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Optimization of asparaginase production from *Zymomonas mobilis* by continuous fermentation

Francieli Bortoluzzi Menegat, Cristiani Baldo, Maria Antonia Pedrine Colabone Celligoi and João Batista Buzato*

[1]*Departamento de Bioquímica e Biotecnologia, Universidade Estadual de Londrina, Rod. Celso Garcia Cid, PR 445, Km 380, Cx. Postal 10011, 86057-970, Londrina, Paraná, Brazil. *Author for correspondence. E-mail: buzato@uel.br*

ABSTRACT. Asparaginase is an enzyme used in clinical treatments as a chemotherapeutic agent and in food technology to prevent acrylamide formation in fried and baked foods. Asparaginase is industrially produced by microorganisms, mainly gram-negative bacteria. *Zymomonas mobilis* is a Gram-negative bacterium that utilizes glucose, fructose and sucrose as carbon source and has been known for its efficiency in producing ethanol, sorbitol, levan, gluconic acid and has recently aroused interest for asparaginase production. Current assay optimizes the production of *Z. mobilis* asparaginase by continuous fermentation using response surface experimental design and methodology. The studied variables comprised sucrose, yeast extract and asparagine. Optimized condition obtained 117.45 IU L^{-1} with dilution rate 0.20 h^{-1}, yeast extract 0.5 g L^{-1}, sucrose 20 g L^{-1} and asparagine 1.3 g L^{-1}. Moreover, carbon:nitrogen ratio (1:0.025) strongly affected the response of asparaginase activity. The use of *Z. mobilis* by continuous fermentation has proved to be a promising alternative for the biotechnological production of asparaginase.

Keywords: sucrose, asparagine, yeast extract, enzyme, microbial production, factorial design.

Otimização da produção de asparaginase de *Zymomonas mobilis* por fermentação contínua

RESUMO. A asparaginase é uma enzima usada em tratamento clínico como agente quimioterapêutico e em tecnologia de alimentos na prevenção de formação de acrilamida em alimentos fritos e assados. Asparaginase é industrialmente produzida por micro-organismos, principalmente bactérias gram negativas. *Zymomonas mobilis* é uma bactéria gram negativa que utiliza glicose, frutose e sacarose como fonte de carbono e é conhecida por sua eficiência para produzir etanol, sorbitol, levana, ácido glicônico, e mais recentemente, tem despertado interesse no uso desse micro-organismo na produção de asparaginase. Este trabalho teve como objetivo otimizar a produção de asparaginase de *Z. mobilis* por fermentação contínua, pelo uso do delineamento experimental e da metodologia da superfície de resposta, testando as variáveis: sacarose, extrato de levedura e asparagina. A condição ótima alcançada, com produção de 117,45 UI L^{-1} foi na taxa de diluição 0,20 h^{-1}, utilizando 0,5 g L^{-1} de extrato de levedura, 20 g L^{-1} de sacarose e 1,3 g L^{-1} de asparagina. Observou-se que a relação carbono:nitrogênio (1:0,025) exerceu forte influência na resposta da atividade de asparaginase. A utilização de *Z. mobilis* por fermentação contínua demonstrou ser uma alternativa promissora na produção biotecnológica da asparaginase.

Palavras-chave: sacarose, asparagina, extrato de levedura, enzima, produção microbiana, delineamento fatorial.

Introduction

Asparaginase (asparagine amidohydrolase EC 3.5.1.1) hydrolyzes the amino acid asparagine into aspartic acid and ammonia. Asparaginase is an intracellular enzyme produced by several different microorganisms, including Gram-negative bacteria, mycobacteria, yeasts and fungi, and may also be extracted from plants and from the plasma of certain vertebrates. It was the first enzyme with antitumor activity to be studied extensively in humans (Maladkar, Singh, & Naik, 1993). Although the bacterium *E. coli* produces antitumor asparaginase (Müller, & Boos, 1998; Shanmugaprakash et al., 2015; Olu et al., 2008; Song et al., 2015), it causes toxic side effects when used continuously (Prakasham, Rao, Rao, Lakshmi, & Sarma, 2007; Kotzia & Labrou, 2005), coupled to high production costs.

Besides its use in antitumor treatments, asparaginase is also employed in food technology to reduce the formation of acrylamide (a toxic carcinogenic compound) in fried and baked food (Kornbrust, Stringer, Lange, & Hendriksen, 2009; Zuo, Zhang, Jiang, & Mu, 2015; Kumar, Shimray, Indrani, & Manonmani, 2014; Batool, Makky, Jalal, & Yusoff, 2016).

Interestingly, L-asparaginases from *Aspergillus niger* and *Aspergillus oryzae* have been commercially

used to decrease the formation of acrylamide (Hendriksen, Kornbrust, Ostergaard, & Stringer, 2009). Asparaginase has been focused in the search for other microorganisms that produce the enzyme with low toxicity and low-cost raw materials in the fermentation process.

Zymomonas mobilis is one of several microorganisms that have been studied extensively in recent years and have aroused interest in the field of biotechnology. Besides its use in the production of ethanol, it provides asparaginase, levan, sorbitol (Ernandes, Boscolo, & Cruz, 2010) and gluconic acid (Silveira et al., 1999). Further, current research group has also studied the utilization of commercial sugar and sugarcane juice for the production of asparaginase by *Z. mobilis* and *Erwinia herbicola* by submerged fermentation. Results revealed that *Z. mobilis* has a great potential for the production of asparaginase (Wietchorek, Buzato, Menegat, & Celligoi, 2013).

Zymomonas mobilis is a Gram-negative bacterium that uses glucose, fructose and sucrose as energy sources (Swings & Ley, 1977). Carbohydrate catabolism and ethanol production occur through the Entner-Doudoroff pathway (Sprenger, 1996). Since continuous fermentation in fermentation processes achieves highest production and yield rates, it may be an interesting approach for the production of asparaginase from *Z. mobilis*.

Thus, the objective of this work was to optimize the production of asparaginase from *Z. mobilis* through continuous fermentation, using the response surface experimental design and methodology, and testing the following variables: sucrose, yeast extract and asparagine. Results show that continuous fermentation is a useful method to produce asparaginase by *Z. mobilis*. To our knowledge, this is one of the few studies to describe the production of asparaginase by *Z. mobilis* using continuous fermentation.

Material and methods

Microorganism and growth media

Zymomonas mobilis ATCC 35001 was used in a slant culture containing glucose (10 g L^{-1}), yeast extract (5 g L^{-1}) and agar (15 g L^{-1}), and stored in a refrigerator (2 to 8°C). Table 1 shows the composition of the fermentation medium. The components were solubilized in distilled water and sterilized separately in groups (sulfates, phosphates, chlorides, sucrose, asparagine and yeast extract) and mixed aseptically.

Table 1. Composition of the fermentation medium.

Component	Concentration (g L^{-1})
$CaCl_2.H_2O$	0.00025
NaCl	0.008
$MgSO_4.7H_2O$	2.0
KH_2PO_4	3.5
Sucrose [a]	10.0, 20.0 and 30.0
Asparagine [a]	0.1, 0.5 and 0.9
Yeast extract [a]	0.5 , 1.0 and 1.5

[a]According to factorial design (Table 2).

Continuous fermentation

Continuous culture was performed in a 0.5-L reactor and a 0.3-L working volume. Temperature was set at 30°C ± 1, with constant agitation. The culture was fed continuously with a new autoclaved medium (from a reservoir) using an adjustable-speed peristaltic pump. Initially, fermentations were carried out in batch mode for approximately 8 hours until the culture reached the exponential growth phase; the culture was afterwards fed continuously. Dilution ratio was set at 0.20 h^{-1} during the phase in which the variables were tested. During the volumetric production stage and medium optimized, the dilution rates 0.20, 0.25 and 0.30 h^{-1} were tested.

Analytical Determinations

Determination of asparaginase activity followed Peterson and Ciegler (1969). Briefly, 0.1 mL of enzymatic extract was added to a solution containing asparagine (0.04 M) and incubated at 37°C. After 30 minutes, the reaction was stopped and 15% of trichloroacetic acid were added. After centrifugation, the supernatant was diluted in distilled water and treated with 0.1 ml of Nessler's reagent. The optical density was measured at 400 nm. One international unit (IU) of L-asparaginase is defined as the amount of enzyme required to release 1 μmol of ammonia per minute at 37°C under pH 8.6 (buffer Tris-HCl 0.05 M). Asparaginase activity was expressed as IU L^{-1} of the fermented broth. Biomass was estimated by spectrophotometry at a wavelength of 610 nm, and the corresponding dry mass was obtained by a calibration curve.

Experimental design

The evaluation of the different conditions for asparaginase production from *Z. mobilis* was evaluated by incomplete 3^3 factorial design, with 3 replications in the central. Variables comprised concentration of yeast extract (X_1), concentration of sucrose (X_2), and concentration of asparagine (X_3), (Table 2). Statistica 7.0 was used for statistical analysis.

Table 2. Box-Behnken design with the variation levels of tested variables.

Assays	X_1	X_2	X_3
1	-1	-1	0
2	1	-1	0
3	-1	1	0
4	1	1	0
5	-1	0	-1
6	1	0	-1
7	-1	0	1
8	1	0	1
9	0	-1	-1
10	0	1	-1
11	0	-1	1
12	0	1	1
13	0	0	0
14	0	0	0
15	0	0	0

Original variables	Variation levels		
	-1	0	1
X_1 Yeast extract (g L^{-1})	0.5	1.0	1.5
X_2 Sucrose (g L^{-1})	10	20	30
X_3 Asparagine (g L^{-1})	0.1	0.5	0.9

Results and discussion

The factorial experimental design has been used by several authors in studies on asparaginase production from *Zymomonas mobilis* (Camilios-Neto, Buzato, & Borsato, 2006; Casotti et al., 2007) and other microorganisms (Prakasham et al., 2007; Hymavathi, Sathish, Subba, & Prakasham, 2009; Kenari, Alemzadeh, & Maghsodi, 2010; Babu, Ramagopal, & Rami-Reddy, 2010; Agarwal, Kumar, & Veeranki, 2011; Usha, Mala, Venil, & Palaniswamy, 2011; Gurunathan, & Sahadevan, 2012; Singh & Srivastav, 2013).

Table 3 presents the design matrix used in this work, along with the experimental values obtained for asparaginase activity. It may be observed that assay 7, with 0.5, 20 and 0.9 g L^{-1} concentrations, respectively for yeast extract, sucrose and asparagine, demonstrated the highest enzyme activity, namely, 99.09 IU L^{-1}. High concentration of asparagine was a factor that improved the rates of asparaginase activity. The presence of amino acid asparagine, related to asparaginase production, was demonstrated by the pioneer works by Galani, Drainas, and Typas (1985) with submerged fermentation from *Z. mobilis* in growth medium, with and without asparagine.

Assay 11 revealed the weakest performance of *Z. mobilis* for enzyme activity and biomass activity. The combination of variables tested showed it to be a low-yield growth medium with unsatisfactory fermentation. Assays 8 and 12, with progressive performance (asparaginase activity of 26.37 and 58.00 IU L^{-1}, respectively), indicated that the ideal ratio between sucrose and yeast extract would feature ever-decreasing amounts of yeast extract. Assay 7 demonstrated that the ideal ratio was 1:0.025 (sucrose : yeast extract) among the four culture variations analyzed. Another observation is that the greater the enzyme activity, the higher the biomass value obtained.

Table 3. Matrix containing numbered assays, decoded variables and results obtained for asparaginase.

Assays	Decoded Variables			Asparaginase Activity (IU L^{-1})
	Yeast extract (g L^{-1})	Sucrose (g L^{-1})	Asparagine (g L^{-1})	
1	0.5	10	0.5	4.69
2	1.5	10	0.5	21.19
3	0.5	30	0.5	12.55
4	1.5	30	0.5	78.96
5	0.5	20	0.1	17.33
6	1.5	20	0.1	24.00
7	0.5	20	0.9	99.09
8	1.5	20	0.9	26.37
9	1.0	10	0.1	7.44
10	1.0	30	0.1	55.19
11	1.0	10	0.9	14.47
12	1.0	30	0.9	58.00
13	1.0	20	0.5	49.45
14	1.0	20	0.5	46.21
15	1.0	20	0.5	44.20

Table 4 shows the four results obtained in the assays with asparagine concentration in the upper limit of the tested condition (0.9 g L^{-1}). Amounts of yeast extract and sucrose used, as well as the biomass and ratio between yeast extract and sucrose.

Table 4. Decoded variables and obtained results.

Assay	Yeast extract (g L^{-1})	Sucrose (g L^{-1})	Asparaginase (IU L^{-1})	Biomass (mg mL^{-1})	YE:S ⋆
7	0.5	20	99.09	38.33	0.025
8	1.5	20	26.37	20.41	0.075
11	1.0	10	14.47	11.93	0.1
12	1.0	30	58.00	33.79	0.033

⋆YE:S – Yeast extract : sucrose ratio.

Previous research have shown that the carbon source:yeast extract ratio had a strong effect on enzyme ativity response. Research by Camilios-Neto et al. (2006) yielded a maximum rate (16.55 IU L^{-1}) of asparaginase from *Z. mobilis*, even though the above authors used different concentrations of molasses and yeast extract as variables. Lower asparaginase rates seem to be influenced by the high concentration of total reducing sugars in the molasses. Lee, Tribe, and Rogers (1979) reported that glucose at high concentrations produced incomplete fermentations since the sugar was not fully metabolized. Moreover, the minerals in the molasses could have inhibited growth, particularly magnesium salts in the substrate. Casotti et al. (2007) used sugarcane juice and yeast extract at high concentrations as variables, and asparagine at 1.0 g L^{-1}. The authors obtained 9.75 IU L^{-1} as the best asparaginase activity which proved that, although sugarcane juice and yeast extract

were rich substrates and represented an excellent medium for the growth of microorganisms, they did not favor asparaginase production, but merely stimulated alcohol fermentation (90% sugar intake) and biomass production.

The results of the activity were analyzed by response surface methodology (Figure 1). The equation below fitted the model using the incomplete 3^3 factorial design:

$$Y = 54.2067 + 0.121X_1 + 2.4988X_2 + 12.337X_3 - 10.6758X_1^2 - 5.1858X_2^2 + \\ + 3.1967X_3^2 + 12.4750X_1X_2 - 23.8175X_1X_3 + 8.1775X_2X_3 \quad (01)$$

where: Y_1 represents the expected asparaginase activity; X_1 is the concentration of yeast extract; X_2 is sucrose concentration; X_3 is the concentration of asparagine.

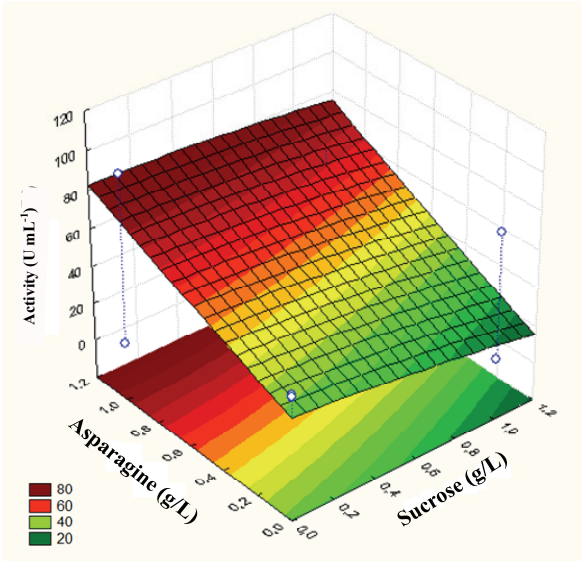

Figure 1. Response surface showing the effect of asparagine and sucrose on asparaginase activity.
Source: Authors.

The surface response graph indicates that, to increase asparaginase activity, the concentration of sucrose and yeast extract must remain at low levels, while the amount of asparagine must increase.

So that the volumetric production stage could be evaluated, an assay was performed with an asparagine concentration of 1.3 g L^{-1} at different dilution rates: 0.20, 0.25 and 0.30 h^{-1}. Table 5 shows the obtained results.

Activity and yield values were 15.6% higher for dilution rate 0.20 h^{-1} in an equal volume of produced fermented broth as that obtained from assay 7, with less asparagine. Results confirm that increase in asparagine stimulates asparaginase activity.

Table 5. Values obtained in optimized culture of *Z. mobilis* at different dilution rates.

D (h^{-1})	Biomass (mg mL^{-1})	Asparaginase (IU L^{-1})	Fermented Broth (mL)	Yield (IU L^{-1} h^{-1})
0.20	42.51	117.45	60	7.05
0.25	32.77	86	75	6.45
0.30	30.68	60	90	5.40

Nevertheless, activity, biomass and yield rates decrease as dilution rate increases. Dilution rates that exceeded maximum growth rate of the microorganism produced lower biomass rates. These data characterize cell wash since the microorganism cannot reproduce at the same speed as growth medium renovation.

Conclusion

Among the evaluated conditions, the best asparaginase production (117.45 IU L^{-1}) occurred when the medium under optimum conditions contained 0.5 g L^{-1} yeast extract, 20 g L^{-1} sucrose and 1.3 g L^{-1} asparagine. Dilution rate over 0.20 h^{-1} resulted in decreased production parameters. In fact, the carbon:nitrogen ratio greatly affected asparaginase activity response, with the best ratio (1:0.025).

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