A Novel L-Asparaginase from *Enterobacter* sp. Strain M55 from Maras Salterns in Peru

This work is licensed under a Creative Commons Attribution 4.0 International License

A. Hurtado,[a] J. C. Flores-Santos,[a] C. N. Flores-Fernández,[a,*] S. Saavedra,[a] J. H. P. M. Santos,[b] A. Pessoa-Júnior,[b] M. E. Lienqueo,[c] M. J. Bayro,[d] and A. I. Zavaleta[a]

[a]Laboratorio de Biología Molecular, Facultad de Farmacia y Bioquímica, Universidad Nacional Mayor de San Marcos, Lima-Peru

[b]Department of Biochemical and Pharmaceutical Technology, School of Pharmaceutical Sciences, University of São Paulo, São Paulo-Brazil

[c]Centre for Biotechnology and Bioengineering, Universidad de Chile, Santiago-Chile

[d]Faculty of Natural Sciences, Universidad de Puerto Rico, Puerto Rico

doi: https://doi.org/10.15255/CABEQ.2022.2064

Original scientific paper
Received: February 10, 2022
Accepted: April 19, 2022

L-Asparaginase (ASNase) is used in medicine for neoplasms treatment and in food industry for mitigation of acrylamide in high-temperature processed food. In medicine, commercial ASNases have exhibited side effects and L-glutaminase (GLNase) activity affecting the clinical treatment. The aim of this work was to study a novel ASNase from *Enterobacter* sp. M55 isolated from Maras Salters in Peru, which was purified and biochemically characterised. This ASNase exhibited a *K*m of 5.71 mM and a *V*max of 0.16 μmol mL^{-1} min^{-1}, as well as an optimum temperature and pH of 37 °C and 6, respectively. Moreover, a good activity (80 %) was observed at physiological pH. Likewise, the enzyme increased its activity by around 50% in presence of urea, glutathione, and glucose. Whilst in presence of serum compounds, it kept more that 60 % of activity. In addition, this ASNase showed low GLNase activity.

Keywords:
L-asparaginase, L-glutaminase, *Enterobacter*, saline environments, clinical treatment

Introduction

L-Asparaginase (ASNase) (E.C. 3.5.1.1), specially from bacteria, is being widely studied owing to its potential applications in medicine and food industry[1,2]. In medicine, it is used for the treatment of non-Hodgkin lymphoma, acute lymphoblastic leukaemia (ALL), and other malignant tumours. ASNase catalyses the breakdown of the circulating L-asparagine, a fundamental amino acid for most malignant cells, in L-aspartate and ammonia. In food industry, this enzyme inhibits acrylamide formation in a variety of starch-rich products after grilling, baking, or frying[3–5].

ASNase is also included in the World Health Organization's List of Essential Medicines[6]. Medical ASNase formulations from *Escherichia coli* and *Erwinia chrysanthemi* are available on the market. However, they have caused clinical hypersensitivity in treated patients[7]. Other side effects such as pancreatitis, aphasia, liver disorders, deep vein thrombosis, hyperglycaemia, and neurological crisis have been also reported in cases of long-term administration of this drug. Additionally, these commercial ASNases have presented L-glutaminase (GLNase) activity, the deamination of L-glutamine to L-glutamic acid and ammonia[8–10]. The clinical importance of GLNase activity in ASNases is still under debate, with opposite reports in the literature about its effect in neoplasms treatment. Some studies have reported that GLNase activity contributes to the cytotoxicity of ASNase on neoplastic cells. However, other reports have indicated that GLNase activity is not essential for anticancer activity. What is clear is that low GLNase activity has been correlated with a significant reduction in toxicity in patients[11–13].

To overcome the problems previously described, research is focused on looking for novel ASNases from extreme environments with low GLNase activity to be used as potentially safer drugs. Thus, saline environments represent a valuable source of bacterial strains with potential biotechnological applications in pharmaceutical industry[1,14]. Halophilic and halotolerant bacteria have exhibited great potential for the production of ASNase with enhanced

*Corresponding author: E-mail: carol.fernandez.18@ucl.ac.uk

properties such as activity and stability in either acid or alkaline conditions, as well as absence of GLNase activity[15]. Bacteria including *Bacillus, Staphylococcus, Pseudomonas, Halomonas* and *Idiomarina* isolated from saline environments have been reported as L-asparaginase producers[1,16–18].

The aim of this study was to study a novel L-asparaginase from *Enterococcus* sp. strain M55, isolated from Maras Salterns in Peru. The enzyme was purified and biochemically characterised by determining its kinetic parameters, the effect of temperature, pH, ions, and serum components on ASNase activity as well as substrate specificity. Our findings have demonstrated that this ASNase presents potential for biotechnological applications in medicine.

Materials and methods

Bacterial strain and L-asparaginase production

Enterobacter sp. strain M55 was isolated from Maras Salterns in Peru by members of the Laboratorio de Biología Molecular, Facultad de Farmacia y Bioquímica, Universidad Nacional Mayor de San Marcos. The strain was grown from a glycerol stock on Luria Bertani (LB) broth at 37 °C for 24 h. The culture was then centrifuged at 5000×g for 5 min, and the pellet was transferred into LB broth supplemented with 10 g L^{-1} glucose. The culture was incubated at 37 °C and 160 rpm for 24 h. The cells were centrifuged, washed twice with 145 mM NaCl, and the final pellet was transferred into the ASNase production medium. ASNase was produced by submerged fermentation in a modified medium containing, in g L^{-1} [19,20]: 10, glucose; 10, yeast extract; 1, L-asparagine; 3, Na_2HPO_4; 6, NaH_2PO_4; 0.5, NaCl; 0.012, $CaCl_2{\cdot}2H_2O$; and 0.12, $MgSO_4{\cdot}7H_2O$, at pH 7.0. The starting culture presented an initial OD600 of 0.2, and was incubated at 37 °C and 160 rpm for 24 h. After this time, cells were harvested by centrifugation (5000×g for 20 min), washed twice with 145 mM NaCl, and resuspended in lysis buffer containing 50 mM Tris-HCl and 1 mM EDTA, pH 7.0. Sonication was carried out at 4 °C for 2 min at 30 % amplitude with 5 s ON and 30 s OFF. The clarified lysate was obtained by centrifugation at 11200×g for 40 min at 4 °C.

L-Asparaginase activity assay

The ASNase activity was determined using two methods. Although Nessler assay based on ammonia quantification is the most used, it might have some interferences. In this assay, potassium, mercury, and iodine reacted in proportion to ammonia concentration giving a coloured compound that was quantified spectrophotometrically. Thus, for instance, the presence of compounds such as potassium phosphate could be an interference. Therefore, the L-aspartic acid β-hydroxamate (AHA) assay was also used[21].

L-Aspartic acid β-hydroxamate (AHA) assay

L-Aspartic acid β-hydroxamate (AHA) colorimetric assay for the determination of ASNase activity was carried out following the method reported by Magri *et al.*[22] with some modifications. The reaction consisted of 700 µL of 50 mM Tris-HCl (pH 7.5), 100 µL of 100 mM of L-asparagine, 100 µL of 1 M hydroxylamine (pH 7), and 100 µL of clarified lysate. The mixture was incubated at 37 °C for 30 min. The reaction was then stopped by adding 250 µL of a solution containing 0.62 M ferric chloride, 0.306 M trichloroacetic acid, and 0.33 M HCl. The supernatant was separated by centrifugation at 2800×g for 5 min, and the product was quantified at 500 nm (Genesys® 10 UV spectrophotometer, Thermo®). A standard curve using different AHA concentrations was carried out. One unit of ASNase activity (U) was defined as the amount of the enzyme that produces 1 µmol of AHA per minute at pH 7.5 and 37 °C. The specific activity (U mg^{-1}) was defined as the ratio between the ASNase activity (U mL^{-1}) and total protein concentration (mg mL^{-1}).

Nessler assay

Nessler assay quantifies the ammonia released after ASNase catalysis. The realised ammonia reacts with Nessler reagent to produce a coloured solution measured at 436 nm[22,23]. The reaction included 100 µL of clarified lysate, 100 µL of 189 mM L-asparagine and 400 µL of 50 mM Bis-Tris-HCl, pH 6.0. The mixture was incubated at 37 °C for 30 min. After this time, 250 µL of Nessler reagent was added, and the coloured solution was measured spectrophotometrically. A standard calibration curve using different ammonium sulphate concentrations was performed. One unit of ASNase activity (U) was defined as the amount of enzyme that produces 1 µmol of ammonia per minute at pH 6 and 37 °C.

Protein quantification

Protein was quantified by bicinchoninic acid protein assay reported by Smith *et al.*[24] with some modifications. The reaction consisted of 200 µL of bicinchoninic acid in copper sulphate (50:1 v/v) and 25 µL of sample. The mixture was incubated at 37 °C for 25 min, and the absorbance was measured at 562 nm. Protein concentration (mg mL^{-1}) was determined based on a calibration curve using bovine serum albumin.

Purification of L-asparaginase from *Enterobacter* sp. strain M55

ASNase purification was carried out by ion exchange and size exclusion chromatography using an AKTA® AVANT 25 FPLC (GE Healthcare) system equipped with Unicorn software. For ion-exchange chromatography, the clarified lysate was loaded onto a DEAE Sepharose column (Sigma-Aldrich) (CV 5 mL), previously equilibrated with 25 mM Bis-Tris buffer, pH 6.0. The column was washed with the same buffer, and the ASNase was eluted with a salt gradient from 0 to 0.5 M NaCl at a 1 mL min^{-1} flow rate. Fractions were collected and tested for ASNase activity. Those exhibiting ASNase activity were concentrated for further purification via size exclusion chromatography. Size exclusion chromatography was performed using a Superdex 200 10/300 GL column (GE Healthcare), equilibrated with 10 mM phosphate buffer containing 140 mM NaCl, pH 7.4, at a flow rate of 0.5 mL min^{-1}. The flow-through was collected in fractions of 13.6 mL. The clarified lysate and the collected fractions from both purification steps were run in an SDS-PAGE gel according to the Laemmli protocol[25].

Characterisation of L-asparaginase from *Enterobacter* sp. strain M55

Enzyme characterisation included kinetic assay, as well as effect of pH, temperature, ions, inhibitors, and serum components on enzyme activity. Substrate specificity assays were also tested. ASNase kinetic parameters were determined at 37 °C using L-asparagine as substrate at a concentration ranging from 2 to 20 mM. The effect of pH on ASNase activity was evaluated at 37 °C and pH values ranging between 4 and 9 using the following 50 mM buffers: sodium acetate (pH 4–5.5), potassium phosphate (pH 6–7.5), Tris–HCl (pH 8 and 9). The effect of temperature on enzyme activity was tested at pH 7.5 between 10 °C and 70 °C. For testing the effect of pH and temperature on the enzyme, activity was measured by the AHA assay, and expressed as residual ASNase activity (%) from the optimum.

To determine the effect of ions on enzyme activity, ASNase was pre-incubated with several ions (chloride forms) such as Mg^{2+}, Ca^{2+}, Ba^{2+}, Mn^{2+}, Cu^{2+}, Co^{2+}, Na^{+} and K^{+} at 100 mM for 2 h at 4 °C. Similarly, the effect of inhibitors such as urea, PMSF (phenylmethylsulphonyl fluoride), EDTA, EGTA, 2-mercaptoethanol, DL-dithiothreitol, SDS (all at 10 mM), and glutathione (0.5 mM) were tested. In the same way, the effect of serum components (10 mM) including glucose, galactose, arabinose, citrate, haemoglobin, lactate, and pyruvate was evaluated. ASNase activity was determined by the Nessler and AHA assays and expressed as relative activity (%) from a control without the presence of ion, inhibitor or serum component.

Substrate specificity was evaluated by measuring enzyme activity using several amino acids and other compounds at 10 mM, including L-asparagine, L-glutamine, L-histidine, L-glutamate, L-aspartate, D-aspartate, and urea as described by Husain *et. al.*[9] For this study, enzyme activity was determined by the Nessler assay, and expressed as residual activity (%) from the activity using L-asparagine.

Results and discussion

Purification of L-asparaginase

The clarified lysate of ASNase from *Enterobacter* sp. strain M55 was purified, and a molecular weight of around 140 kDa was observed in Native-PAGE. In SDS-PAGE, a molecular weight of around 35 kDa was observed, indicating that this enzyme is tetrameric (data not shown). ASNases from different sources have been reported as monomeric, dimeric, tetrameric, and hexameric, and microbial ASNases have exhibited a molecular weight ranging from 32 to 205 kDa[26].

Purification results of ASNase from *Enterobacter* sp. strain M55 are summarised in Table 1. The final purification factor was 16-fold with around 36 % recovery yield and a specific activity of 0.7 U mg^{-1}.

Table 1 – *Purification of L-asparaginase from Enterobacter sp. strain M55*

Step	Total activity (U)	ASNase activity (U mL^{-1})	Total protein (mg mL^{-1})	ASNase Specific activity (U mg^{-1})	Yield (%)	Fold purification
Clarified lysate	2.85	0.20	4.55	0.04	100	1.0
Anion Exchange chromatography (DEAE)	1.45	1.45	7.28	0.2	51.01	4.53
Size exclusion chromatography (Superdex 200)	1.03	0.92	1.32	0.7	36.26	15.94

L-Asparaginase kinetic assay

The enzyme exhibited Michaelis-Menten kinetics, and the kinetic parameters were a *K*m of 5.71 Mm, and a *V*max of 0.16 μmol mL^{-1} min^{-1} (Fig. 1). Previous studies have reported close *K*m values for ASNases from *Escherichia coli* and *Erwinia*[27,28].

Effect of temperature and pH on L-asparaginase activity

The effect of temperature on ASNase activity is shown in Fig. 2. The enzyme exhibited maximum activity at 37 °C, and maintained around 50 % of its activity at 30 and 45 °C. Likewise, Fig. 3 illustrates the effect of pH. The optimum activity of ASNase was observed in a pH range of 6 and 6.5. In addition, the enzyme kept more than 50 % of its optimum activity at pH from 7 to 9. It is important to mention that at physiological pH, the enzyme conserved around 80 % of the optimum.

Effect of ions, inhibitors, and serum components on L-asparaginase activity

As shown in Fig. 4, the enzyme kept 100 % of its activity in presence of Na^+ and K^+. However, the other ions caused enzyme inhibition with a residual activity less than 20 %. Variable results have been reported about the effect of ions on bacterial ASNases activity. In some of those, ions such as Na^+, K^+, Mg^{2+}, Mn^{2+} and Co^{2+} enhanced the enzymatic activity [29,30].

Results on the effect of inhibitors are presented in Fig. 5. Compounds such as urea and glutathione increased the activity by around 50 %, whilst no significant loss of activity was observed in presence of PMSF (serine protease inhibitor) as well as EDTA and EGTA (chelating agents). In contrast, the enzyme was completely inhibited by 2-mercaptoethanol, DL-dithiothreitol and SDS. Some studies of ASNases from *Enterobacter* have reported that

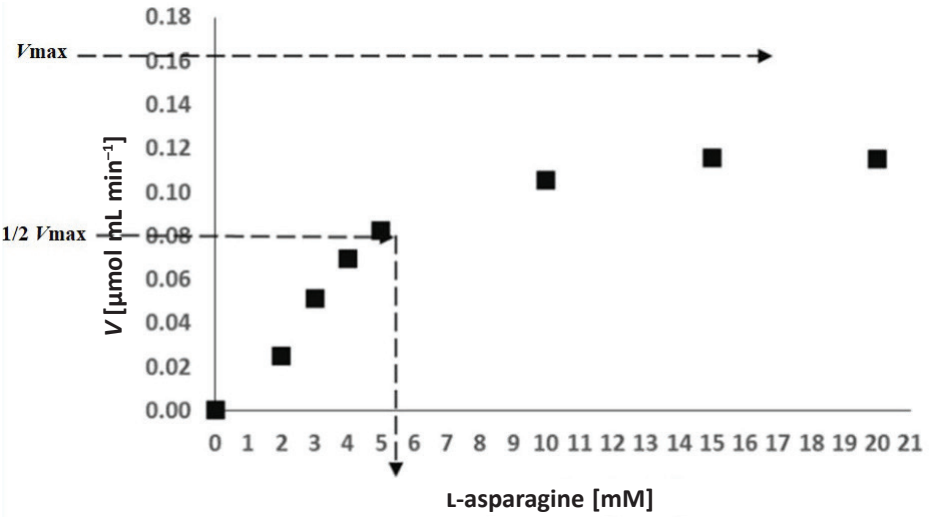

Fig. 1 – *Michaelis-Menten kinetics of L-asparaginase from Enterobacter sp. strain M55*

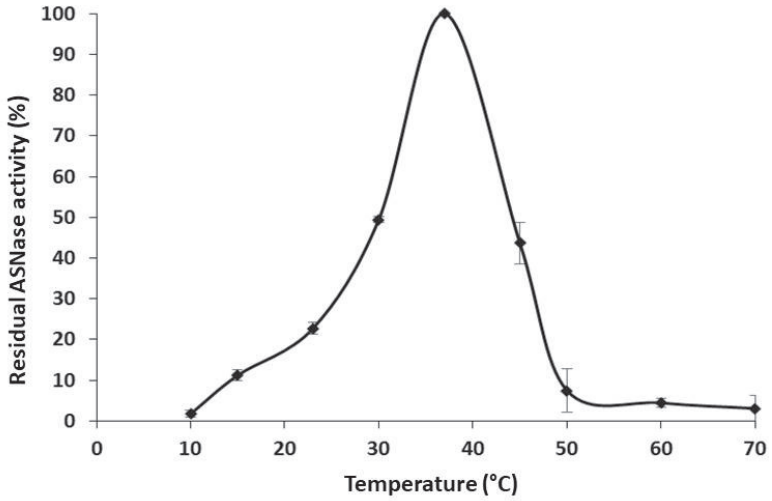

Fig. 2 – *Effect of temperature on L-asparaginase from Enterobacter sp. strain M55*

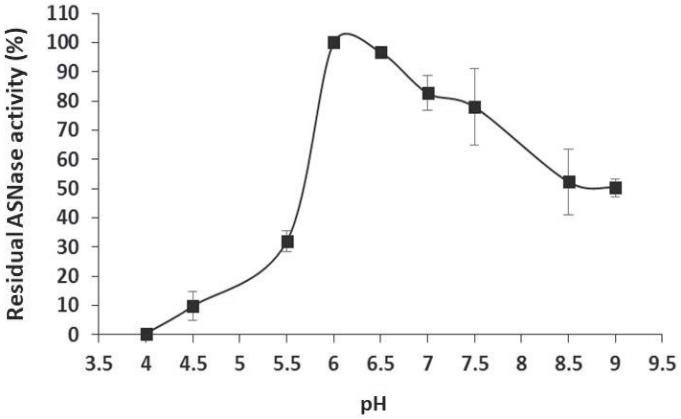

Fig. 3 – *Effect of pH on L-asparaginase from Enterobacter sp. strain M55*

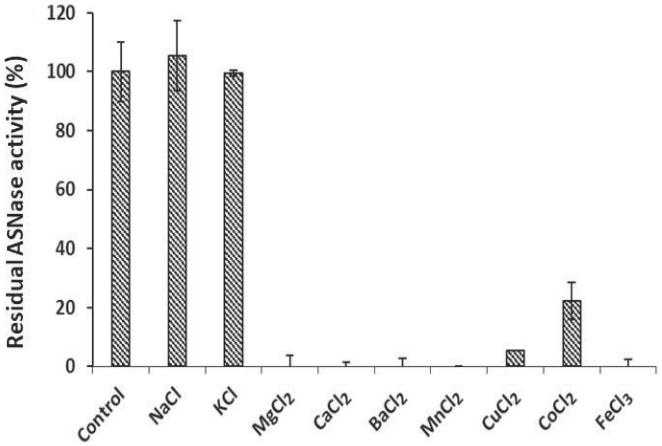

Fig. 4 – *Effect of ions on L-asparaginase from Enterobacter sp. strain M55*

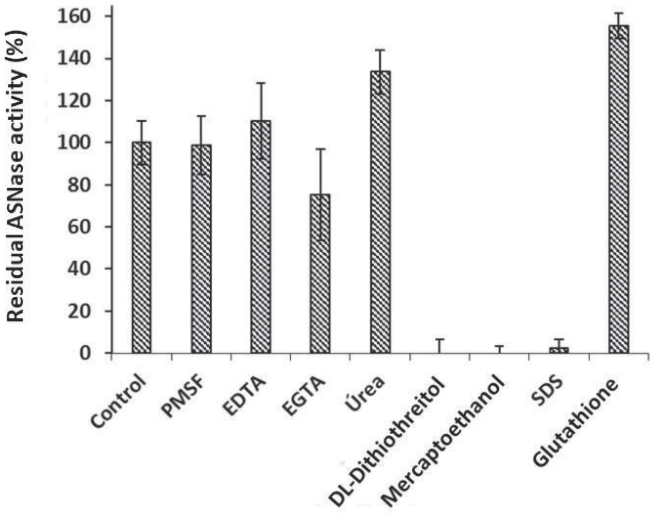

Fig. 5 – *Effect of inhibitors on L-asparaginase from Enterobacter sp. strain M55*

2-mercaptoethanol, DL-dithiothreitol, and SDS had no significant effect on the enzyme activity[9].

Regarding the effect of serum compounds on ASNase activity (Fig. 6), it was observed that glucose increased the activity by around 50 %. In presence of the other tested compounds, the enzyme kept more than 60 % of its activity. Since glucose is the most abundant sugar in the blood serum, its positive effect on this ASNase is relevant for a subsequent potential therapeutic application of the enzyme.

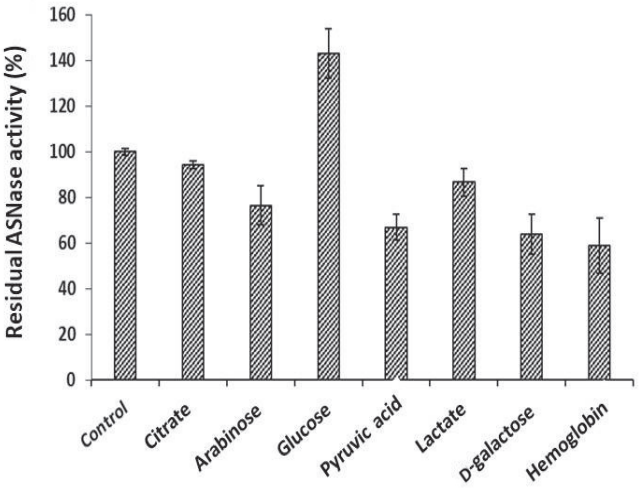

Fig. 6 – *Effect of serum components on L-asparaginase from Enterobacter sp. strain M55*

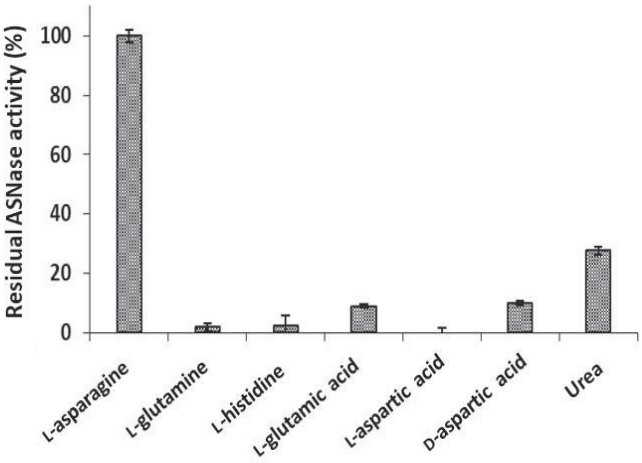

Fig. 7 – *Substrate specificity of L-asparaginase from Enterobacter sp. strain M55*

L-Asparaginase substrate specificity

ASNase activity was measured in presence of various compounds to determine substrate specificity (Fig. 7). It is fundamental to mention that GLNase activity of the ASNase from this study was 1.8 %. ASNases from *Escherichia coli* and *Erwinia chrysanthemi* approved by the Food and Drug Administration (FDA), have shown GLNase activities of 2 and 10 %, respectively. These ASNases have been implicated in side effects related to the disruption of protein synthesis induced by GLNase activi-

ty of ASNase drugs. In addition, some patients tend to discontinue the treatment due to such toxicity[10–13]. These findings are promising for subsequent ASNase clinical applications in neoplasms treatment such as AAL.

Conclusions

This work contributes to the knowledge of ASNases providing information on a novel enzyme isolated from a bacterium from an extreme environment. Characterisation studies are fundamental for subsequent studies and application of the enzymes. This ASNase demonstrated good activity at physiological conditions as well as low GLNase activity. These findings support the potential application of this ASNase in medical treatment.

ACKNOWLEDGMENTS

This research was supported by Grant 169-2017-FONDECYT-CONCYTEC, Peru, as well as by Science, Technology and Research Trust award 2020-00128, and the Institutional Funds for Research Program of University of Puerto Rico, Puerto Rico.

References

1. *Qeshmi, F. I., Homaei, A., Fernandes, P., Javadpour, S.*, Marine microbial L-asparaginase: Biochemistry, molecular approaches and applications in tumour therapy and in food industry, Microbiol. Res. **208** (2018) 99. doi: https://doi.org/10.1016/j.micres.2018.01.011
2. *Ray, M., Adhikari, S., Kundu, P.,* Isolation and characterization of microbial asparaginase to mitigate acrylamide formation in food, in Kundu, R., Narula, R. (eds) Advances in Plant & Microbial Biotechnology, Springer, Singapore, 2019, pp 95-100. doi: https://doi.org/10.1007/978-981-13-6321-4_1
3. *Cachumba, J. J., Antunes, F. A., Peres, G. F., Brumano, L. P., Santos, J. C., Da Silva, S. S.,* Current applications and different approaches for microbial L-asparaginase production, Braz. J. Microbiol. **47** (2016) 77. doi: https://doi.org/10.1016/j.bjm.2016.10.004
4. *Xu, F., Oruna-Concha, M. J., Elmore, J. S.,* The use of asparaginase to reduce acrylamide levels in cooked food, Food Chem. **210** (2016) 163. doi: https://doi.org/10.1016/j.foodchem.2016.04.105
5. *Wang, Y., Xu, W., Wu, H., Zhang, W., Guang, C., Mu, W.*, Microbial production, molecular modification, and practical application of L-asparaginase: A review, Int. J. Biol. Macromol. **186** (2021) 975. doi: https://doi.org/10.1016/j.ijbiomac.2021.07.107
6. URL: https://www.who.int/publications/i/item/WHOM-VPEMPIAU2019.06 (08.2.2022.)
7. *Brigitha, L. J., Fiocco, M., Pieters, R., Albertsen, B. K., Escherich, G., Lopez-Lopez, E., Mondelaers, V., Vora, A., Vrooman, L., Schmiegelow, K., van der Sluis, I. M., Ponte di Legno Toxicity Working Group*, Hypersensitivity to Pegylated *E. coli* asparaginase as first-line treatment in contemporary paediatric acute lymphoblastic leukaemia protocols: A meta-analysis of the Ponte di Legno Toxicity working group, Eur. J. Cancer **162** (2022) 65. doi: https://doi.org/10.1016/j.ejca.2021.11.016
8. *Cecconello, D. K., Magalhães, M. R., Werlang, I. C. R., Lee, M. L. M., Michalowski, M. B., Daudt, L. E.*, Asparaginase: An old drug with new questions, Hematol. Transfus. Cell Ther. **42** (2020) 275. doi: https://doi.org/10.1016/j.htct.2019.07.010
9. *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** (2016) e0148877. doi: https://doi.org/10.1371/journal.pone.0148877
10. *Chan, W. K., Lorenzi, P. L., Anishkin, A., Purwaha, P., Rogers, D. M., Sukharev, S., Rempe, S. B., Weinstein, J. N.,* The glutaminase activity of L-asparaginase is not required for anticancer activity against ASNS-negative cells, Blood **123** (2014) 3596. doi: https://doi.org/10.1182/blood-2013-10-535112
11. *Nguyen, H. A., Su, Y., Lavie, A.*, Design and characterization of *Erwinia chrysanthemi* L-asparaginase variants with diminished L-glutaminase activity, J. Biol. Chem. **291** (2016) 17664. doi: https://doi.org/10.1074/jbc.M116.728485
12. *Nguyen, H. A., Su, Y., Zhang, J. Y., Antanasijevic, A., Caffrey, M., Schalk, A. M., Liu, L., Rondelli, D., Oh, A., Mahmud, D. L., Bosland, M. C., Kajdacsy-Balla, A., Peirs, S., Lammens, T., Mondelaers, V., De Moerloose, B., Goossens, S., Schlicht, M. J., Kabirov, K. K., Lyubimov, A. V., Merrill, B. J., Sauntharajah, Y., Vlierberghe, P. V., Lavie, A.*, A novel L-asparaginase with low L-glutaminase coactivity is highly efficacious against both T- and B-cell acute lymphoblastic leukemias *in vivo*, Cancer Res. **78** (2018) 1549. doi: https://doi.org/10.1158/0008-5472.CAN-17-2106
13. *Narta, U. K., Kanwar, S. S., Azmi, W.*, Pharmacological and clinical evaluation of L-asparaginase in the treatment of leukemia, Crit. Rev. Oncol. Hematol. **61** (2007) 208. doi: https://doi.org/10.1016/j.critrevonc.2006.07.009
14. *de Lourdes, M., Pérez, D., García, M. T., Mellado, E.,* Halophilic bacteria as a source of novel hydrolytic enzymes, Life **3** (2013) 38. doi: https://doi.org/10.3390/life3010038
15. *Shirazian, P., Asad, S., Amoozegar, M. A.*, The potential of halophilic and halotolerant bacteria for the production of antineoplastic enzymes: L-Asparaginase and L-glutaminase, EXCLI J. **15** (2016) 268. doi: https://doi.org/10.17179/excli2016-146
16. *Alrumman, S. A., Mostafa, Y. S., Al-izran, K. A., Alfaifi, M. Y., Taha, T. H., Elbehairi, S. E.,* Production and anticancer activity of an L-asparaginase from *Bacillus licheniformis* Isolated from the Red Sea, Saudi Arabia, Sci. Rep. **9** (2019) 3756. doi: https://doi.org/10.1038/s41598-019-40512-x
17. *Han, S., Jung, J., Park, W.*, Biochemical characterization of L-asparaginase in NaCl-tolerant *Staphylococcus* sp. OJ82 isolated from fermented seafood, J. Microbiol. Biotechnol. **24** (2014) 1096. doi: https://doi.org/10.4014/jmb.1405.05021
18. *Zolfaghar, M., Amoozegar, M. A., Khajeh, K., Babavalian, H., Tebyanian, H.,* Isolation and screening of extracellular anticancer enzymes from halophilic and halotolerant bacteria from different saline environments in Iran, Mol. Biol. Rep. **46** (2019) 3275. doi: https://doi.org/10.1007/s11033-019-04787-7

19. *Qeshmi, F. I., Rahimzadeh, M., Javadpour, S., Poodat, M.*, Intracellular L-asparaginase from *Bacillus* sp. PG02: Purification, biochemical characterization and evaluation of optimum pH and temperature, Am. J. Biochem. Biotechnol. **12** (2016) 12.
doi: https://doi.org/10.3844/ajbbsp.2016.12.19
20. *Jia, M., Xu, M., He, B., Rao, Z.,* Cloning, expression, and characterization of L-asparaginase from a newly isolated *Bacillus subtilis* B11-06, J Agric. Food Chem. **61** (2013) 9428.
doi: https://doi.org/10.1021/jf402636w
21. *de Freitas, M. M., Souza, P. M., Cruvinel, K., Barros, T., Santos, S. N., Long, P. F., Pessoa, A., Magalhães, P. O.*, Interferences that impact measuring optimal L-asparaginase activity and consequent errors interpreting these data, Appl. Microbiol. Biotechnol. **103** (2019) 5161.
doi: https://doi.org/10.1007/s00253-019-09890-0
22. *Magri, A., Soler, M. F., Lopes, A. M., Cilli, E. M., Barber, P. S., Pessoa, A. J., Pereira, J. F. B.*, A critical analysis of L-asparaginase activity quantification methods—colorimetric methods versus high-performance liquid chromatography, Anal. Bioanal. Chem. **410** (2018) 6985.
doi: https://doi.org/10.1007/s00216-018-1326-x
23. *Shifrin, S., Parrott, C. L., Luborsky, S. W.,* Substrate binding and intersubunit interactions in L-asparaginase, J. Biol. Chem. **249** (1974) 1335.
24. *Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., Klenk, D. C.*, Measurement of protein using bicinchoninic acid, Anal. Biochem. **150** (1985) 76.
doi: https://doi.org/10.1016/0003-2697(85)90442-7
25. *Laemmli, U. K.,* Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature **227** (1970) 680.
doi: https://doi.org/10.1038/227680a0
26. *Jia, R., Wan, X., Geng, X., Xue, D., Xie, Z., Chen, C.*, Microbial L-asparaginase for application in acrylamide mitigation from food: Current research status and future perspectives, Microorganisms **9** (2021) 1659.
doi: https://doi.org/10.3390/microorganisms9081659
27. *Willis, R. C., Woolfolk, C. A.,* Asparagine utilization in *Escherichia coli*, J. Bacteriol. **118** (1974) 231.
doi: https://doi.org/10.1128/jb.118.1.231-241.1974
28. *Kamble, V. P., Rao, R. S., Borkar, P. S., Khobragade, C. N., Dawane, B. S.*, Purification of L-asparaginase from a bacteria *Erwinia carotovora* and effect of a dihydropyrimidine derivative on some of its kinetic parameters, Indian J. Biochem. Biophys. **43** (2006) 391.
29. *Mahajan, R. V., Kumar, V., Rajendran, V., Saran, S., Ghosh, P. C., Saxena, R. K.,* 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** (2014) e99037.
doi: https://doi.org/10.1371/journal.pone.0099037
30. *El-Naggar, N. E., Deraz, S. F., El-Ewasy, S. M., Suddek, G. M.*, Purification, characterization and immunogenicity assessment of glutaminase free L-asparaginase from *Streptomyces brollosae* NEAE-115, BMC Pharmacol. Toxicol. **19** (2018) 51.
doi: https://doi.org/10.1186/s40360-018-0242-1