USE OF ORGANIC SOLVENTS IN THE PURIFICATION OF *Bacillus subtilis* RECOMBINANT L-ASPARAGINASE

Enzo Corvello[1]*, Gabriela B. de Paiva[1], Danielle B. Pedrolli[1], Valéria C. S. Ebinuma[1] & Heitor B. S. Bento[1]

[1] *Department of Bioprocess Engineering and Biotechnology, School of Pharmaceutical Sciences - UNESP, Araraquara, Brazil.*
**E-mail: enzo.corvello@unesp.br*

ABSTRACT

L-asparaginase (L-ASNase, L-asparagine-amydohidrolase, EC 3.5.1.1) is an important enzyme that can be used as biopharmaceutical for treating certain types of cancer. However, for its safe use, this protein must be purified from the crude enzymatic extract generally obtained by microorganism's cultivation. Precipitation is widely applied as an initial protein purification step, in which organic solvents can be used for the separation of many undesired contaminants. Thus, this study evaluated the feasibility of methanol and acetone as precipitating agents of L-ASNase produced by a genetically modified *Bacillus subtilis* and presented in the enzymatic extract after cell disruption, at different ratios and their effect at the recovered enzyme-specific activity. Results showed that increasing the volume of crude extract improved the purification fold, especially with the 4:1 methanol ratio achieving a value of 1.99. However, excessive solvent led to reduced activity compared to the initial crude extract. Beyond that, these solvents were analyzed regarding to be a Nessler interferents, what showed that methanol and acetone causes inaccuracies in the analysis, preventing an accurate assessment of supernatant activity using this method. These findings suggest that precipitating with lower volumes of organic solvent ratio holds promise for the initial purification of the L-ASNase produced by microorganisms.

Keywords: L-Asparaginase. Precipitation. Organic Solvents. Nessler reagent.

1 INTRODUCTION

L-asparaginase (L-ASNase, L-asparagine-amydohidrolase, EC 3.5.1.1) is an enzyme that catalyze the asparagine hydrolysis reaction, generating aspartate and ammonium as result. Due to this ability, it has a huge industrial importance. L-ASNase can be produced at a large scale by microorganisms and commercialized for different destinations. At the pharmaceutical industry, it is used as a biopharmaceutical against Acute Lymphoblastic Leukemia (ALL) therapy, leading the tumorous cells to death because of the reduced levels of exogenous asparagine used to maintain their metabolism[1]. At the food industry, the L-ASNase can be used to reduce acrylamide formation, a carcinogenic compound generated by Maillard reaction between asparagine and reducing sugars at processed starchy foods[2].

Due to the required applications, it is necessary to purify the microbial L-ASNase from the crude extract that contains metabolites, such as other proteins considered impurities, to apply it for biopharmaceutical purposes[3]. A commonly used initial step purification involves the precipitation technique using organic solvents. This process disrupts the hydrophilic hydration around hydrophobic regions of the protein, facilitating interactions between regions with different charges and leading to protein aggregate formation. This step is useful to concentrate the desired enzyme and enables subsequent specific purification processes, such as chromatography techniques. Although organic solvent precipitation is a known method, the lack of standard methodology and different specifications of microbial extracts regarding each source, makes the study of this method essential to develop an industrial process, since these compounds can denature the enzyme leading to low recovery levels[4].

Nessler colorimetric method is widely used to evaluate L-ASNase activity due its great sensibility to react with ammonia, producing an orange compound that can be analyzed by spectrophotometry, allowing to obtain ammonia concentration and consequently the enzyme activity. However, this method has some limitations, there are some compounds that may be interferent in the analysis, which can over or underestimate the real value, making it necessary to evaluate the used compounds in order to avoid any kind of interference at the analysis[5].

Within this context, this research aimed to evaluate the potential of methanol and acetone to precipitate the L-ASNase produced by a genetically modified *Bacillus subtilis* in specific conditions and to evaluate possible interferences related to them with the Nessler methodology.

2 MATERIAL & METHODS

L-Asparaginase production

The inoculum was prepared by activation of stock culture of engineered *B. subtilis* (20% v/v in glycerol aqueous solution maintained at -80°C) in 100 mL of Luria-Bertani (LB) medium supplemented with erythromycin 1 µg/mL in 500 mL Erlenmeyer type flasks. Cells were grown overnight (16-18h) in orbital shaker (INNOVA40, New Brunswick, USA) at 37°C and 220 rpm. The production started with the transferring of the inoculum to the stirred tank bioreactor (Minifors II, Infors, New Jersey/USA), with two Rushton impeller and 4 L of volume. The initial optical density (OD 600 nm) was adjusted to 0.1 absorbance units (A.U) and the production was carried for 24 hours at 30°C, 300 rpm, aeration of 1.5 L/min and 30% of dissolved oxygen. Aqueous xylose

solution (50% wt) was used as inducing agent at 0.5% wt at the point when the OD achieved 0.8 A.U. After the bioprocess, the cells were recovered by centrifugation (13000 *xg*, 10 min, 4°C) and washed with saline phosphate buffer (PBS) pH 8. The resulting pellets were disrupted by sonication (Eco-Sonics-Ultronic, São Paulo, Brazil) with 15 sets of 2 min with 45% power and 3 min of interval between them. This process was conducted under ice batch and with a 1:20 proportion of PBS to the corresponding inoculum produced pellet. The enzymatic extract was recovered by centrifugation at the same condition cited before.

Precipitation

The enzymatic extract was precipitated with methanol and acetone. This step was conducted for 1h, at -18°C and constant homogenization, using different ratios: 1:1, 2:1, 4:1, 1:2 and 1:4 (crude extract:organic solvent), fixing the crude extract volume at 2 mL. The precipitate was recovered by centrifugation (18800 *xg*, 20 min, 4ºC), dried with nitrogen and then it was solubilized in 1 mL of PBS pH 8.0. The supernatant and the precipitate were stored at -18°C prior the enzymatic activity test.

Interference analysis

The organic solvent interference with Nessler reagent was checked by mixing 0.5 mL of water, 0.45 mL of the organic solvent, 0.05 mL of an ammonium sulfate solution (0.1 mg/mL) and 0.25 mL of Nessler reagent. The colorimetric quantification was performed after 20 minutes of reaction at 436 nm in spectrophotometer (Genesys 10S UV-Vis).

Enzymatic activity

The L-ASNase activity was measured using the Nessler reagent, which is based on the ammonia release in the medium due the asparagine hydrolysis by the enzyme. The first assay step consisted in the L-asparagine hydrolysis at 37°C for 10 min, that was performed by the addition of 0.5 mL of Tris-HCl pH 8.6, 0.5 mL of the enzymatic sample and 0.05 mL of L-asparagine 189 mM solution in a 1.5 mL microtube. The reaction was stopped with the addition of 0.25 mL trichloroacetic acid (TCA) 1.5 M solution and then centrifuged. In a new microtube was added 0.5 mL of the hydrolysis supernatant, 0.5 mL of water and 0.25 mL of Nessler reagent. After 20 min the colorimetric quantification was made in a spectrophotometer at 436 nm (Genesys 10s UV-Vis, Thermo Scientific). The L-ASNase activity was calculated according to equation 1:

$$L\text{-}ASNase\ activity\ (U\ mL^{-1}) = \frac{c_{NH_4^+}.\ V_r.V_{Nessler}}{V_t.t_r.V_E} \quad (1)$$

Where: $c_{NH_4^+}$= ammonium concentration (µmol/mL), V_r = enzymatic reaction volume (mL), $V_{Nessler}$ = sample volume for Nessler's ammonium quantification (mL), V_t= amount of V_r used for Nessler quantification (mL), T_r= enzymatic reaction time (min) and V_E= enzymatic extract volume used in the reaction (mL).

Total protein concentration

The BCA protein assay test kit (Thermo-Scientific) was used to quantify the total protein concentration, which 25 µL of diluted sample was added to 200 µL of BCA working reactant and incubated at 37°C for 30 min. The absorbance was measured at 562 nm in a microplate reader (Multimode Plate Reader-EnSpire, PerkinElmer) and the concentration was calculated according to a standard calibration curve using bovine serum albumin.

Purification fold

The efficiency of each precipitation was evaluated by the purification fold, which indicates if the desired enzyme is more concentrated or not at the precipitate. This parameter was calculated according to equation 2:

$$Purification\ fold = \frac{Precipitate_{specific\ activity\ (U\ mL^{-1})}}{Crude\ extract_{specific\ activity\ (U\ mL^{-1})}} \quad (2)$$

3 RESULTS & DISCUSSION

Nessler interference test

The Nessler method is used to indirectly evaluate the L-ASNase activity, but it has a plenty of interferents that can make the analysis unprecise[6]. Thus, both methanol and acetone were tested to evaluate their capability to be a Nessler interferent. The results are shown at figure 1 and table 1.

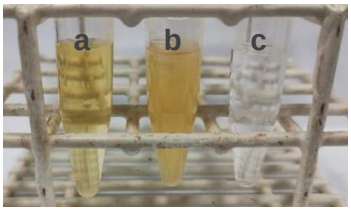

Figure 1 Interference reaction with Nessler reagent of a) water, b) methanol and c) acetone.

Table 1. Interference effect of methanol and acetone on ammonia quantification by Nessler reagent method.

Sample	NH_3 (mg mL^{-1}) *	Relative error (%)
Water	0.0102 ± 0,0003	-4.8
Methanol	0.0244 ± 0,0011	226
Acetone	0.0014 ± 0,0004	-86.9

*0,01075 mg mL^{-1} ammonia stardard solution

According to the results, both organic solvents are interferents to the method by different forms. The methanol overestimated ammonia concentration at 226% and the acetone underestimated the analysis by avoiding the formation of the usual orange color that is a result of dimercurioammonium iodide formation by the reaction between mercury iodide and ammonia. This results support the studies reported by Zhao *et al* (2019), which analyzed both solvents at 40% v/v and achieved a relative error of 174% for methanol and -86% for acetone.

Organic solvent precipitation

The enzymatic extract containing L-asparaginase was submitted to precipitation with methanol and acetone as precipitating agents, aiming to improve the purity and the specific activity of the L-ASNase produced. Since methanol and acetone interfere with the Nessler method, the supernatant was discarded and the pellet was analyzed in terms of L-ASNase activity and total protein. The results are shown in table 2.

Table 2. Activity, total protein, specific activity, and purification fold from the resulting methanol and acetone precipitates

Solvent	Sample *	Activity (U mL^{-1})	Total protein (mg mL^{-1})	Specific activity (U mg^{-1})	Purification fold
Methanol	Crude extract	0.633 ± 0.029	8.26 ± 0.08	0.077	n.a.**
	1:1	0.676 ± 0.009	8.88 ± 0.22	0.076	0.99
	2:1	0.418 ± 0.015	5.55 ± 0.19	0.075	0.98
	4:1	0.182 ± 0.002	1.19 ± 0.01	0.153	1.99
	1:2	0.395 ± 0.011	9.59 ± 0.10	0.041	0.54
	1:4	0.386 ± 0.064	7.96 ± 0.04	0.048	0.63
Acetone	Crude extract	0.755 ± 0.089	8.11 ± 0.04	0.093	n.a. **
	1:1	1.089 ± 0.137	10.61 ± 0.63	0.103	1.10
	2:1	0.253 ± 0.012	2.13 ± 0.07	0.119	1.28
	4:1	0.108 ± 0.034	1.54 ± 0.18	0.070	0.75
	1:2	0.515 ± 0.063	7.28 ± 0.01	0.071	0.76
	1:4	0.612 ± 0.026	7.99 ± 0.62	0.077	0.82

*Crude extract: solvent ratio used in precipitation, **n.a: non-applicable

Analyzing the results, there was a slight improvement in the specific activity of the precipitate when there was less organic solvent in the medium, with a better partial L-asparaginase purification with methanol at a 4:1 ratio for 1h. The time and ratio used are important factors, since the results may indicate an enzyme denaturation in the presence of excess solvent[7] and a possibility to let the reaction for longer periods to improve the enzyme precipitation.

4 CONCLUSION

In conclusion, the use of a lower solvent volume is a better option at the initial purification, in which methanol 4:1 precipitation promoted a high value of specific activity by almost doubling the purification fold. In addition, it is clear that a Nessler interference analysis is indispensable to avoid false positive or negative results, as could be seen for methanol and acetone, that can interfere with the analysis.

REFERENCES

[1] BENTO, H. B. S., PAIVA, G.B., ALMEIDA, M.R., SILVA, C.G., CARVALHO, P.J., TAVARES, A.P.M., PEDROLLI, D.B., SANTOS-EBINUMA, V.C. 2022. Bioprocess Biosyst Eng. 45 (10).1635-1644.
[2] KOSZUCKA, A., NOWAK, A., NOWAK, I., MOTYL, I.2020. Crit Rev Food Sci Nut. 60 (10).1677-1692.
[3] SCHEINER, M., RAZDAN S., MILLER, A.M., BRICENO, M.E., BARUA, S. 2020. Biotechnol Bioeng.117 (8). 2588-2609.
[4] TUNDISI, L. L., COÊLHO, D.F., ZANCHETTA, B., MORIEL, P., JUNIOR, A.P., TAMBOURGUI, E.B., SILVEIRA, E., MAZZOLA, P.G. 2017. Separation & Purification Reviews. 46 (1). 35-43.
[5] ZHAO, Y., SHI, R., BIAN, X., ZHOU, C., ZHAO, Y., ZHANG, S., WU, F., WATERHOUSE, G.I.N., WU, L.Z., TUNG, C.H., ZHANG, T. 2019. Adv Sci. 6 (8).
[6] FREITAS, M. M., SOUZA, P.M., CRUVINEL, K., BARROS, T., SANTOS, S.N., LONG, P.F., PESSOA, A., MAGALHÃES, P.O. 2019. Appl Microbiol Biotechnol. 103 (13). 5161-5166.
[7] MAGSUMOV, T., ZIYING, L., SEDOV, I. 2020. Int J Biol Macromol. 160. 880-888.

ACKNOWLEDGEMENTS

FAPESP (process 2023/01368-3, 2023/14606-0, 2021/01284-9, 2021/06686-8, 2020/15513-7); CAPES Finance code 001.