**Title: Biochemical characterization of extremozyme L-asparaginase from *Pseudomonas* sp. PCH199 for therapeutics**

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**Fig. S1** Qualitative estimation of L-ASNase production by PCH199. The strain *Pseudomonas* sp. PCH199 was streaked on M9 medium supplemented with indicator dye phenol red. After 24h of incubation at 28 ℃, color change around the bacterial colony from yellow to pink has indicated L-asparagine hydrolysis by L-ASNase due to change in pH.

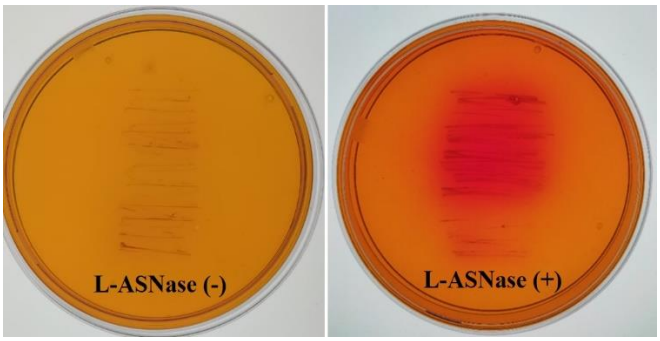


**Fig. S2** Phylogenetic tree of *Pseudomonas* sp. PCH199 with related strains based on 16S rRNA gene sequence analysis. The tree was constructed by maximum likelihood method using MEGA11 with 1000 boot-strapping replication. PCH199 showed 99.06 % sequence similarity to *Pseudomonas glycinae* MS586(T).

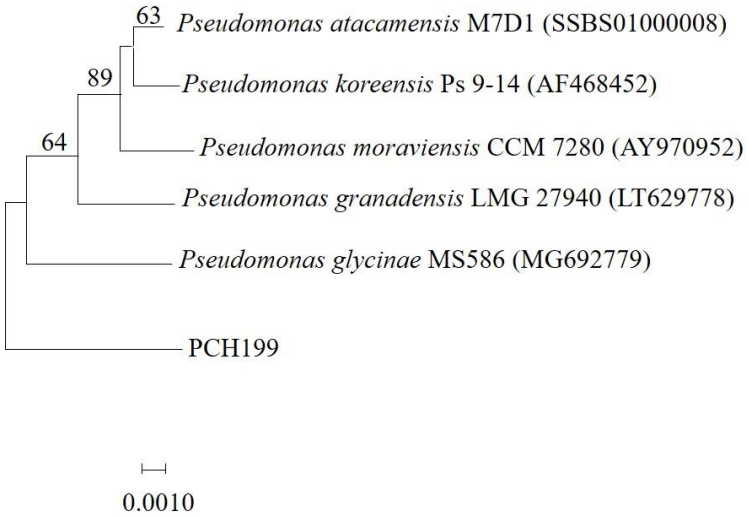

**Fig. S3** Graphical representation of kinetic study of PCH199 L-ASNase. Determination of $K_m$ and $V_{max}$ of purified L-ASNase for L-glutamine by non-linear regression analysis of experimental steady-state data. (a) Plot of the reaction velocities (V) versus substrate concentration (S: 0.02 – 0.5 mM) fitted to the Michaelis-Menten equation. (b) The corresponding Lineweaver-Burk plot ($K_m$ = 0.034 mM and $V_{max}$ = 57.98 U/mg) of L-ASNase catalyzed reaction.

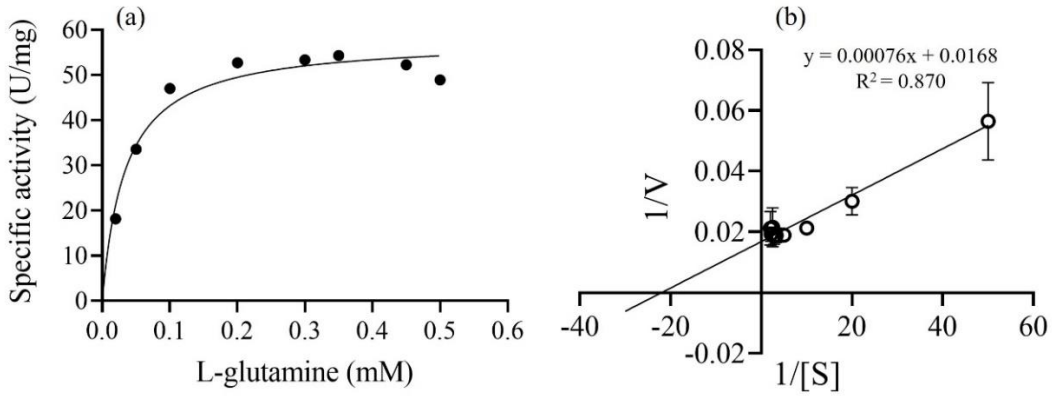


**Fig.S4** Graphical representation of periplasmic L-ASNase activity when PCH199 was cultured at M9 minimal medium with various pH (5.8-7.5) and periplasmic L-ASNase activity corresponding to each pH was represented.

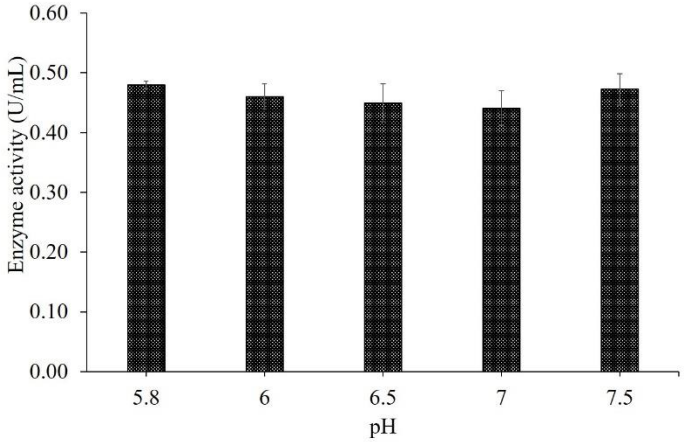

**Table S1** Central Composite Design of selected variables and the responses thereof. The maximum response obtained with a specific condition is marked in bold font.

| Run Order | Buffer concentration (mM) | L-asparagine concentration (%) | Glucose concentration (%) | Response (U/mL) |
|---|---|---|---|---|
| 1 | 50 | 2.732 | 0.25 | 0.541 |
| 2 | 50 | 2.732 | 0.25 | 0.664 |
| **3** | **100** | **2.0** | **0.5** | **0.766** |
| 4 | 0 | 2.0 | 0 | 0.038 |
| 5 | 0 | 2.0 | 0.5 | 0.009 |
| 6 | 100 | 2.0 | 0 | 0.635 |
| 7 | 50 | 1.0 | 0.25 | 0.456 |
| 8 | 50 | 1.0 | 0.25 | 0.444 |
| 9 | 50 | 1.0 | -0.183 | 0.328 |
| 10 | -36.602 | 1.0 | 0.25 | 0.232 |
| 11 | 50 | 1.0 | 0.683 | 0.437 |
| 12 | 136.603 | 1.0 | 0.25 | 0.553 |
| 13 | 50 | 1.0 | 0.25 | 0.456 |
| 14 | 50 | 1.0 | 0.25 | 0.451 |
| 15 | 50 | -0.732 | 0.25 | 0.048 |
| 16 | 50 | -0.732 | 0.25 | 0.036 |
| 17 | 100 | 0 | 0.5 | 0.026 |
| 18 | 0 | 0 | 0.5 | -0.024 |
| 19 | 0 | 0 | 0 | -0.019 |
| 20 | 100 | 0 | 0 | 0.046 |

**Table S2** Analysis of variance of second-order polynomial model for the effect of different variables on L-ASNase production.

| Source | Term df | Error df | F-value | p-value | Remark |
|---|---|---|---|---|---|
| Whole-plot | 2 | 3.46 | 9.51 | 0.0394 | Significant |
| b-Asparagine concentration | 1 | 3.38 | 16.97 | 0.0205 | |
| $b^2$ | 1 | 3.53 | 2.05 | 0.2347 | |
| Subplot | 7 | 6.55 | 14.40 | 0.0016 | Significant |

| | | | | |
|---|---|---|---|---|
| A-Salt concentration | 1 | 7.20 | 56.62 | 0.0001 |
| C-Glucose concentration | 1 | 7.20 | 1.45 | 0.2662 |
| Ab | 1 | 7.20 | 42.13 | 0.0003 |
| AC | 1 | 7.20 | 0.9731 | 0.3559 |
| bC | 1 | 7.20 | 0.1425 | 0.7167 |
| $A^2$ | 1 | 3.83 | 2.28 | 0.2089 |
| $C^2$ | 1 | 3.83 | 2.52 | 0.1906 |
| $R^2$ (predicted) = 0.954 | | | | |
| $R^2$ (adjusted) = 0.892 | | | | |

df, indicates for degree of freedom.