitro cell culture

L-asparaginase (EC 3.5.1.1) is an extensively used oncotherapeutic enzyme against various hematopoietic malignancies. The medicine accounts for ~40% of the total enzyme needs worldwide and one-third of the world's requirement for anticancer agents¹. Presently, it is commercially produced from either *Escherichia coli* or *Erwinia chrysanthemi*. Further, pegylated *E. coli* L-asparaginase variant is also available forming the first, second, and third-line treatment regimens². Unfortunately, these forms have limits in certain cancer patients with hypersensitivity, pancreatitis, coagulation disorders, diabetes, and kidney and liver dysfunctions due to immunogenicity or concurrent L-glutaminase and urease activities, so supplementary purification protocols are done to minimize adverse effects³. Hence, the key focus in the current asparaginase exploration is to delineate other sources for an L-asparaginase free of L-glutaminase and urease actions⁴. The limitation in long-term administration due to immunogenicity also necessitates identifying alternative microbial resources producing serologically distinct L-asparaginases⁵.

¹Marine Biotechnology, Fish Nutrition and Health Division, ICAR-Central Marine Fisheries Research Institute (CMFRI), Ernakulam North P.O, Post Box No. 1603, Kochi 682 018, India. ²Cochin University of Science and Technology Kochi, Kochi, Kerala 682022, India. ³Kerala university of fisheries & ocean studies, Panangad P.O., Kochi, Kerala 682 506, India. ✉email: sumithravet@gmail.com; sumithra.G@icar.org.in

In the search for novel microbial sources, screening for type II L-asparaginases with low K_m (high affinity) is crucial, as only enzymes active at the micromolar L-asparagine levels found in human blood are therapeutically effective⁶. Type II enzymes are advantageous due to their periplasmic secretion, enabling easier large-scale production, especially from Gram-positive bacteria, which lack a periplasmic space⁶. Since bacterial growth conditions contribute to 30 to 40% yield variation, culture optimization is essential. Traditional media optimization is inefficient, making statistical approaches like response surface methodology (RSM) preferable. Recombinant production can also enhance scalability and cost-effectiveness^{7,8}. Stability improvements of the enzyme have been achieved through nanoparticle immobilization, chemical modifications, and protein/media engineering⁹. Marine microbes represent promising sources of novel biocatalysts^{8,10} and recent studies have identified marine asparaginases without L-glutaminase activity from *Bacillus licheniformis* and *Bacillus velezensis*^{11,12} and without L-glutaminase and urease activity from *Trichosporon asahii*, *Chryseomicrobium amylolyticum*, *Bacillus altitudinis*, and *Priestia megaterium*^{4,13,14}.

The present study investigates the antineoplastic competence of a previously identified type II L-asparaginase from *Chryseomicrobium amylolyticum* (CamASNase), shown to lack L-glutaminase and urease activity⁴. Enzyme production was optimized using statistical tools, including Plackett-Burman and RSM-Central Composite Design. The enzyme was then purified and characterized for its physicochemical and kinetic properties. Additionally, the complete sequence of the *ansB* gene encoding CamASNase was obtained for the first time and subjected to in silico analysis. Briefly, in vitro cell culture-based research (using a breast cancer cell line (MCF-7), two leukaemia cell lines (THP-1 and MOLT-4), and normal human fibroblast cells (HDF) and in silico research (sequence analysis, 3D protein structure modelling, and molecular docking studies) were instrumented to validate the antineoplastic competence of the enzyme.

Results

Revival of the bacterial strain and confirmation of L-asparaginase production in control conditions

The CMFRI/ChAm-01 produced 115.69 ± 1.44 IU/ml after 24 h in the control conditions. The isolate was deposited in microbial type culture collection at the Institute of Microbial Technology, Chandigarh (MTCC ID: 13184).

Statistical optimization of L-asparaginase production

Plackett-Burman (PB) design

The activity during PB experiments ranged from 19.75 to 152.9 IU/ml (Table 1). Statistical analysis showed that the four variables, namely peptone, inoculum concentration, incubation temperature, and speed of agitation positively affected L-asparaginase production. Whereas, initial medium pH, NH_4Cl and NaCl showed negative effects. The Pareto chart illustrated that the initial medium pH was the most significant variable (Fig. 1A). ANOVA of the model had a F -value of 45.68 ($p=0.0003$), R^2 value of 0.98, and adjusted R^2 value of 0.96. The strong correlation between the observed and predicted values (Fig. 1B), and the residual plot indicating an even dispersion of residual data around the x-axis (Fig. 1C) demonstrated the accuracy. The generated polynomial equation was $Y = 98.83 - 22.75(B) + 7.38(C) + 11.77(D) - 15.59(G) + 12.72(H) - 18.62(I)$; where Y = L-asparaginase production, and B, C, D, G, H, and I are initial pH, incubation temperature, inoculum concentration, NaCl, peptone, and NH_4Cl , respectively. Experimental verification of the model in a medium (initial pH=6) with 1.05 mM L-asparagine, 3.7% inoculum concentration, 0.15% K_2HPO_4 , 1.13% NaCl, 0.18% peptone, and 10 mM NH_4Cl after incubation at 30 °C with 125 rpm agitation for 24 h resulted in 155.44 IU/ml enzyme production and the model accuracy was 98.87%.

Centre composite design (CCD)

The activity ranged from 24 to 282 IU/ml (Table 2). The model had a F -value of 58.13 ($p<0.0001$) (Table 2), R^2 value of 0.97, and adjusted R^2 value of 0.96. The strong correlation between the observed and predicted values (Fig. 2A) and normal probability plot of residuals (Fig. 2B) displayed the accuracy. Based on the observed factors and predicted responses, the second-order polynomial equation predicted was $Y = 264.89 + 9.33 A + 11.82B + 13.68 C + 7.62D - 17.25E + 18.92 BD + 8.41AD + 13.73 CD - 9.90 DE - 36.88A^2 - 26.11B^2 - 15.75C^2 - 54.39 D^2 - 40.78 E^2$, where Y = L-asparaginase production, A, B, C, D, and E are peptone, incubation temperature, inoculum concentration, initial medium pH, and NH_4Cl concentration, respectively. The response surface plot between incubation temperature (B), and pH (D) indicated a synergistic action of these two factors with the maximal production at a temperature of 30.5 °C and pH of 6.6 (Fig. 2C). Similarly, there was a synergistic interaction between inoculum concentration (C) and pH (D) (Fig. 2D), between peptone concentration (A) and pH (D) (Fig. 2E), and antagonistic interaction between pH (D) and NH_4Cl concentration (E) (Fig. 2F) with the highest production at the median levels of each variable. Experimental verification of the optimal conditions was done by checking the L-asparaginase production in a medium (initial pH=6.7) with 1mM L-asparagine, 4.5% inoculum concentration, 0.2% K_2HPO_4 , 1% NaCl, 0.37% peptone, and 6.9 mM NH_4Cl after incubation at 30.6 °C with 150 rpm agitation for 24 h resulted in the production of 273.77 IU/ml and the model accuracy was 96.96%.

Purification of CamASNase

The specific activity, fold purification, and % yield during each step is given in SFig. 1 and Table S4. In brief, the enzyme had a specific activity of 173.69 IU/mg protein and was 34.6 times purified.

		A	B	C	D	E	F	G	H	J	K	L	L-asparaginase activity (U/ml)		
Std	Run	A (%)	pH	Temp (°C)	Inoculum (%)	Agitation (rpm)	K ₂ HPO ₄ (%)	NaCl (%)	Peptone (%)	NH ₄ Cl (%)	D1	D2	Experimental	Predicted	Residuals
2	1	-1	1	1	-1	1	1	1	-1	-1	-1	1	67.01 ± 0.53	62	5.01
11	2	1	-1	1	1	1	-1	-1	-1	1	-1	1	130 ± 1.053	124.98	5.02
12	3	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	127.9 ± 0.42	123.98	3.98
5	4	-1	-1	1	-1	1	1	-1	1	1	1	-1	128.7 ± 0.87	126.88	1.82
7	5	1	-1	-1	-1	1	-1	1	1	-1	1	1	118 ± 0.31	118.19	- 0.18
10	6	-1	1	1	1	-1	-1	-1	1	-1	1	1	138.9 ± 0.76	142.16	- 3.26
9	7	1	1	1	-1	-1	-1	1	-1	1	1	-1	19.75 ± 1.22	24.76	- 5.01
3	8	1	-1	1	1	-1	1	1	1	-1	-1	-1	152.9 ± 0.55	156.49	- 3.59
4	9	-1	1	-1	1	1	-1	1	1	1	-1	-1	69.8 ± 1.22	58.98	10.82
8	10	1	1	-1	-1	-1	1	-1	1	1	-1	1	61 ± 0.49	66.61	- 5.61
ANOVA table															
Factor				Coefficient estimate			Standardized effect			Contribution (%)			p-value		
Intercept				98.83											
Model													0.003		
B-Media pH				- 22.75			- 45.5067			34.77			0.0002		
C-Temperature				7.38			14.76			3.658			0.0241		
D-Inoculum				11.77			23.54			9.3			0.0038		
G-NaCl				- 15.59			- 31.1733			16.31			0.0011		
H-Peptone				12.72			25.44			10.867			0.0027		
J-NH ₄ Cl				- 18.62			- 37.24			23.29			0.0005		
R ²				0.9821											
Adjusted R ²				0.9606											
Predicted R ²				0.8968											

Table 1. Statistical optimization of *C. amylolyticum* type II L-asparaginase production using Plackett–Burman design. A, L-asparagine; RPM, rotations per min; D1 and D2, dummy variables; NaCl, sodium chloride; Temp, temperature.

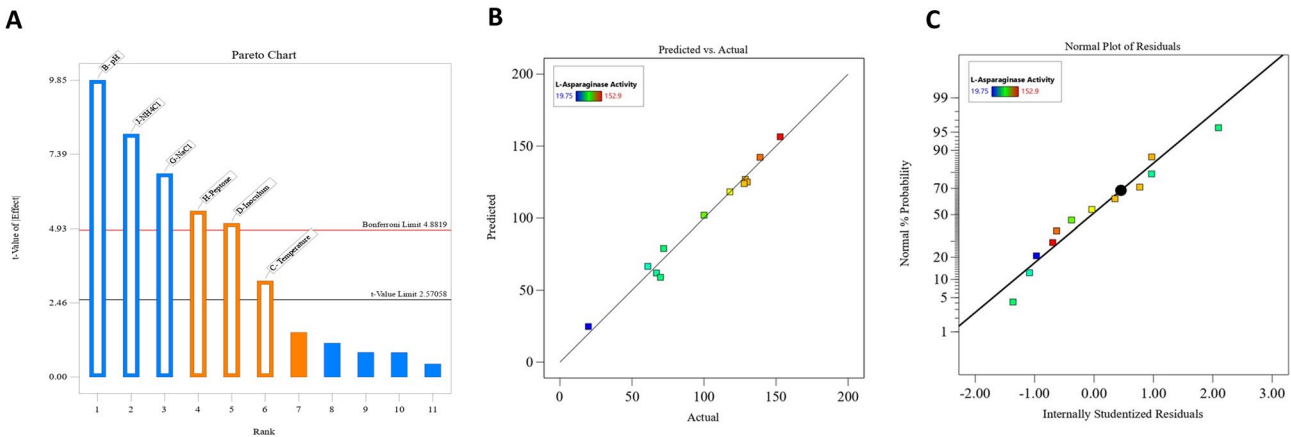


Fig. 1. Plackett-Burman (PB) design-based media optimization for *C. amylolyticum* type II L-asparaginase production. (A) Pareto Chart showing the order of significance of the variables affecting the enzyme production. Nine independent factors were screened with two unassigned/ dummy parameters. The independent factors are: A: L-asparagine (mM), B: medium pH, C: incubation temperature (°C), D: Inoculum (%), E: Agitation (rpm), F: K₂HPO₄ (%), G: NaCl (%), H: Peptone (%), J: NH₄Cl (mM), K: Dummy 1, L: Dummy 2. Orange bars denote those variables with significant ($p < 0.05$) positive effects and blue bars denote those variables with significant ($p < 0.05$) negative effects; (B): Correlation plot of predicted values against actual values of the enzyme activity; (C): Normal probability plot of the internally studentized residuals.

Std	Block	Run	Coded variables					L-Asparaginase activity (U/ml)		
			A	B	C	D	E	Experimental	Predicted	Residual
24	B1	1	Peptone (%)	Temperature (°C)	Inoculum (%)	pH	NH ₄ Cl (%)			
11	B1	2	0	0	0	0	0	276.7	270.73	5.97
10	B1	3	− 1	1	− 1	1	1	39.7	71.31	− 31.61
8	B1	4	1	− 1	− 1	1	1	56.4	28.49	27.91
13	B1	5	1	1	1	− 1	− 1	102.2	98.74	3.46
16	B1	6	− 1	− 1	1	1	1	57.9	64.65	− 6.75
21	B1	7	1	1	1	1	1	150.9	144.79	6.11
15	B1	8	0	0	0	0	0	278.5	270.73	7.77
4	B1	9	− 1	1	1	1	− 1	194	180.43	13.57
7	B1	10	1	1	− 1	− 1	1	77	84.15	− 7.15
6	B1	11	− 1	1	1	− 1	1	67	65.4	1.6
2	B1	12	1	− 1	1	− 1	1	84	98.25	− 14.25
20	B1	13	1	− 1	− 1	− 1	− 1	92.88	113.03	− 20.15
22	B1	14	0	0	0	0	0	222.7	270.73	− 48.03
12	B1	15	0	0	0	0	0	278.6	270.73	7.87
14	B1	16	1	1	− 1	1	− 1	149.5	144.26	5.24
5	B1	17	1	− 1	1	1	− 1	120	137.6	− 17.6
19	B1	18	− 1	− 1	1	− 1	− 1	106.33	94.29	12.04
3	B1	19	0	0	0	0	0	282	270.73	11.27
18	B1	20	− 1	1	− 1	− 1	− 1	83.11	80.19	2.92
23	B1	21	0	0	0	0	0	281.6	270.73	10.87
1	B1	22	0	0	0	0	0	281.1	270.73	10.37
9	B1	23	− 1	− 1	− 1	− 1	1	102.66	79.69	22.97
17	B1	24	− 1	− 1	− 1	1	− 1	53.66	64.13	− 10.47
36	B2	25	0	0	0	0	0	276.8	270.73	6.07
30	B2	26	0	0	0	0	0	251.2	259.04	− 7.84
35	B2	27	0	0	2	0	0	222.8	223.41	− 0.6107
ANOVA table										
Source			Coefficient estimate		Sum of squares		F-value		P-value	
Intercept			264.89							
Block			B1 5.58 B2 −5.85		1094.03					
Model					2.50E + 05		58.13		<0.0001 (Significant)	
A-Peptone			9.33		2087.68		6.31		0.0202	
B-Temperature			11.82		3353.57		10.14		0.0045	
C-Inoculum			13.68		4492.51		13.59		0.0014	
D-pH			7.62		1393.55		4.21		0.0528	
E-NH4Cl			-17.25		7138.74		21.59		0.0001	
BD			18.92		5726.71		17.32		0.0004	
CD			13.73		3015.66		9.12		0.0065	
DE			-9.90		1568.16		4.74		0.0410	
AD			8.41		1131.65				0.0418	
A ²			-36.88		43515.94		131.60		<0.0001	
B ²			-26.11		21809.51		65.95		<0.0001	
C ²			-15.75		7935.69		24.00		<0.0001	
D ²			-54.39		94661.08		286.26		<0.0001	
E ²			-40.78		53207.03		160.26		<0.0001	
Residual					6944.25					
Lack of fit					4108.23		0.8914		0.5903 (not significant)	
Pure error					2836.03					
Cor total					2.58E + 05					
Fit Statistics										
R ²			0.9730							
Adjusted R ²			0.9562							
Predicted R ²			0.9308							

Table 2. Statistical optimization of *C. amylolyticum* type II L-asparaginase production using centre composite design.

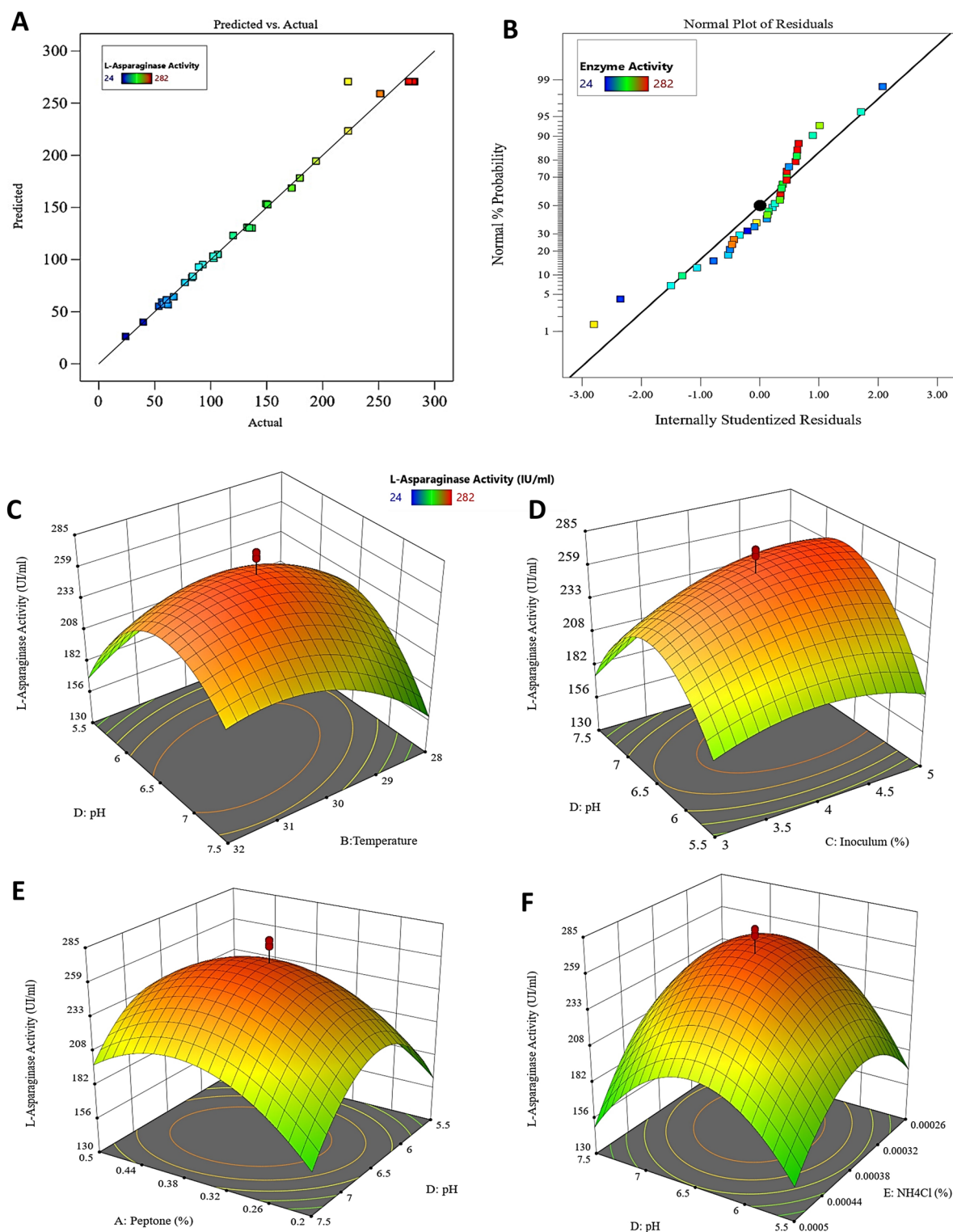


Fig. 2. Centre composite design-based media optimization for *C. amylolyticum* type II L-asparaginase production. (A): Correlation plot between the actual and predicted values for L-asparaginase activity; (B): Normal probability plot of internally studentized residuals; (C): Response surface plot showing the interaction between different incubation temperature (B) and initial medium pH (D); (D): showing the interaction between different inoculum concentration (C) and initial medium pH (D); (E): Response surface plot showing the interaction between different peptone concentration (A) and initial medium pH (D); (F) Response surface plot showing the interaction between different NH_4Cl concentrations (E) and initial medium pH (D).

Biochemical characterization

Stability evaluation

The purified enzyme retained >95% of its activity during the initial two hours of incubation, after which a significant ($p < 0.001$) decrease was noticed. There was no significant difference ($p = 0.95$) between one and two hours of incubation. Further, the enzyme retained >80% and >75% of its activity up to 12 and 24 h, respectively after which a steep decline was recorded (Fig. 3A). However, the purified enzyme retained only 36.76% of its activity after lyophilization showing a significant decrease ($p < 0.001$) in the activity after lyophilization (63.93 IU/mg protein).

Effect of ions, protein modifying agents, and surfactants

Two-way ANOVA showed a significant influence of the type of ions, its concentration, and ion type-concentration interaction on the activity ($p < 0.001$). Fe^{2+} acted as an enhancer in all the tested concentrations, with significantly higher ($p < 0.001$) activity at 10 mM compared to 5 and 15 mM. Three ions, namely Co^{2+} , Mn^{2+} and NH_4^+ ions acted as enhancers at 5 and 10 mM concentrations. Fe^{3+} and Na^+ acted as enhancers at 5 and 10 mM concentration, respectively. The ions, viz. K^+ , Zn^{2+} , Mg^{2+} , and Cu^{2+} ions were inhibitors in all the tested concentrations (Fig. 3B). Among different ion-concentration combinations, the maximal activity was caused by Fe^{3+} ions at 5 mM concentration. Two-way ANOVA showed the significant influence of the type of surfactants, its concentration, and surfactant type-concentration interaction on the activity ($p < 0.001$). All the evaluated surfactants showed a significant augmentation at 0.05% concentration, but retarded activity at 0.1% (Fig. 3C). The maximal augmentation was caused by PEG immediately followed by Triton X-100, both at 0.05% concentration. Regarding protein modifying agents also, two-way ANOVA showed the significant influence of the type, its concentration, and type-concentration interaction on the activity ($p < 0.001$). The agents tested, namely, β -mercaptoethanol, DMSO, DTT, and SDS caused an improved activity at 5 mM, but caused a dip at 10 mM, except β -mercaptoethanol (Fig. 3D). β -mercaptoethanol caused augmentation at both tested concentrations. The presence of PMSF completely inactivated the CamASNase at all the tested concentrations. The maximal augmentation of the activity was caused by DTT, immediately followed by β -mercaptoethanol both at 5 mM concentration (Fig. 3D).

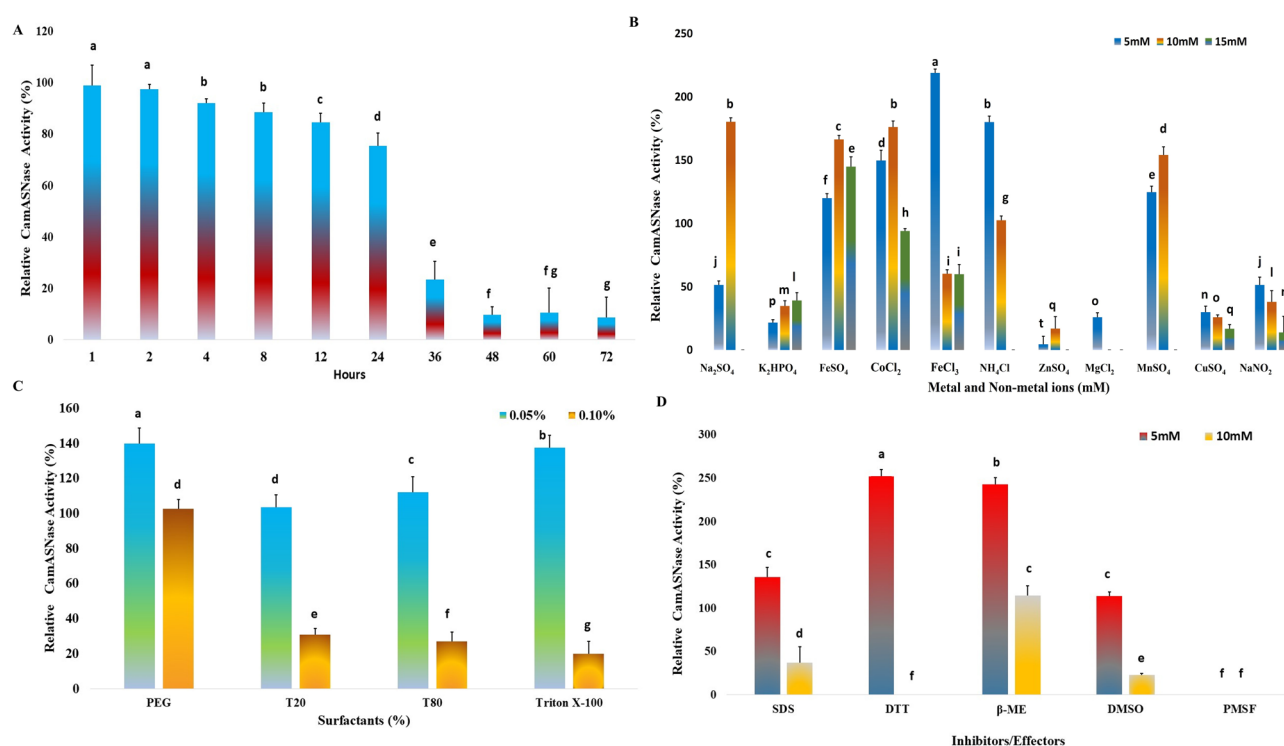


Fig. 3. Stability evaluation and effect of ions, surfactants, and protein modifying agents for *C. amylolyticum* type II L-asparaginase activity. (A): Stability of the enzyme at human physiological conditions (pH = 7.4; temperature = 37 °C); (B): Effect of different metal and non-metal ions on the activity; (C): Effect of different surfactants on the activity; (D): Effect of protein modifying agents on the activity. Data are presented as mean $\pm$ SEM (standard error of the mean). Error bars represent the SEM of enzymatic activity. Specifically, three biological replicates were analysed, each with three technical replicates (totalling nine measurements). First, the mean and standard deviation (SD) of the technical replicates were calculated for each biological replicate. Subsequently, the mean of these biological replicate averages was determined, and the SEM was calculated by dividing the SD by the square root of 3 ($\sqrt{3}$). Different alphabets indicate different levels based on statistical significance ($p < 0.05$).

Substrate specificity and kinetic parameters

There was a significant difference in the enzyme activity between substrates ($p < 0.001$) (Fig. 4A). In detail, the purified CamASNase was inactive towards L-glutamine, D-glutamine, L-glutamic acid, and urea, but displayed 5.7% relative activity with Boc-L-asparagine compared to L-asparagine. In the kinetic studies, the CamASNase activity increased gradually with the increase in the substrate concentration from 0.3 to 0.019 mM; thereafter, the activity decreased with increase in the substrate (Fig. 4B). The optimum substrate concentration was 0.019 mM. The K_m and V_{max} were calculated as 6.364 μ M and 909.09 μ M/min, respectively. The K_{cat} was 222.82/sec.

In vitro serum and trypsin half-life

The presence of both trypsin and serum significantly reduced the enzymatic activity, and the inhibition was significantly increased as the time progressed ($p < 0.001$). The trypsin half-life was ~ 27 min (Fig. 4C). The enzyme lost complete activity after 50 min of incubation in the presence of trypsin. The CamASNase retained $> 70\%$ activity in the serum for 24 h. The enzyme retained 50% of its activity for 39 h (Fig. 4D).

Structural modelling and in silico analysis

The gene-specific PCR from cDNA produced a single band of ~ 266 bp and sequence analysis confirmed the specific amplification. The generated full-length sequence was 981 bp that coded for 326 amino acid-protein, and submitted to NCBI GenBank (Accession number WQG15662). The phylogenetic analysis confirmed the tight clustering of asparaginase encoding genes based on their type rather than their host source (SFig. 2). The amino acid percentage identity of type II gene of CMFRI/ChAm-01 with type II asparaginase coding genes of *Chryseomicrobium excrementi* (WP100352642.1), *Chryseomicrobium aureum* (WP204589139.1), *Chryseomicrobium* sp. FSL W7-1435 (WP342525734.1), *E. coli* (WP160472400.1), and *D. chrysanthemi* (WP226052626.1) was 93.6%, 79.7%, 79.5%, 31.6% and 32.2%, respectively. The predicted physicochemical parameters of CamASNase compared to the commercial formulations (*E. coli* and *D. chrysanthemi* type II asparaginases) are given in Table S5. The presence of signal peptide was predicted from the sequence. CamASNase was predicted as a non-allergenic, whereas the other two commercial asparaginases were predicted as allergenic. The ToxinPred server showed the absence of toxic peptide motifs in the L-asparaginase proteins of all three microbes.

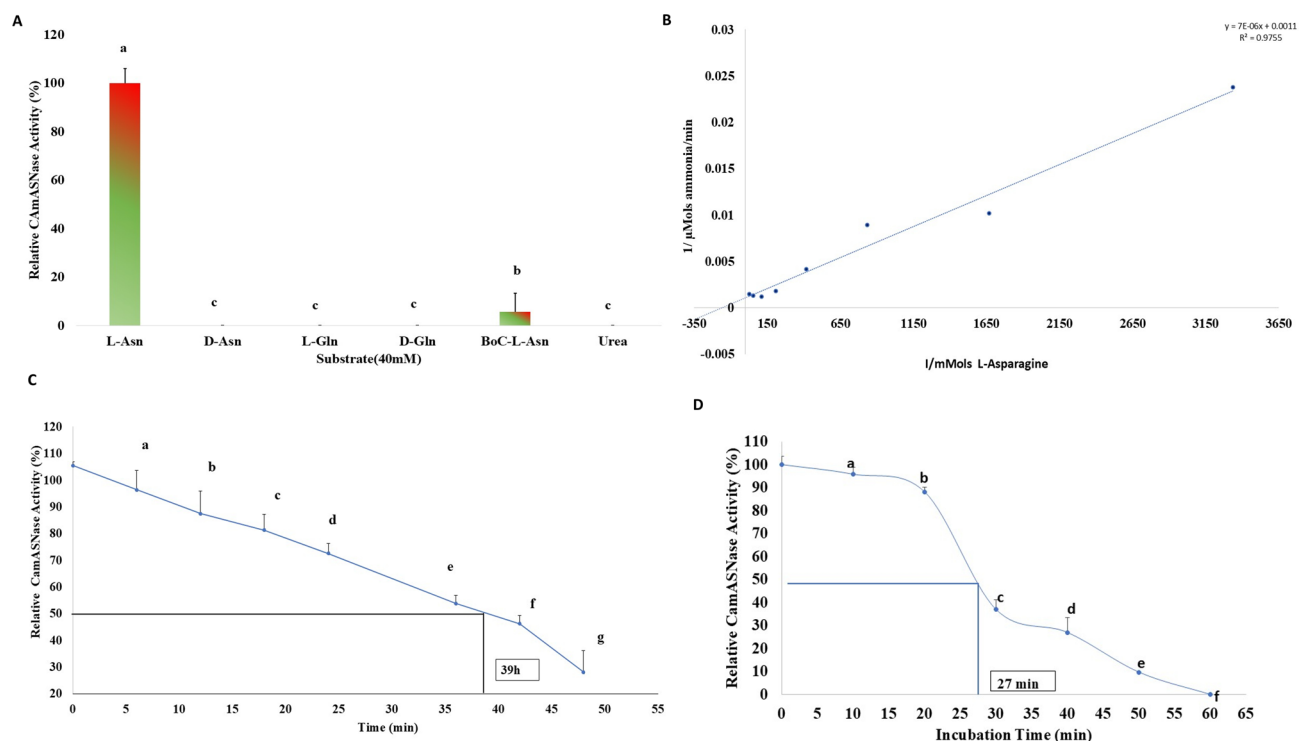


Fig. 4. Substrate specificity, kinetic parameters, in vitro serum and trypsin half-life of *C. amylolyticum* type II L-asparaginase. (A): Enzyme activity towards various substrates; (B): Impact of varying L-asparagine concentrations on the activity; (C): In vitro serum half-life of the enzyme; (D): In vitro trypsin half-life of the enzyme. Data are presented as mean $\pm$ SEM (standard error of the mean). Error bars represent the SEM of enzymatic activity. Specifically, three biological replicates were analysed, each with three technical replicates (totalling nine measurements). First, the mean and standard deviation (SD) of the technical replicates were calculated for each biological replicate. Subsequently, the mean of these biological replicate averages was determined, and the SEM was calculated by dividing the SD by the square root of 3 ($\sqrt{3}$). Different alphabets indicate different levels based on statistical significance ($p < 0.05$).

The secondary structure prediction tool showed CamASNase had 35.28% α -helix (115 amino acids), 46.93% random coils (153 amino acids), and 17.79% extended strands (58 amino acids).

The C-score of the predicted 3D structure of the monomer through i-TASSER was 1.38. The Ramachandran plot illustrated that 90.7% of amino acids fell in the favoured region and 7.8% residues fell in the additionally allowed region (Fig. 5). The model had an overall ProSA z-score of -9.34 , QMEANDisCo score of 0.71 ± 0.05 , and ERRAT score of 91.47. The predicted 3D structure through AlphaFold v 2.0 (Fig. 5) had the ERRAT score of 93.99, ProSA z-score of -11.3 and QMEANDisCo score of 0.78 ± 0.05 . The Ramachandran plot illustrated that 95.4% of the amino acids fell in favoured region, 3.6% residues fell in the additionally allowed region, and 0.3% residues fell in the generously allowed region (Fig. 5). The alignment between the models made through i-TASSER model and AlphaFold 2.0 had a RMSD value of 0.845. The predicted 3D structure of the CamASNase tetramer (Fig. 5) had the ProSA z-score of -9.81 , QMEANDisCo score of 0.73 ± 0.05 , and ERRAT score of 87.77. The Ramachandran plot (PROCHECK) illustrated that 92.8% of the amino acids fell in the favoured region, 6.8% residues fell in the additionally allowed region and 0.4% residues fell in the generously allowed region (Fig. 5).

Molecular docking studies

Two active site signature patterns (PS00144 and PS00917) were identified. The amino acid patterns for PS00144 and PS00917 were GVVITHGDTL and VIATGGTIA, respectively. The binding pocket residues for

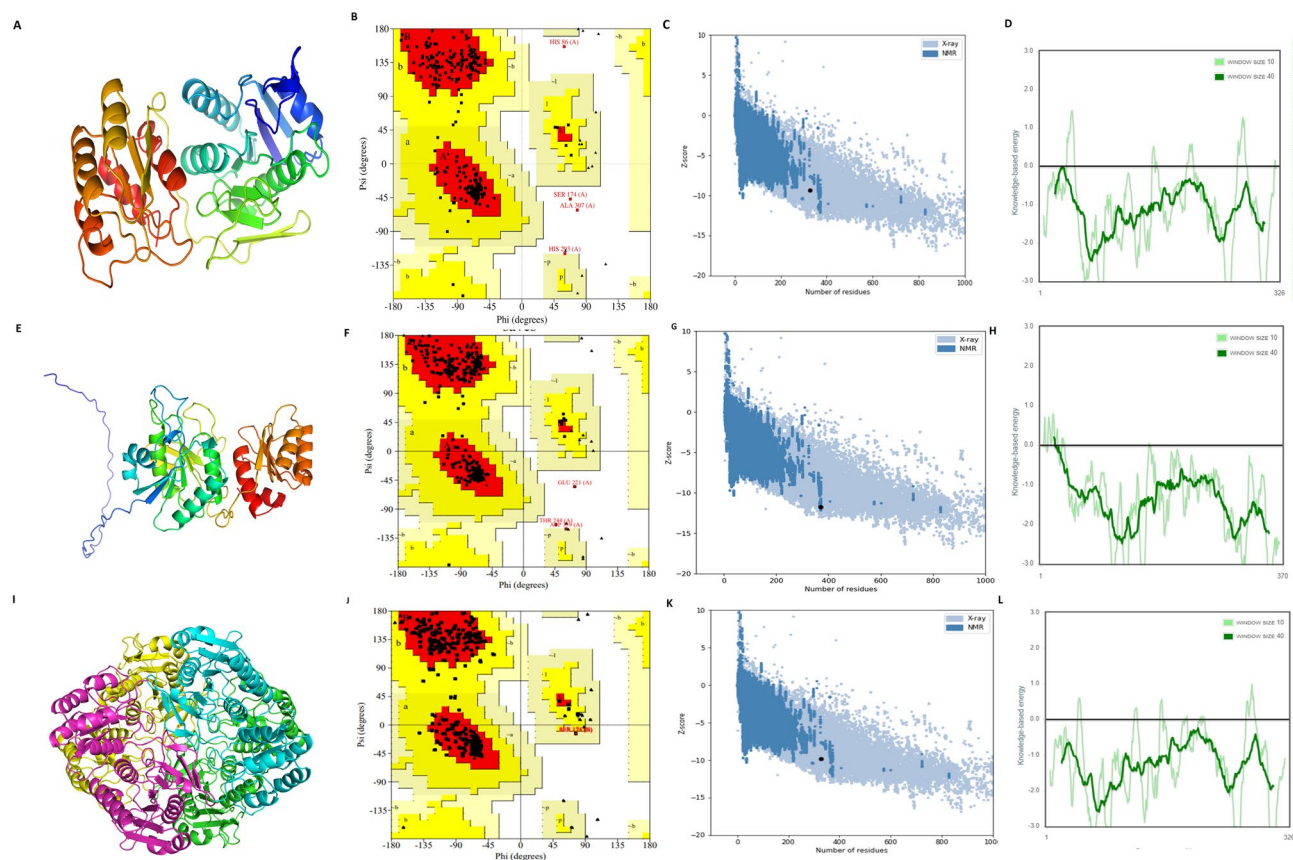


Fig. 5. Three-dimensional structure of type II L-asparaginase from *Chryseomicrobium amylolyticum* through different modelling servers and their validation. (A): 3D monomer structure through the ‘iTASSER server’; (B): Ramachandran plot-based validation of the ‘iTASSER’ based structure; (C): ProSA-based quality evaluation ‘iTASSER’ model; (D): ProSA estimated energy profile of the ‘iTASSER’ model; (E): 3D monomer structure through the ‘AlphaFold’; (F): Ramachandran plot-based validation of the ‘AlphaFold’ based structure; (G): ProSA-based quality evaluation of the ‘AlphaFold’ model. (H): ProSA estimated energy profile of the ‘AlphaFold’ model; (I): 3D structure of the tetramer through the ‘AlphaFold’; (J): Ramachandran plot-based validation of the tetrameric structure through the ‘AlphaFold’; (K): ProSA-based quality evaluation of the ‘AlphaFold’ tetramer structure; (L): ProSA estimated energy profile of the ‘AlphaFold’ tetramer structure. In (B, F and J) figures, the alphabets, (A, B, and L) designate amino acids in favored regions; a, b, l, and p designate amino acids in additional allowed regions; ~a, ~b, ~l, ~p designate amino acids in generously allowed regions. The red color shows the most favored regions. In (C, G, and K) figures, ProSA-web z-scores of all protein chains in PDB identified by X-ray crystallography (light blue) or NMR spectroscopy (dark blue) are depicted. The z-scores of the studied protein are represented by a black dot. In D, H and L: ProSA analysis showed that the knowledge-based energy values of most of the amino acid residues are negative, indicating the reliability and quality of the built 3-D structures.

Ligands	Parameter	Amino acids residues involved
L-asparagine	Hydrogen bond	T12, D89, T88, G87, A113(Chain-C), Y277(Chain-A)
	Active site residues	G11, T12, S55, D89, T88, G87, A113, M114, K161(Chain-C), Y277(Chain-A)
	Binding affinity (kcal/mol)	-5.0
L-glutamine	Hydrogen bond	D89(Chain-A), Q239, N241(Chain-C)
	Active site residues	T12, D89, T88, G87, M114, K161(Chain-A), Q239, G238, N241, V274(Chain-C)
	Binding affinity (kcal/mol)	-4.6
Urea	Hydrogen bond	V263, S264, H293, G294, N296 (Chain-C)
	Active site residues	V263, N268, H293, G294, N296, L272 (Chain-C)
	Binding affinity (kcal/mol)	-3.5

Table 3. Molecular Docking results of *C. amylolyticum* type II L-asparaginase with L-asparagine, L-glutamine, and urea.



Fig. 6. Molecular interactions between *C. amylolyticum* type II L-asparaginase and L-asparagine. (A): L-asparagine (ligand) occupied at the active site; (B): Molecular interactions between the enzyme and the ligand; (C): Shows the prominent amino acid residues of the enzyme interacting with the bound ligand.

L-asparagine, L-glutamine, and urea are given in Table 3. Validation through the consurf server showed that the binding pocket residues were conserved and occupied in the two active site signature patterns. Compared to the other two substrates, the amino acid composition of the urea-binding site was unique. Moreover, it failed to demonstrate the involvement of any significant functional residues. The optimal ligand docking pose (Fig. 6) suggested that L-asparagine (-5.0 kcal/mol) had the maximum binding affinity (shown through low binding energy) for CamASNase, followed by L-glutamine (-4.6 kcal/mol), and urea (-3.0 kcal/mol) (Table 3).

Anticancer activity of CamASNase

Cytotoxicity evaluation using MTT assay

A dose-dependent increase in cytotoxicity was observed in all the cell lines ($p < 0.001$) (Fig. 7) and the IC₅₀ values against MCF7, MOLT4 and THP-1 cell lines were found to be 0.043 IU/ml, 0.041 IU/ ml, and 0.05 IU/ ml, respectively. There was only ~ 12% viability in MCF cell line at 0.14 IU/ml, ~ 29% cell death in MOLT4 at 0.17 IU/ml and 100% cell death in THP-1 cell line at 0.1 IU/ml.

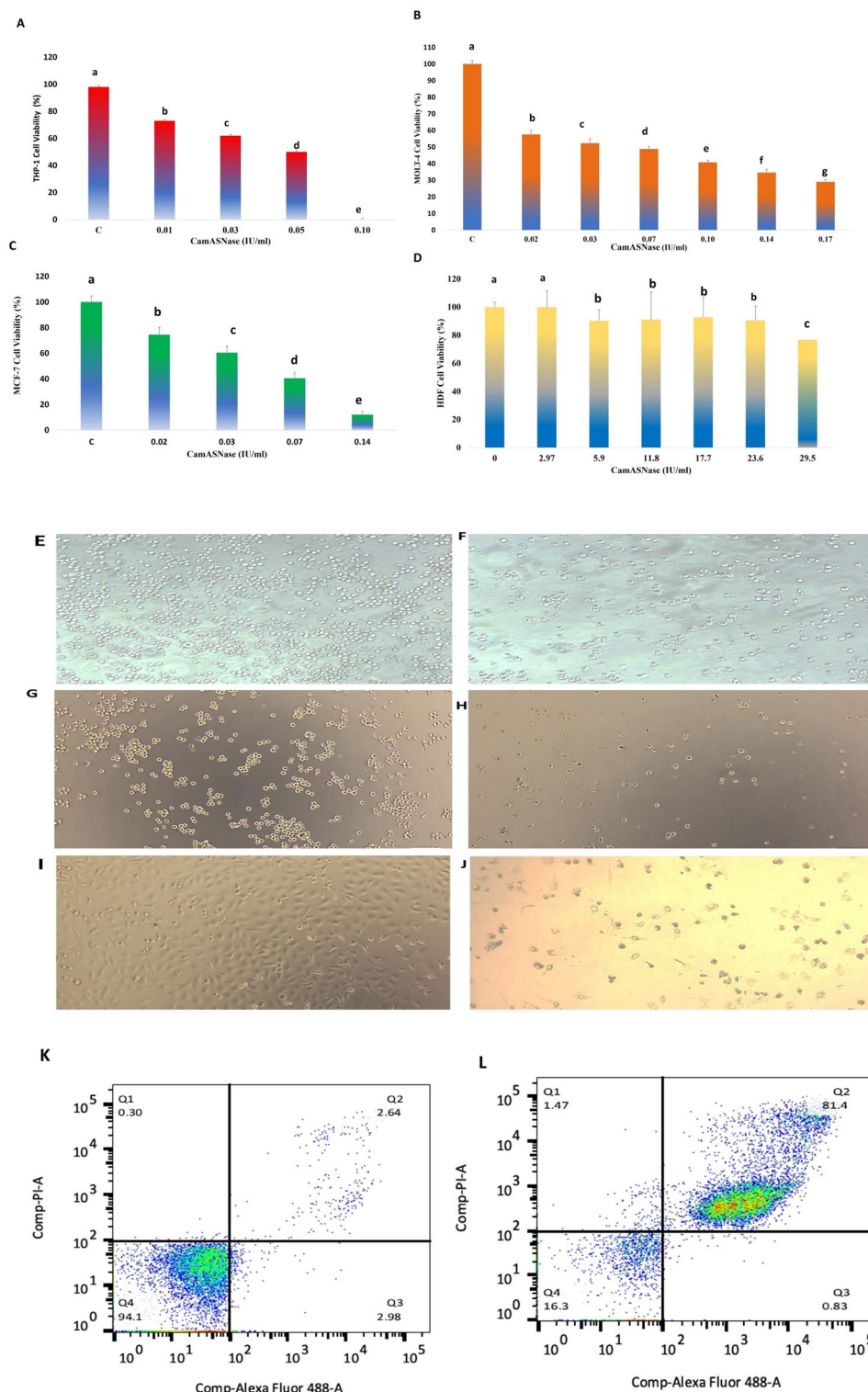
Assessment of cell morphology and apoptosis assay

The morphological changes in THP-1, MCF7 and MOLT4 cells treated with CamASNase included membrane blebbing, cell shrinkage, reduction in size, an increase in the area without the presence of cells, and a rough appearance on their surface (Fig. 7). However, the untreated cells showed no significant morphological alterations. Further, the morphological alterations were increased with increasing doses of CamASNase.

During apoptosis assay, the Annexin V/PI staining showed $78 \pm 2.9\%$ apoptotic cells in the MOLT-4 leukemic cells after 24 h of CamASNase exposure (Fig. 7), with a highly significant difference from the negative control ($p < 0.001$).

Toxicological evaluation on noncancerous human cells

CamASNase demonstrated a dose dependent cytotoxicity in normal cells ($p < 0.001$). CamASNase did not affect normal cell viability up to 2.97 IU/ml ($p > 0.05$) (Fig. 7D). Further, >90% of cells were viable even at 23.6 IU/ml with no significant difference between concentrations ranging from 5.9 to 23.6 IU/ml ($p > 0.05$). No hemolytic activity was observed with purified CamASNase in the blood agar (SFig. 3). The purified enzyme had no hemolytic activity even at the maximum tested concentration (14 IU/ml).



Discussion

There is an urgent need for new L-asparaginase preparations with clinically desirable features due to the numerous adverse effects associated with the currently used formulations. Complete characterization of the enzymatic parameters, molecular features and efficacy against various human carcinoma cell lines of the novel enzymes is critical to contextualize them within the therapeutic landscape. Further large-scale manufacturing requires production maximization through optimizing the bioprocessing parameters. The present study focuses on these critical aspects of one novel type-II L-asparaginase (CamASNase) from a gram-positive bacterium, *C. amylolyticum* CMFRI/Ch Am-01, which was identified from a unique niche, the haemolymph of mud crab (*Scylla serrata*)⁴.

In the first step, we optimized the enzyme production through the Plackett-Burmann (PB) followed by Response Surface Methodology using Central Composite Design (CCD). There was a wide variation (19.75 to

◀ **Fig. 7.** Anticancer activity of *C. amylolyticum* type II L-asparaginase in different cell lines after 24 h treatment. (A) Cytotoxicity evaluation in THP-1 cell lines; (B) Cytotoxicity evaluation in MOLT-4 cell lines; (C) Cytotoxicity evaluation in MCF-7 cell lines (D) Cytotoxicity evaluation in human adult dermal fibroblast cells; (E): Morphology of THP-1 cells (negative control); (F): Morphology of THP-1 cells after 24 h of purified enzyme treatment; (G): Morphology of MOLT-4 cells (negative control); (H): Morphology of MOLT-4 cells after 24 h of purified enzyme treatment; (I): Morphology of MCF-7 cells (negative control); (J): Morphology of MCF-7 cells after 24 h of purified enzyme treatment; (K): Results of apoptosis assay in MOLT-4 leukemia cells (negative control); (L): Results of apoptosis assay in MOLT-4 cells treated with the purified enzyme at IC50 concentration). In figures (A to D): Error bars indicate the standard deviation from the average enzyme activity. Letters starting from 'a' to 'g' indicate different levels based on statistical significance ($p < 0.05$). Figures (F, H, and J) show the cells undergoing apoptosis and are characterized by cellular rounding up, shrinkage, membrane blebbing and loss of cell adhesion. In figure (K and L), the quadrant Q1, Q2, Q3, and Q4 shows the percentage of dead cells, late apoptotic, early apoptotic, and live cells, respectively.

152.9 IU/ml) in L-asparaginase activity during PB experiments, reflecting the significance of the optimization process. The PB results showed five variables; peptone concentration, inoculum density, incubation temperature, agitation speed, and initial medium pH, positively affected L-asparaginase production, while two, NH_4Cl and NaCl concentrations, negatively affected it. Our results on the positive effect of organic nitrogen source (peptone) and negative impact of inorganic nitrogen source (NH_4Cl) on L-asparaginase production were similar to the previous report on L-asparaginase production by different bacteria^{14,15}. They hypothesised that the stimulatory influences of various amino acids and short peptides in the peptone might be the reason for improved L-asparaginase production. The stimulatory effects of inoculum concentration and agitation speed and the negative effect of NaCl on L-asparaginase production by *Streptomyces brolosae* were reported¹⁶, supporting our results. The positive impact of agitation might be due to the homogeneous distribution of the growth medium and enhanced oxygen transfer to the growing cells through adequate mixing, as previously hypothesized¹². Since the lower inoculum density gives insufficient biomass for enzyme formation, the positive influence of inoculum concentration was expected. However, the higher inoculum density can deplete the substrate and nutrients leading to the accumulation of toxic and production-inhibitory substances. El-Naggar et al.¹⁶ reported the negative influence of temperature and pH on the L-asparaginase production by *Streptomyces brolosae* in contradiction to our results. Such difference in the effects of various variables on the enzyme production by different species is always expected and the same warrants the research on maximizing the production of each industrially significant enzyme through statistical optimization. The PB model had the R^2 value of 0.98 demonstrating its accuracy. The predicted optimal conditions resulted in the production of 155.44 IU/ml which was 1.34 times higher than the L-asparaginase production before applying the PB model (115.69 IU/ml).

In the second step, CCD was performed to identify the optimum values of the significant variables identified through the PB model. The strong correlation between the observed and predicted values, the residual plot, and the normal probability plot displayed the model's accuracy. The CCD model had the R^2 value of 0.97. Four factors, viz. peptone concentration, incubation temperature, inoculum concentration, NH_4Cl concentration and three interactions between the factors, viz. incubation temperature and pH, inoculum concentration and pH, and pH and NH_4Cl concentration were identified as the significant terms. The predicted optimal levels were pH (6.5), temperature (30°C), inoculum concentration (4%), peptone (0.35%) and NH_4Cl (7.5 mM). In this context, it is important to note that the optimal temperature was 30°C, which is advantageous for industrial applications, as it reduces the thermal denaturation risk during production. The CCD optimization resulted in the production of 273.77 IU/ml which is 2.37 times higher than the production before optimization (115.69 IU/ml). The validation of the results revealed a good degree of precision of the predicted model. In consonance with the results, 2.41 and 2.65 times increase in L-asparaginase activity by *Acinetobacter baumannii* and *Fusarium foetens* has been reported with CCD optimization^{17,18}.

As the molecular basis behind the CamASNase is not known, we generated the complete sequence of *ansB* gene of *C. amylolyticum* CMFRI/Ch Am-01. The generated sequence showed a lower percentage identity with *E. coli*, and *D. chrysanthemi* (31.63%, and 32.7%, respectively) suggesting a novel serologically different enzyme from the current therapeutic preparations, which is one of the priority requirements for the novel L-asparaginases. The results suggested the CamASNase application as an alternative during the elicitation of the immunological responses due to the long-term administration of the current preparations⁵. In silico sequence analysis revealed that the monomeric molecular weight of CamASNase was 35.58 kDa, consistent with other reports on bacterial asparaginases having molecular weights of 25 to 42.59 kDa⁵. Most mesophilic bacterial type II L-asparaginases occur as tetramers with 140 to 160 kDa molecular weights, supporting our results. Molecular weights of the current therapeutic *E. coli* and *Erwinia chrysanthemi* type II L-asparaginase are of 141 kDa¹⁹ and 150 kDa²⁰, respectively.

The present study employed a multi-step purification protocol for the enzyme, involving ammonium sulfate precipitation, dialysis, and sequential ultrafiltration, to ensure adequate purity and functionality prior to downstream functional and biochemical characterization. Recognizing the importance of streamlining the protocol for large-scale applications, future work will focus on simplifying the process to enhance scalability and industrial applicability. The purified CamASNase showed specific activity of 173 IU/mg at 37 °C, which is better than the L-asparaginases produced by *Helicobacter pylori* (31.2 IU/mg)²¹, *B. licheniformis* (36.08 IU/mg)¹¹, *E. cloacae* (105.07 IU/mg)²². CamASNase retained > 75% of its activity up to 24 h at human physiological conditions. The K_m value was 6.36 μM , which was lower than that for *E. coli* (12.5 μM) and *E. chrysanthemi* (18 μM) asparaginase^{23,24}. As the blood contains ~50 μM of L-asparagine, the K_m of an L-asparaginase needs to be

< 50 μm to become therapeutically effective. The K_{cat} was 222.82 /sec which is 5-times higher than *E. coli* type II asparaginase (48.9/ sec) and comparable to *D. chrysanthemi* asparaginase (207.5/sec)²⁵. Accordingly, the K_m and K_{cat} values indicated the strong L-asparagine affinity of CamASNase, suggesting an excellent candidate for cancer research. In vitro serum and trypsin half-life were ~ 36 h and ~ 27 min, respectively, which are another two characteristics for evaluating the therapeutic efficacy. The values were higher than the in vitro serum (20 h) half-life and similar to the trypsin half-life (26 min) of the commercial native *E. coli* type II asparaginase²⁶, suggesting a better plasmatic half-life of CamASNase. CamASNase showed stringent specificity towards L-asparagine without any obvious activity on L-glutamine and urea, reinforcing the previous results of CamASNase⁴. As the associated glutaminase and urease activities are one of the reasons for the reported side effects connected with the present therapeutic formulations¹⁴, CamASNase would pose fewer side effects.

The information on the impact of different effectors that can stimulate or impede an enzyme can be utilized to improve its catalytic efficiency. However, L-asparaginases from different sources respond differently to the same effector, necessitating research in each enzyme. The results showed that Co^{2+} , Mn^{2+} , NH_4^+ , Fe^{3+} and Na^+ ions acted as enhancers. Three ions, viz. K^+ , Zn^{2+} , Mg^{2+} , and Cu^{2+} ions were inhibitors. Divalent ions, viz. Co^{2+} , Cu^{2+} , Mn^{2+} , Mg^{2+} , and Fe^{2+} were reported as inhibitors of bacterial L-asparaginases²⁷. The binding of metal ions to the enzyme may disrupt or activate the catalytic process depending on the concentration, enzyme, and ions might explain the obtained results. Regarding the protein-modifying agents, the activity was increased in the presence of β -mercaptoethanol, SDS, DMSO, and DTT, which might be due to decreased intermolecular disulfide bridge formation resulting in decreased protein aggregation as reported earlier²⁸. The results are similar to *Pectobacterium carotovorum*²⁸ and *Erwinia carotovora* L-asparaginases²⁹. However, one study³⁰ reported the complete loss of asparaginase activity in the presence of SDS, contrary to the present findings. The complete loss of CamASNase activity in the presence of PMSF was similar to that of *V. cholerae* asparaginases³¹, but contrary to *B. subtilis* and *Pseudomonas* sp. asparaginases^{32,33}. The loss of activity in the presence of PMSF suggested the existence of serine in the active site³⁴ which was then verified during the in-silico analysis of active site (S55). Briefly, the results showed the differential influence of effectors on different L-asparaginases depending on type and concentration and the generated data can be used to improve the catalytic efficiency of CamASNase.

To validate in vitro characterization results, a thorough in silico investigation was conducted on the *ansB* of *C. amylolyticum*. The investigation predicted the physicochemical, functional, and structural features. The predicted pI of CamASNase was 6.27 indicated that the enzyme is acidic in blood, parallel to *E. coli* asparaginase³⁵. The predicted instability index of 32.13 suggested the stable nature of the enzyme, aligning with our in vitro stability characterization results on retaining > 75% of its activity up to 24 h and a better in vitro serum half-life. However, the enzyme lost $\sim 60\%$ of its initial activity after lyophilization, suggesting that lyophilization with a suitable cryoprotectant needs to be optimized for CamASNase in the future study. Further, the influence of freeze-thaw cycles on the CamASNase activity is another area to be explored to ensure long-term preservation. The negative GRAVY index of CamASNase (-0.038) showed the hydrophilic ability, which is necessary for functioning in the human body. The predicted signal sequence validated the extracellular secretory nature of CamASNase observed during the enzyme quantification. ToxinPred predicted the absence of toxin peptide in the sequence suggesting its nontoxic structure. Further, CamASNase was projected as nonallergenic by 'AllerTOP v2.0 tool', in contrast to *E. coli* and *D. chrysanthemi*. The prediction might be a contributory reason for the reported side effects associated with the enzymes of *E. coli* and *D. chrysanthemi*. However, validation of the safety features is warranted in future through in-vivo animal models and clinical trials.

The secondary structure prediction of CamASNase revealed α -helix (35.28%), random coils (46.93%), and extended strands (17.79%) with no β -turns. The findings align with previous studies indicating the existence of multiple random coils in L-asparaginases³³. Further, the projected active site signature patterns of CamASNase are well analogous with previous reports on the glutaminase-free L-asparaginase of *Cobetia amphilecti*³³, and glutaminase and urease free type II asparaginase from *B. altitudinis*¹³.

In the next step of in silico analysis, we followed a virtual screening approach on the major three ligands of L-asparaginases, namely urea, L-asparagine, and L-glutamine for finding the most suitable compound for CamASNase, thus to verify the results of in vitro substrate specificity. Template-based homology modelling was not possible for CamASNase due to low amino acid sequence identity (34.88%) in the RCSB PDB database. Hence, the ab initio modelling approach was used through the i-TASSER. The generated model had a C-score of 1.38, illustrating the overall quality. The model accuracy was indicated by ProSA z-score, Ramachandran plot analysis, QMEANDisCo score, and ERRAT score. Simultaneously, the AlphaFold 2.0 that builds the protein structure on the basis of its amino acids, was used to model the 3D structure of CamASNase. The model quality analysis scores illustrated the overall quality of this model. When two 3D models of CamASNase were aligned through the 'PyMOL', the RMSD value of the overlapping dimers was 0.84. As the low RMSD (between 0 and 5) indicates a decent configuration standard, the results indicated an exceptional agreement between the two built models. Further, the analysis revealed the homotetramer feature of CamASNase as described earlier, we also generated a 3D structure model of the tetramer. The generated tetrameric model had ERRAT score of 87.76, proSA z-score of -9.7, a QMEANDisCo score of 0.7 ± 0.5 , and 100% amino acid residues in the permitted region (Ramachandran plot analysis), demonstrating its suitability for downstream investigation.

Molecular docking was done to study the interaction of CamASNase with the reported major three ligands of L-asparaginases, namely urea, L-asparagine, and L-glutamine. In all the type II bacterial L-asparaginases, the tetrameric protein is designed by the two tight dimers and the residues within the tight dimer form enzymatically functional active sites. Accordingly, a dimeric form of the enzyme was applied in molecular docking studies. The results showed that the CamASNase had maximum binding affinity (represented through less binding energy) for L-asparagine (-5.0 kcal/mol), followed by L-glutamine (-4.6 kcal/mol) and then urea (-3.0 kcal/mol). The amino acid sequence of the active site revealed that L-asparagine and L-glutamine attach to the same active site, while urea binds to a completely different and insignificant site, without the involvement of any important

functional residues, an observation similar to the urea docking studies with urease free type II L-asparaginase of *B. altitudinis*¹³. Lack of involvement of major functional residues in the binding and the less binding affinity to urea can explain the absence of urease activity during substrate-specificity experiments. Further, the better binding affinity of the same active site to L-asparagine compared to L-glutamine might explain the absence of L-glutaminase action in the substrate specificity.

The study evaluated the anticancer activity of CamASNase in three different cancer cell lines (MCF7, MOLT4 and THP-1) and toxicity in noncancerous human cells (human adult dermal fibroblast cell line and blood cells). The IC₅₀ values against MCF7, MOLT4 and THP-1 cell lines were 0.043, 0.041, and 0.05 IU/ml, respectively. The IC₅₀ values of *D. chrysanthemi* and *E. coli* L-asparaginases against the MOLT-4 cell line were 1.0 IU/ml and 7.5 to 10.0 IU/ml, respectively³⁶, showing the better anticancer activity (lower IC₅₀) of CamASNase. The values were also better than the L-asparaginase of other reported microbes like, *Enterobacter cloacae* (7.1 IU/ml) in MOLT-4²⁵ and *Streptomyces rochei* in MCF-7 (4.95 IU/ml)¹⁶. It is important to note that a direct comparative assay using commercial asparaginases alongside CamASNase under identical experimental conditions was not performed in this study. The IC₅₀ values of the commercial enzymes were obtained from literature, though we used the same MOLT-4 cell line and similar assay conditions³⁶ for CamASNase. This represents a limitation of the present work, and direct head-to-head comparative studies under strictly identical conditions are warranted in future investigations. Further, supporting the results on cytotoxicity, morphological changes were observed in all the tested cell lines after CamASNase treatment through phase contrast microscopy. Quantitative confirmation of the apoptotic process using flow cytometry showed 78% apoptotic cells in the MOLT-4 cells after 24 h of CamASNase exposure at IC₅₀ concentration, which is higher than reported the apoptotic cell percentage of 14.84% for the standard drug, doxorubicin³⁸, further signifying the potent anticancer activity against leukemic cells. While checking the toxicities in noncancerous cells, CamASNase did not affect the HDF cells at the estimated IC₅₀ of THP-1, MOLT-4, and MCF-cell lines. Further, the enzyme did not affect normal cell viability up to 2.97 IU/ml and >90% of cells were viable even at 23.6 IU/ml. As hemolysis is a reported issue with L-asparaginases, we checked the hemolytic activity of purified CamASNase in the blood agar and through the quantitative hemolytic assay which was negative even at the highest tested concentration (14 IU/ml) used, suggesting the hemocompatible nature. The results further showed that CamASNase activity is highly specific to soluble substrates, like L-asparagine and does not involve interaction with or disruption of cellular membranes. Noteworthy, asparaginase is used with agents like doxorubicin, vincristine, and prednisone in the pediatric ALL chemotherapy³⁷. Synergistic effects have also been reported with methotrexate, cytarabine, temozolomide, the glutaminase inhibitors, and the mTOR inhibitor rapamycin³⁸. Accordingly, exploration of pharmacokinetics and therapeutic potential of CamASNase in combination with immunotherapy and novel targeted formulations is a future direction of the present study.

In conclusion, this study characterizes a novel and safe type II L-asparaginase from *Chryseomicrobium amylolyticum* (CamASNase), which exhibits potent cytotoxic and pro-apoptotic effects against MCF-7, MOLT-4, and THP-1 cancer cell lines, with no toxicity observed in normal human fibroblasts at specific concentrations. The IC₅₀ values for MCF-7, MOLT-4, and THP-1 were 0.043, 0.041, and 0.05 IU/ml, respectively, with CamASNase inducing 81.4% apoptosis in MOLT-4 cells. The enzyme displayed clinically desirable features, including high specificity for L-asparagine, absence of L-glutaminase and urease activity, type II nature, extended in vitro serum half-life, structural distinctiveness from commercial enzymes, and predicted non-allergenic, non-toxic properties. Molecular docking confirmed a stronger binding affinity for L-asparagine compared to L-glutamine and urea. Kinetic analysis revealed a low Km (6.364 µM), high Vmax (909.09 µM/min), and Kcat (222.82 s⁻¹), indicating strong substrate affinity and catalytic efficiency. Enzyme production was enhanced 2.37-fold (from 155.44 to 281.6 IU/ml) using Plackett–Burman and Central Composite Design-based statistical optimization. The enzyme was purified through multistep procedure and the purified enzyme showed a specific activity of 173.69 IU/mg protein, with trypsin and serum half-lives of 27 min and 39 h, respectively. The generated information on the different effectors on the enzymatic activity can be explored to improve the catalytic efficiency. These findings underscore the therapeutic potential of CamASNase and support further in vivo studies to advance L-asparaginase-based cancer therapies.

Materials and methods

Revival of the bacterial strain and confirmation of L-asparaginase production in control conditions

A type II L-Asparaginase-producing bacterium, viz. *C. amylolyticum* strain CMFRI/ChAm-01 (NCBI GenBank Accession Number: MZ707763, Microbial Type Culture Collection (MTCC) ID: 13184) identified through the earlier investigation⁴ was used. The bacterium was originally obtained from the haemolymph of giant mud crab, *Scylla serrata* (Forskål, 1775) and maintained in the marine microbial culture collection of Fish Microbiology Lab, ICAR-CMFRI, Kochi as glycerol stocks at -80°C. The revival from the glycerol stock was done by subculturing onto Zobell's Marine Agar (ZMA) plates and incubating at 30 °C for 24 h. The L-asparaginase production from the revived culture was evaluated by cultivating the strain in the modified glycerol asparagine medium as described previously⁴. The enzyme activity in the supernatant of the culture medium was quantitated through the revised Nesslerization method²⁸. The results were expressed as the specific enzyme activity (IU/mg of protein) after estimating the protein through the Lowry method³⁹.

Statistical optimization of L-asparaginase production

The culture suspension was prepared in a 250 ml flask having 50 ml Zobell's marine broth (Himedia, India). After incubation for 18–24 h in a rotatory shaker incubator at 30 °C and 150 rpm the culture suspension was prepared in phosphate-buffered saline (pH = 7.4) to get the OD of 1 at 600 nm and was used as an inoculum for the succeeding experiments.

Plackett–Burman (PB) design

Plackett–Burman (PB) design was initially applied to screen and evaluate the significant parameters affecting the CamASNase production. Nine different parameters were screened with two unassigned/ dummy parameters (Table S1). Based on the 11 input parameters, Design Expert 13 (Stat-Ease Inc., Minneapolis, MN, USA) recommended 12 experimental runs with the minimal run resolution (Table S2). The runs were executed in triplicates and the average yield was treated as responses. The effect of individual factors on CamASNase production was calculated by the Eq. 1: $E(x) = (\Sigma M^+ - \Sigma M^-)/N$; where, $E(x)$ represents the result of the factor being studied and M^+ and M^- are responses at higher and lower levels of the parameter, respectively and N is the total number of trials.

Centre composite design (CCD)

Based on PB results nine factors were whittled down to five, namely, incubation temperature, pH, incubation period, agitation rate, and NH_4Cl concentration. Central composite design (CCD) was then applied, which resulted in an experimental plan consisting of 36 trials with 30 factorial points (-1 and $+1$) and six central points ($0, 0$). The significant factors having a positive impact in PB design were kept at a high level, and those factors having a negative impact were maintained at a low level. The data was analysed using Design Expert 13.0. The experiments were conducted in triplicates and the average of L-asparaginase activity obtained was used as the results. The second-order polynomial equation was used to fit the CCD experimental findings into the response surface regression model. The coefficient of determination (R^2) was used to represent the quality of fit of the polynomial model equation. The goodness of fit of the statistical model was validated in terms of CamASNase production within the predicted conditions of the model.

Purification of CamASNase

The bacterial culture supernatant prepared through the optimised conditions (crude enzyme preparation) was combined with an equivalent amount of 50 mM phosphate buffer (pH 7.4) and purified through a step-by-step procedure. Briefly, the crude enzyme was precipitated with 80% ammonium sulphate, collected by centrifugation (15000 rpm for 15 min), and dissolved in the buffer. The sample was dialyzed using 10 kDa cut-off dialysis tubing (SIGMA, USA) for 24 h. The dialyzed enzyme was ultrafiltered in three phases using the Amicon Ultra-1 centrifugal filter Ultracel-10 K (Millipore, Ireland) in the first phase (to avoid contaminant proteins of < 10 kDa) followed by Amicon Ultra-1 centrifugal filter Ultracel-30 K (Merk Millipore, Ireland) to avoid the proteins of < 30 kDa. Amicon Ultra-1 centrifugal filter Ultracel-50 KDa (Merk Millipore, Ireland) was used to ultrafiltrate the retentate to avoid the proteins of < 50 kDa and to concentrate the target protein. L-asparaginase activity and protein concentration was evaluated in the retentate and permeate obtained in each ultrafiltration phase. DEAE-Sephadex anion exchange chromatography (SIGMA, USA) was then used to purify the portion having the L-asparaginase activity (permeate in the last step). The flow rate was 2 ml min⁻¹. Enzyme activity and protein concentration were determined for each portion. The fractions with maximum enzyme activity were combined, dialyzed with 50 mM Tris-HCl (pH = 7.4), and lyophilized before downstream analysis. The purity and molecular weight of the enzyme were checked by non-reductive polyacrylamide gel electrophoresis.

Biochemical characterization

Stability evaluation

The purified enzyme preparations were exposed to pH 7.4 at 37 °C for 72 h. The enzyme activity was estimated at 1, 2, 4, 8, 12, 24, 36, 48, 60, and 72 h of incubation under the standard assay conditions. The formula (Enzyme activity of sample/enzyme activity of control) $\times 100$ was applied to estimate the relative enzyme activity%. Further, the specific activity of the purified enzyme was estimated before and after lyophilization (done without using any cryoprotectant) and relative enzyme activity was measured.

Effect of ions, protein modifying agents, and surfactants

The effects of ten ions, viz. Na^+ , K^+ , Ca^{2+} , Co^{2+} , Mg^{2+} , Fe^{2+} , Fe^{3+} , Zn^{2+} , Mn^{2+} , and Cu^{2+} , five protein modifying agents, viz. phenylmethanesulfonyl fluoride (PMSF), sodium dodecyl sulfate (SDS), ethylene diamine tetra acetic acid (EDTA), Dimethyl Sulfoxide (DMSO), and β -mercaptoethanol, and four surfactants, viz. tween 20, tween 80, PEG-5000, and Triton-X-100 on CamASNase activity were estimated. Following a pre-incubation of the enzyme with individual metal ions, surfactants, or protein modifying agents for 60 min at 37 °C, the residual enzyme activity was calculated under the routine assay conditions and the results were recorded as the relative activity. The effect of ions and protein modifying agents was studied at 5-, 10- and 15-mM concentrations. Similarly, three different concentrations, viz. 0.05%, 0.1% and 1% were evaluated for surfactants.

Substrate specificity and kinetic parameters

The affinity of CamASNase towards five substrates, namely D-asparagine, L-glutamine, D-glutamine, Boc-asparagine, and urea was checked by replacing 40 mM L-asparagine with the equal concentration of the evaluated substrate in the routine assay. The results were expressed as relative activity%. The kinetics of purified enzyme were estimated by calculating the enzyme activities under varied L-asparagine concentrations (1 to 80 μM) at human physiological conditions (37 °C and pH=7.4). The maximum velocity (V_{max}) and Michealis-Menten constant (K_m) were calculated using the non-linear regression analysis from the Lineweaver-Burk plot. The turnover number (K_{cat}) was estimated through the formula; $K_{\text{cat}} = V_{\text{max}} / [E]$, where $[E]$ is the enzyme concentration used. The experiments were conducted in triplicates and the mean values were taken.

In vitro serum and trypsin half-life

For trypsin half-life, the mixture comprising 50 IU purified enzyme and 50 IU trypsin in 50 mM phosphate buffer (pH 7.4) was homogenized and incubated at 37 °C for 60 h. Enzyme activity was estimated at 10 min intervals and used for calculating the relative enzyme activity%. For *in vitro* serum half-life, the purified CamASNase enzyme solution (50 IU) was mixed with 2.5 ml human serum and incubated at 37 °C. Asparaginase activity was estimated at different periods (0, 6, 12, 18, 24, 36, 42, and 48 h).

Structural modelling and in silico analysis

The total RNA was extracted from the culture pellet using NucleoSpin RNA, Mini kit (Macherey-Nagel, Germany). Reverse transcription was carried out using total RNA (1 µg) with a 'PrimeScript 1st strand cDNA synthesis kit' (Takara, Japan). A set of gene-specific primers were designed (Table S3) from the published asparaginase gene of *Chryseomicrobium excrementi* (NCBI Accession number: NZ_PCGR01000001.1:512568–513548) with the 'Primer3 web version 4.1.0'. The purified amplicon was sequenced at Genespec Labs. The obtained sequence was employed to design different primers (Table S3) for subsequent PCR. The sequences obtained from the various fragments were assembled using Editseq (DNASTAR, Lasergene, USA) to generate the complete cDNA sequence. Finally, the complete gene was amplified using ansB-F1 and RC-AnsCa-R2 primers, cloned into a 'pMD20-T vector' (Takara), and sequenced.

The DNA sequence was converted into corresponding amino acid sequence. The homology analysis was done with counterparts reported from the same genus and type I and type II asparaginase sequences from *E. coli* and *D. chrysanthemi*. Initially, multiple sequence alignment was done using CLUSTALW and the data was used for phylogenetic analysis using Molecular Evolutionary Genetics Analysis, version 10 (MEGA 10)⁴⁰. After calculating the evolutionary distances using the Poisson model and they were expressed through the units of the number of amino acid substitutions per site. The neighbour-joining (NJ) method was used to make the phylogenetic tree in which 1000 bootstrap replicates estimated tree topology.

The physicochemical parameters of *C. amycolyticum* asparaginase were analysed through the 'ExPASy-ProtParam tool'⁴¹. The allergens and toxic peptides were screened through the web servers, viz. 'AllerTOP v 2.0'⁴² and ToxinPred⁴³. The analysis for the signal peptide and the prediction of the signal peptide cleavage site was done using SignalP 5.0⁴⁴. InterPro tool of EMBL-EBI were applied for analysing the protein superfamily and domain. The Prosite tool of ExPASy was applied to inspect the protein functional sites and motifs⁴⁴.

The 2D structure of the protein was predicted through the 'SOPMA secondary structure prediction' server⁴⁵. While building the 3D model the protein sequence was searched through the Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB) database to detect suitable templates for homology modelling. As there was only 34.88% identity which was low to perform homology modelling, ab-initio modelling approach was followed using i-TASSER and the model having the maximum C-score was validated by analysing the QMEANDisCo score value, ERRAT 2.0 of SAVES v 6.0, Ramachandran plot, and ProSA z-score. The 3-D structure was also made through 'AlphaFold2-colab' under default settings⁴⁶. The predicted structures were observed by the PyMOL Molecular Graphics System (<https://pymol.org/2/>). The reliability of the model was assessed through the Ramachandran plot and ERRAT 2.0 and through the ProSA plot. The generated models were visualized in PyMOL v 4.3.0⁴⁷. Subsequently, the two 3D designs were aligned through the 'PyMOL' and RMSD values were noted. The homo-tetrameric state of the CamASNase was made through the 'AlphaFold2-colab' under default settings. The model was then validated by the Ramachandran plot, ERRAT 2.0 and through the ProSA plot.

Molecular docking studies

The ligand preparations were done following the earlier protocol¹³. The conserved functional amino acid residues and 3D model were evaluated using the 'ConSurf web server tool' and the model was observed in 'PyMOL v 4.3.0'. The Prosite tool of ExPASy was applied to identify active sites and active site signature patterns of the catalytic centre⁴⁷. 'AutoDock Vina version 1.1.2'⁴⁸ in 'PyRx 0.8'⁴⁹ was used to perform the docking studies with the CamASNase dimer model. Using 'Discovery Studio BIOVIA' (2021), water molecules were first removed and polar hydrogen atoms and Kollman charges were added to make the protein structures. The ligands were transformed into the AutoDock Ligand format (pdbqt), and the energy was minimized by the Universal Force Field of 'Open Babel' tool in the 'PyRx'. The grid region for CamASNase was identified as follows: exhaustiveness = 8.0; size $_x$ = 62.8030; size $_y$ = 67.0575; size $_z$ = 69.7324; center, x = 67.3458; center, y = 20.8509; center $_z$ = 21.9300. The previously described protocol was followed for docking¹³. The energy-based scoring function was used to rank and visualize the interactions between enzymes and ligands. 'PyMOL' was used for visualizing the binding.

Anticancer activity of CamASNase

Cytotoxicity evaluation using MTT assay

The cytotoxicity of the purified enzyme against human cancer cell lines, viz. MCF7 (a breast carcinoma cell line), MOLT4 (a human T-lymphoblast acute lymphoblastic leukemia cell line) and THP-1 cell lines (a human leukaemia monocytic cell line) was estimated using MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay. Cell lines were purchased from the National Centre for Cell Science, Pune, India. The MCF7 and MOLT4 cells were seeded in a 96-well plate at a density of 10×10^4 cells per well and exposed to the different amounts of the purified CamASNase (0.01 to 0.170 IU/µg protein) and incubated at 37 °C in 5% CO₂ for 24 h. The THP-1 cells were seeded at 2×10^6 /well and treated with varied amounts of the purified CamASNase (0.012 to 0.10 IU/µg protein) and incubated at 37 °C in 5% CO₂ for 48 h. Control wells were kept with an equal number of cells in RPMI medium without enzyme. After incubation, cells were treated with 10% MTT dye (5 mg/mL in PBS) for 4 hours in the incubator. Then 100 µL of 10% SDS in PBS was added to each

well and incubated overnight in the dark. The resulting absorbance was measured at 570 nm. The viability was estimated by the equation, % viability = [OD of the test well / OD of the control well] × 100.

Assessment of cell morphology and apoptosis assay

For exploring the morphological changes in THP-1, MCF7 and MOLT4 cells after treatment with CamASNase, the cells seeded as above were exposed to varied amounts of the purified CamASNase (0.01 to 0.170 IU/μg protein). After incubation the cells were examined for apoptosis and documented using phase-contrast optical microscopy at 10X and 40X magnifications.

Apoptosis was assessed by flow cytometry using the Annexin-V/propidium iodide (PI) staining protocol⁵⁰. Briefly, the MOLT-4 cells were seeded at 2.7×10^5 cells/ well, treated with the purified CamASNase (IC_{50} concentration) and incubated at 37 °C in 5% CO₂ for 24 h. The untreated cells, heated cells, and 0.01% Tritan X-100 treated cells were used as negative control, annexin-positive control, and PI-positive control, respectively. After washing thrice with PBS, 100 μl cell suspension was mixed with 100 μl of 1X annexin V binding buffer (BD Pharmingen) containing 5 μl AbFlour 488 conjugated Annexin V dye and 10 μl PI and incubated for 15 min at dark. The flow cytometry was done on the BD FACSCelesta flow cytometer (BD Biosciences, India). All experiments were performed in triplicates.

Toxicological evaluation on noncancerous human cells

The cytotoxicity analyses of CamASNase against noncancerous cells were evaluated using human adult dermal fibroblast cell (HDF) through the MTT assay as described for the human cancer cell lines. The selectivity index (SI) indicating the anticancer selectivity was calculated using the formula: $SI = IC_{50}$ of the enzyme in the normal cell line/ IC_{50} of the enzyme in cancer cell line, where IC_{50} is the enzyme concentration killing 50% of the cells¹².

Further, the haemolytic action of purified CamASNase was qualitatively evaluated by loading the purified enzyme onto wells made on the blood agar plates and incubated at 37 °C for 24 h. The plates were checked for the clearance zone after incubation. For the quantitative haemolytic assay, heparinized human blood cells were washed thrice with sterile saline and resuspended in 100 mM sodium phosphate buffer (pH 7.4). The cells were then incubated with serial dilutions of the purified CamASNase for 24 h at 37 °C. After centrifugation at 2500 rpm for 15 min the optical density of the supernatant was read at 541 nm. The buffer incubated with the washed cells was the blank, and the cells incubated with 1% Triton X-100 was the positive control. All experiments were performed in triplicates.

Statistical analysis

The Shapiro–Wilk test was applied to evaluate the data normality. The data lacking normality were transformed before the analysis. The data (from evaluation of stability, substrate specificity, trypsin and serum half-life, and MTT assay) were compared through one-way ANOVA followed by Tukey's HSD post hoc test. Two-way ANOVA followed by Turkey test was used to analyse the effect of ions, protein modifying agents, and surfactants on the enzymatic activity. The results from the apoptosis assay were compared through the Independent-T test. The SPSS version 16 was used for all statistical analyses. Significant and very significant differences were indicated by p -values < 0.05 and < 0.01, respectively.

Data availability

The authors confirm that the data supporting the findings of this study are available within the article [and/or] its supplementary materials. The generated full-length sequence of the functional gene was submitted to NCBI GenBank (Accession number WQG15662).

Received: 19 February 2025; Accepted: 24 June 2025

Published online: 23 July 2025

References

1. <https://market.us/report/asparaginase-market/>. Global asparaginase market by source (*Escherichia coli*, *Erwinia chrysanthemi*) by route of administration, intravenous, intramuscular) by application (acute lymphoblastic leukemia (all), lymphoblastic lymphoma (lbl), others) by distribution channels.
2. Fonseca, M. H. G., Fiúza, T. S., de Moraes, S. B. & de Souza, T. A. C. B. de & Trevizani, R. Circumventing the side effects of L-asparaginase. *Biomed. Pharmacother.* **139**, 111616 (2021).
3. Souza, P. M. et al. Optimization and purification of L-asparaginase from fungi: A systematic review. *Crit. Rev. Oncol. Hematol.* **120**, 194–202 (2017).
4. Lailaja, V. P. et al. Characterization of novel L-asparaginases having clinically safe profiles from bacteria inhabiting the hemolymph of the crab, *Scylla serrata* (Forskål, 1775). *Folia Microbiol. (Praha)*. **67**, 491–505 (2022).
5. Chakravarty, N., Priyanka, Singh, J. & Singh, R. P. A potential type-II L-asparaginase from marine isolate *Bacillus australimaris* NJB19: statistical optimization, in silico analysis and structural modeling. *Int. J. Biol. Macromol.* **174**, 527–539 (2021).
6. El-Fakharany, E., Orabi, H., Abdelkhalek, E. & Sidkey, N. Purification and biotechnological applications of L-asparaginase from newly isolated *Bacillus halotolerans* OHEM18 as antitumor and antioxidant agent. *J. Biomol. Struct. Dyn.* **40**, 3837–3849 (2022).
7. Beygmoradi, A., Homaei, A., Hemmati, R. & Fernandes, P. Recombinant protein expression: Challenges in production and folding related matters. *Int. J. Biol. Macromol.* **233**, 123407 (2023).
8. Izadpanah Qeshmi, F., Homaei, A., Khajeh, K., Kamrani, E. & Fernandes, P. Production of a novel marine *Pseudomonas aeruginosa* recombinant L-asparaginase: Insight on the structure and biochemical characterization. *Mar. Biotechnol.* **24**, 599–613 (2022).
9. Razzaghi, M., Homaei, A., Hemmati, R., Saberi, D. & Kavousipour, S. Cellulose-gold nanohybrid as an effective support to enhance the catalytic efficiency and stability of α-amylase from *Bacillus aquimaris*. *J. Mol. Liq.* **391**, 123399 (2023).
10. Ghattavi, S., Homaei, A. & Fernandes, P. Marine natural products for biofouling elimination in marine environments. *Biocatal. Agric. Biotechnol.* **61**, 103385 (2024).
11. Alrumman, S. A. et al. Production and anticancer activity of an L-Asparaginase from *Bacillus licheniformis* isolated from the red sea, Saudi Arabia. *Sci. Rep.* **9**, 3756 (2019).

12. Mostafa, Y. et al. Enhanced production of glutaminase-free L-asparaginase by marine *Bacillus velezensis* and cytotoxic activity against breast cancer cell lines. *Electron. J. Biotechnol.* **42**, 6–15 (2019).
13. Lailaja, V. P. et al. In vitro and in silico analysis unravelled clinically desirable attributes of *Bacillus altitudinis* L-asparaginase. *J. Appl. Microbiol.* **135**, (2024).
14. Ashok, A. et al. Microbes producing L-asparaginase free of glutaminase and urease isolated from extreme locations of Antarctic soil and moss. *Sci. Rep.* **9**, 1423 (2019).
15. Venil, C. K. & Lakshmanaperumalsamy, P. Response surface methodology for the optimization of alpha amylase production by *Serratia marcescens* SB08. *J. Sci. Ind. Res.* **51**, 333–339 (2008).
16. El-Naggar, N. E. A. et al. Process development for scale-up production of a therapeutic L-asparaginase by *Streptomyces brolosae* NEAE-115 from shake flasks to bioreactor. *Sci. Rep.* **9**, 13571 (2019).
17. Abhini, K. N., Rajan, A. B., Zuhara, F., Sebastian, D. & K. & Response surface methodological optimization of L-asparaginase production from the medicinal plant endophyte *Acinetobacter baumannii* ZAS1. *J. Genet. Eng. Biotechnol.* **20**, 22 (2022).
18. Parashiva, J., Nuthan, B. R., Rakshith, D. & Satish, S. Endophytic fungi as a promising source of anticancer L-asparaginase: A review. *Curr. Microbiol.* **80**, 282 (2023).
19. Kotzia, G. A. & Labrou, N. E. L-asparaginase from *Erwinia chrysanthemi* 3937: Cloning, expression and characterization. *J. Biotechnol.* **127**, 657–669 (2007).
20. Khushoo, A., Pal, Y., Singh, B. N. & Mukherjee, K. J. Extracellular expression and single step purification of Recombinant *Escherichia coli* L-asparaginase II. *Protein Expr Purif.* **38**, 29–36 (2004).
21. Dhavala, P. & Papageorgiou, A. C. Structure of *Helicobacter pylori* L-asparaginase at 1.4 Å resolution. *Acta Crystallogr. Sect. D Biol. Crystallogr.* **65**, 1253–1261 (2009).
22. Husain, I., Sharma, A., Kumar, S. & Malik, F. Purification and characterization of glutaminase free asparaginase from *Enterobacter cloacae*: In-vitro evaluation of cytotoxic potential against human myeloid leukemia HL-60 cells. *PLoS One*. **11**, e0148877 (2016).
23. Müller, H. Use of L-asparaginase in childhood ALL. *Crit. Rev. Oncol. Hematol.* **28**, 97–113 (1998).
24. Mohan Kumar, N. S. & Manonmani, H. K. Purification, characterization and kinetic properties of extracellular L-asparaginase produced by *Cladosporium* Sp. *World J. Microbiol. Biotechnol.* **29**, 577–587 (2013).
25. Schalk, A. M., Nguyen, H. A., Rigouin, C. & Lavie, A. Identification and structural analysis of an L-asparaginase enzyme from guinea pig with putative tumor cell killing properties. *J. Biol. Chem.* **289**, 33175–33186 (2014).
26. Zhang, Y. Q. et al. Synthesis, characterization and immunogenicity of silk fibroin-L-asparaginase bioconjugates. *J. Biotechnol.* **120**, 315–326 (2005).
27. Zuo, S., Zhang, T., Jiang, B. & Mu, W. Recent research progress on microbial L-asparaginases. *Appl. Microbiol. Biotechnol.* **99**, 1069–1079 (2015).
28. Kumar, S., Venkata Dasu, V. & Pakshirajan, K. Purification and characterization of glutaminase-free L-asparaginase from *Pectobacterium carotovorum* MTCC 1428. *Bioresour Technol.* **102**, 2077–2082 (2011).
29. Warangkar, S. C. & Khobragade, C. N. Purification, characterization, and effect of thiol compounds on activity of the *Erwinia carotovora* L-Asparaginase. *Enzyme Res.* 1–10 (2010).
30. Mahajan, R. V. et al. Purification and characterization of a novel and robust L-asparaginase having low-glutaminase activity from *Bacillus licheniformis*: In vitro evaluation of anti-cancerous properties. *PLoS One*. **9**, e99037 (2014).
31. Radha, R., Arumugam, N. & Gummadi, S. N. Glutaminase free L-asparaginase from *Vibrio cholerae*: Heterologous expression, purification and biochemical characterization. *Int. J. Biol. Macromol.* **111**, 129–138 (2018).
32. Arredondo-Nuñez, A. et al. Characterization of a type II L-asparaginase from the halotolerant *Bacillus subtilis* CH11. *Life* **13**, 2145 (2023).
33. Farhat, W. et al. Advances in biomaterials for the treatment of retinoblastoma. *Biomater. Sci.* **10**, 5391–5429 (2022).
34. Kumar, S. et al. Molecular cloning, characterization, and in-silico analysis of L-asparaginase from Himalayan *Pseudomonas* sp. PCH44. *3 Biotech* **12**, 162 (2022).
35. Mohideen, A. K. Molecular docking study of L-asparaginase I from *Vibrio campbellii* in the treatment of acute lymphoblastic leukemia (ALL). *EuroBiotech J.* **4**, 8–16 (2020).
36. Abakumova, O. Y., Podobed, O. V., Karalkin, P. A., Kondakova, L. I. & Sokolov, N. N. Antitumor activity of L-asparaginase from *Erwinia carotovora* against different human and animal leukemic and solid tumor cell lines. *Biochem. Suppl. Ser. B Biomed. Chem.* **6**, 307–316 (2012).
37. Girish Babu, K., Mathew, J., Doddamani, G., Narasimhaiah, J. & Naik, L. K. Oral health of children with acute lymphoblastic leukemia: A review. *J. Orofac. Sci.* **8**, 3 (2016).
38. Van Trimpont, M. et al. Novel insights on the use of L-Asparaginase as an efficient and safe anti-cancer therapy. *Cancers (Basel)* **14**, 902 (2022).
39. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**, 265–275 (1951).
40. Kumar, S., Stecher, G., Li, M., Knyaz, C. & Tamura, K. MEGA X: molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol.* **35**, 1547–1549 (2018).
41. Gasteiger, E. et al. Protein identification and analysis tools on the ExPASy Server. in *The Proteomics Protocols Handbook*, 571–607 (Humana Press, Totowa, NJ).
42. Dimitrov, I., Bangov, I., Flower, D. R. & Doytchinova, I. AllerTOP v.2-a server for in silico prediction of allergens. *J. Mol. Model.* **20**, 2278 (2014).
43. Gupta, S. et al. In silico approach for predicting toxicity of peptides and proteins. *PLoS One* **8**, e73957 (2013).
44. Sigrist, C. J. A. et al. New and continuing developments at PROSITE. *Nucleic Acids Res.* **41**, D344–D347 (2012).
45. Combet, C., Blanchet, C., Geourjon, C. & Deléage, G. NPS@: Network protein sequence analysis. *Trends Biochem. Sci.* **25**, 147–150 (2000).
46. Varadi, M. et al. AlphaFold protein structure database: Massively expanding the structural coverage of protein-sequence space with high-accuracy models. *Nucleic Acids Res.* **50**, D439–D444 (2022).
47. Schrödinger, L., DeLano, W. & PyMOL (2020). Retrieved from <http://www.pymol.org/pymol>.
48. Trott, O., Olson, A. J., AutoDock & Vina Improving the speed and accuracy of Docking with a new scoring function, efficient optimization, and multithreading. *J. Comput. Chem.* **31**, 455–461 (2010).
49. Dallakyan, S. & Olson, A. J. Small-molecule library screening by Docking with pyrx. in 243–250 (2015). https://doi.org/10.1007/978-1-4939-2269-7_19
50. van Engeland, M., Nieland, L. J. W., Ramaekers, F. C. S., Schutte, B. & Reutelingsperger, C. P. M. Annexin V-Affinity assay: A review on an apoptosis detection system based on phosphatidylserine exposure. *Cytometry* **31**, 1–9 (1998).

Acknowledgements

The authors are grateful to the Director, ICAR-Central Marine Fisheries Research Institute, Kochi for providing the necessary facilities. LVP acknowledges Kerala State Council for Science Technology and Environment (KSCSTE), Kerala, India, for funding under the Back to Lab-Post-Doctoral fellowship for Women Scientists Scheme (No. 441/2020/KSCSTE). The authors acknowledge Amala Cancer Research Institute, Kerala, India for provid-

ing the facilities to conduct cytotoxicity evaluation experiments using THP-1 cell lines. The authors acknowledge Rajeev K Sukumaran, Principal Scientist, CSIR- National Institute for Interdisciplinary Science and Technology for the guidance on the statistical optimization of the media. This work was supported by the KCSTEC funded project “Investigation for L-Asparaginase Free of Clinical Toxicity from Microbial Associations of Marine Crabs” (No. 441/2020/KSCSTE), ICAR-CMFRI funded project “Health Management in selected finfish and shellfish & bio-prospecting from marine resources” (MBT/HLT/23).

Author contributions

L.V.P. executed the experimental design and combined the observations. S.T.G. conceived the presented idea, supervised the project, facilitated the experiments, and wrote the manuscript. A.M.M., U.S., and A.V.N. provided technical support to carry out experiments. S.R.K. helped in procuring the fund and in drafting the manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1038/s41598-025-08962-8>.

Correspondence and requests for materials should be addressed to T.G.S.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License, which permits any non-commercial use, sharing, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if you modified the licensed material. You do not have permission under this licence to share adapted material derived from this article or parts of it. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by-nc-nd/4.0/>.

© The Author(s) 2025