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RESEARCHES ON THE QUANTIFICATION OF L-ASPARAGINASE PRODUCED BY MICROORGANISMS

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Abstract

L-asparaginase is an enzyme that is mainly used in the medical industry to treat various cancers and in the food industry to reduce acrylamide in starch-containing foods. In order to be used successfully, L-asparaginase must have a certain concentration and a certain purity to give the best results and minimize side effects in the medical industry.
The aim of this paper was the screening of different strains of Bacillus, Streptomyces, yeasts and bacterial endophytes for L-asparaginase biosynthesis, and to highlight the methods used to determine the level of L-asparaginase. Several bacterial strains and two yeast strains were selected and the level of L-asparaginase was quantified. At least two microbial strains will be used in further experiments to optimize the enzyme production.

Key words*: L-asparaginase, enzyme producing microorganisms.*

INTRODUCTION

L-asparaginase (L-asparagine amidohydrolase; EC 3.5.1.1) is an enzyme commonly found in the world of plants, animals and microorganisms. It is able to catalyze the hydrolysis of L-asparagine into aspartic acid and ammonia (Huang et al., 2013). The enzyme L-asparaginase is found in two forms: a cytoplasmic L-asparaginase (type I), which can hydrolyze both L-asparagine and L-glutamine, and a periplasmic L-asparaginase (type II), with a higher substrate affinity. Nowadays, this enzyme is used in medicine to treat acute lymphoblastic leukemia (Yim & Kim, 2019).
Since the early 1970s, L-asparaginase has been approved by the Food and Drug Administration as enzymatic drug for cancer treatment, being used in conjunction with traditional chemotherapy (Lorenzi et al., 2008). Some tumor cells need extracellular L-asparagine to grow because they are deficient in L-asparagine synthetase. Treatments based on L-asparaginase deplete the concentration of L-asparagine in the blood, thereby 'starving' the tumor cells that need this amino acid (Newton, 2012). Such procedure selectively inhibits the growth of malignant cells (Krasotkina et al., 2004; Kotzia & Labrou, 2007). For medical purposes, L-asparaginase produced by *Escherichia coli* (EcAII) and *Erwinia crysanthemi* (ErA) is used (Goswami et al., 2019). The EcAII isoenzyme differs from EcAI by its wide pH activity profile and high affinity for the substrate (Cornea et al., 2002).
The use of L-asparaginase in the treatment of acute lymphoblastic leukemia has some limitations due to drug resistance and adverse reactions. In addition to allergic reactions, L-asparaginase can cause blood clotting abnormalities, liver and pancreatic dysfunction and immunosuppression (Reinert et al., 2006). These side effects have been shown to be caused by the activity of glutaminase and urease. The absence of these two enzymes is therefore, important in therapy.
During microbial L-asparaginase synthesis, the presence of glutaminase and urease in the culture supernatant leads to additional purification steps. The process becomes expensive and laborious (Mahajan et al., 2013; Ashok et al., 2019). Therefore, when searching new microbial strains competitive in L-asparaginase production, the lack of glutaminase and urease production is required.
In some therapeutic studies, adverse reactions such as allergic or hypersensitivity reaction could also be reduced or even eliminated by trapping the enzyme in a polymeric shell (Teodor et al., 2008).

The use of L-asparaginase in the treatment of acute lymphoblastic leukemia must be based on well-established parameters such as quantification and determination of enzyme activity, purification, biochemical properties, stability and activity on the tumor (Costa-Silva, et al., 2020).

L-asparaginase also plays an important role in the food industry because it inhibits the reaction of L-asparagine with reducing sugars, preventing the formation of acrylamide (Mahajan et al., 2013). Acrylamide is considered a toxic substance for the nervous and reproductive system (Dias et al., 2017). It is formed as a result of the Maillard reaction between reducing carbohydrates and L-asparagine, during the thermal preparation of food at over 120ºC (Negoiță et al., 2017). L-asparaginase is applied at the beginning of the thermal preparation of different foods, minimizing the formation of acrylamide and maintaining food quality at economic viable costs (Yim & Kim, 2019). Several formulations are mentioned in the food industry for similar products (Alexe & Dima, 2014; Cercel et al., 2017).

Microbial L-asparaginase production is a laborious process influenced by the substrate composition, especially by the source of carbon and nitrogen as well as other growth parameters. In order to optimize microbial enzyme production, appropriate growth conditions should be provided to the microorganism during fermentation (Souza et al., 2017).

Studies have showed that L-asparaginase derived from bacterial cultures has common biochemical properties. However, some enzymes have optimal operating temperatures at 40ºC while others at 80-85ºC (Muneer et al., 2020). Differences regarding the pH range were also noticed when studying L-asparaginase activity on agar plates, in the presence of pH-indicator dyes. Research has shown that the hydrolytic action of L-asparaginase can be increased at pH between 6.0 and 9.5 (Mihooliya et al., 2017).

The aim of this study was L-asparaginase detection and quantification in various microbial cultures. The purpose was to select potential microbial strains with biotechnological attributes in L-asparaginase production, lacking glutaminase and urease activity.

MATERIALS AND METHODS

Microbial strains

Seventeen yeasts and bacterial strains were evaluated for their ability to produce L-asparaginase, glutaminase and urease. Among these microorganisms, there were five yeast strains, four streptomycete, five rhizobacteria and three endophyte bacteria (Table 1).

These microorganisms were grown on usual culture media in order to refresh the strains after storage.

Table 1. Microbial strains and culture media

Microbial category	Strain	Usual growth media	Specific culture media
Yeasts	*Candida albicans* ATCC10231	YPG	MCD
	Candida parapsilosis CSB 604		
	Pichia anomala 88		
	Metschnikowia sp. CPM1		
	Metschnikowia sp. SG1		
Actinomycete	*Streptomyces* sp. P1S7	Gauze	ISP5
	Streptomyces sp. P2S10		
	Streptomyces sp. Strepto SS16		
	Streptomyces sp. C11		
Rhizobacteria	*Bacillus subtilis* B5	LB	M9
	Bacillus subtilis B6		
	Bacillus sp. OS15		
	Bacillus amyloliquefaciens BPA		
	Bacillus amyloliquefaciens BIR		
Endophytic bacteria	LT MYM1 strain isolated from lavender stem		
	LFF MYM1 strain isolated from lavender leaves and flowers		
	LFF MYM5 strain isolated from lavender leaves and flowers		

Agar plate technique for qualitative-enzyme detection

Screening of enzymes producing microorganisms was performed on specific culture media based on asparagine, glutamine,

urea or $NaNO_3$ as single nitrogen source. These solid media were supplemented with pH indicator dyes, such as phenol red or bromothymol blue. The yeasts were grown on MCD medium, pH 5.5, containing: Na_2HPO_4 6 g/L, KH_2PO_4 2 g/L, NaCl 0.5 g/L, glycerol 0.02 g/L, $MgSO_4$ 0.2 g/L, $CaCl_2$ 0.005 g/L, agar 2%, nitrogen source 0.5%, and pH indicator dyes. The colorants used were either phenol red 0.009% or bromothymol blue 0.007%. Streptomycetes were studied for enzyme production on ISP5 medium containing: 1% glycerol, 0.1% KH_2PO_4, 0.1% microelement solution, 2% agar, 0.5% nitrogen source and 0.009% phenol red, pH 7.2. The micronutrient solution was prepared of 0.01% $FeSO_4$, 0.01% $MnSO_4$, and 0.01% $ZnSO_4$. Rhizobacteria and endophytic bacteria were evaluated for enzyme production on M9 medium containing: 0.2% Na_2HPO, 0.3% KH_2PO_4, 0.05% NaCl, 2% $MgSO_4$, 0.1% $CaCl_2$, 0.2% glucose, 2% agar, 0.5% nitrogen source and 0.009% phenol red at pH = 7.

All microorganisms were inoculated in spots using fresh microbial biomass. Test plates were incubated for 3 days at 28°C. L-asparaginase, glutaminase, and urease activity was visually appreciated through this qualitative screening method.

Quantitative estimation of L-asparaginase activity

The microorganisms revealing only L-asparaginase activity were refreshed on specific culture media with 0.5% L-asparagine to stimulate microbial enzyme production. The obtained cultures were used to inoculate M9, ISP5 and MCD liquid media containing L-asparagine 5 g/L. No pH indicator dyes were needed for this test. The inoculum consisted of 4% (v/v) cells suspension prepared on sterile distilled water.

Inoculated flasks were incubated on a rotary shaker at 120 rpm, at 28°C, for 7 days.

L-asparaginase activity was evaluated using Nesslerization spectrophotometric method. According to this, yellow-colored compounds are produced due to Nessler reagent reaction with the free ammonia released by enzymatic degradation of L-asparagine. The ammonia amount is positively correlated to the optical density measured by spectrophotometry at 425 nm.

Culture samples were centrifuged at 10,000 RCF for 10 minutes at 4°C, to obtain the crude enzyme extract. In order to evaluate the amount of L-asparaginase, 1 volume of crude enzyme extract was added to 9 volumes of specific substrate prepared from 0.04 M L-asparagine in 50 mM TRIS buffer, pH = 8.6. The mixture was incubated at 37°C to allow the enzymatic reaction. After 30 min the reaction was stopped by adding 15% TCA solution. To remove any precipitate, samples were centrifuged 5 min at 10,000 RCF. The supernatant, 100 µl/sample, was diluted in 800 µl distilled water and mixed with 100 µl of Nessler reagent. Samples were maintained at room temperature for 10 min before optical density readings. The spectrophotometric measurements were carried out at 425 nm. Samples were compared to a control reaction were enzymatic activity was stopped with TCA before incubation.

A standard curve was prepared using ammonium sulfate solution in 0.2N H_2SO_4. Enzyme activity (EA) was calculated as the amount of ammonia released during the enzymatic degradation of L-asparagine from the substrate, as shown in the following equation (Ashok et al., 2019):

$$EA\ (U/ml) = \frac{Amount\ of\ NH_4\ liberated\ (\mu mols)}{Incubation\ time\ (min) \times ml\ of\ Enzyme}$$

Quantification of glutaminase and urease activity was also attempted. For these enzymes we used the same culture crude extract and the Nesslerization method as for L-asparaginase activity. Differences consisted in the substrate used. For glutaminase activity we used 0.04M of glutamine in TRIS buffer (pH = 8.6), and for urease we used 0.04M urea in the same buffer.

Dynamic estimation of L-asparaginase activity

The bacteria's capacity to produce L-asparaginase was also dynamically evaluated at 24 h, 48 h and 72 h of culture incubation. Enzyme activity was evaluated using Nesslerization spectrophotometric method, as previously described.

RESULTS AND DISCUSSIONS

Selection of L-asparaginase producing microorganisms

Qualitative determination of L-asparaginase, glutaminase and urease production is a rapid screening method based on a pH sensitive color reaction. The pH indicator dye changes color if tested microorganisms are enzyme producers, able to hydrolyze the nitrogen sources included in the substrate. When using phenol red culture media, the enzyme producing strains change the pH of the medium, turning the color from yellow to bright pink. When using bromothymol blue culture media, the enzyme producing strains modify the color from yellow to green or greenish-blue at neutral pH, or bright aquamarine at alkaline pH levels.

Enzymes production was visually evaluated based on color change, color intensity and halo diameter around microbial growth (Table 2). Distinctive symbols were designated according to the pH-sensitive enzymatic activity, as follows: "– = no color change; "± = slight color change under the imprint of microbial growth, + = slight color change around the colony, ""++ = moderate color change around the colony; "+++ = intense color change around the colony.

Table 2. Qualitative evaluation of enzyme producing microorganisms

Microbial strain	Hydrolyzed nitrogen sources			
	L-asparagine	Glutamine	Urea	$NaNO_3$
ATCC10231	+++	–	–	+
CSB 604	+ to ++	–	–	–
P.a. 88	–	–	–	–
CPM1	++ to +	–	–	–
SG1	++ to +	–	–	–
P1S7	++	+	–	+
P2S10	–	–	–	–
Strepto SS16	++	+	–	–
C11	+++	+++	++	++
B5	+++	±	–	–
B6	+++	±	–	–
OS15	+++	±	–	+
BPA	+++	±	–	–
BIR	+	++	+	–
LT MYM1	+++	–	–	+
LFF MYM1	+++	±	+	–
LFF MYM5	++	+	–	++

Among the tested yeasts, best results regarding L-asparaginase production were obtained with *Candida albicans* ATCC10231. However, this strain also revealed the possibility to use $NaNO_3$. On L-asperagine medium *Metschnikowia* sp. CPM1 and SG1 strains revealed moderate color change around the colony within the first 24 h of incubation (Figure 1), but after two days of incubation the colored halos decreased their width and color intensity. According to this colorimetric assay, CPM1 and SG1 strains were not able to use glutamine, urea or $NaNO_3$. *Candida parapsilosis* CSB 604 produced only L-asparaginase, after 2 days of incubation.

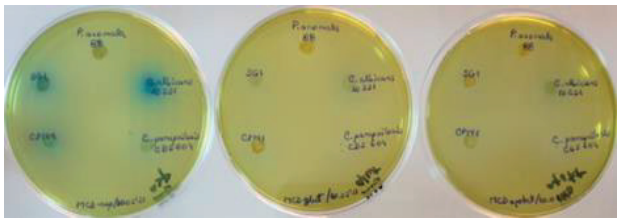

Figure 1. Yeasts strains on CDM media containing L-asparagine (left), glutamine (center), or $NaNO_3$ (left) and bromothymol blue as pH indicator dye (after 24 h of incubation at 28°C)

Among tested streptomycetes, only P1S7 and Strepto SS16 strains revealed L-asparaginase production. However, a weak color change was seen also on glutamine supplemented ISP5 medium after 48 h of incubation (Figure 2).

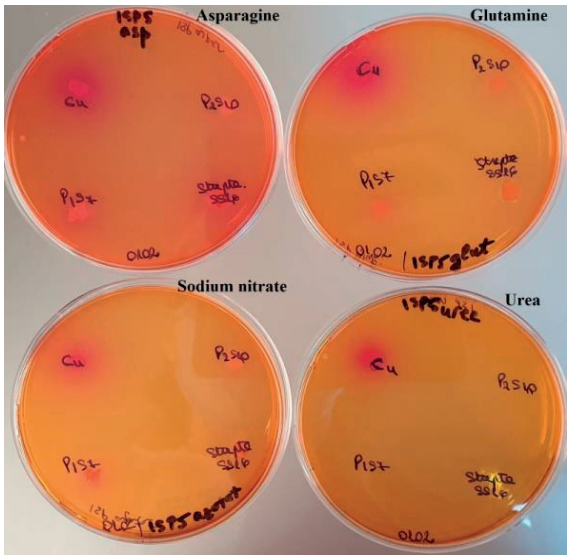

Figure 2. Streptomycete strains on ISP5 media supplemented with phenol red as pH indicator dye (after 48 h incubation at 28°C)

Best results regarding L-asparaginase production were obtained with rhizobacterial and endophytic bacterial strains (Figure 3).

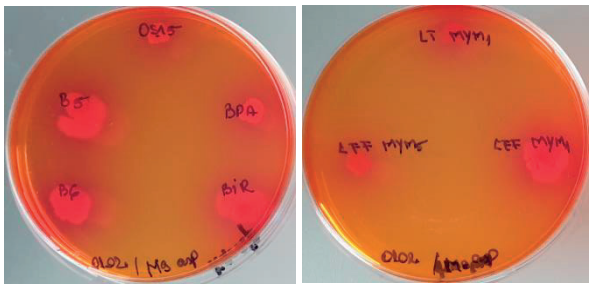

Figure 3. Rhizobacteria and endophytic bacterial strains on M9 media with L-asparagine and phenol red (after 48 h of incubation at 28°C)

Considering the results obtained from the qualitative tests, only strains revealing L-asparaginase production, free of urease and glutaminase, were selected. Some exceptions were made for a few strains that revealed a weak, pale color change after 48 h to 72 h of incubation on glutamine, urea or ammonia nitrate. As a result, in the following quantitative evaluation of the enzymatic activity, only ten strains were selected.

For enzymatic quantification of L-asparaginase, glutaminase and urease activity the selected strains were: two yeasts (*C. albicans* ATCC10231 and *C. parapsilosis* CSB604), two streptomycetes (P1S7 and Strepto SS16), four rhizobacteria (*Bacillus* spp. B5, B6, OS15, BPA), and two endophytes (LT MYM1 and LFF MYM1).

Quantitative evaluation of L-asparaginase activity

Quantitative evaluation of L-asparaginase activity after 7 day of microbial incubation on 0.5% L-asparagine containing media revealed that *Bacillus* sp. OS15 culture contained the highest amount of this enzyme (0.423 U/ml).

No significant differences were seen among BPA, LT MYM1 and B6 strains regarding L-asparaginase activity, after one week of growth. For these, the amount of enzyme was between 0.399 U/ml (in B6) and 0.408 U/ml (in BPA).

Compared to the L-asparaginase producing bacteria, tested yeasts released significant lower amounts of this enzyme. In 7 days old cultures of *C. parapsilosis* CSB604 we detected 198 U/ml, and in *C. albicans* ATCC10231 only 0.039 U/ml.

No detectable L-asparaginase was detected after one week in the culture supernatant of *Streptomyces* spp. P1S7 and Strepto SS16.

Regarding glutaminase and urease activity, the amount of these enzymes in 7 days old culture supernatant was too low to be detected.

Considering the above, dynamic estimation of L-asparaginase activity was performed only for the rhizobacteria and edophyte bacterial strains. The enzyme production was quantified after 24 h and 72 h of microbial incubation and the results were compared with the previous test performed on 7 days old cultures. Results showed that not all tested strains were able to produce L-asparaginase in the first 24 h of growth. Similar aspects were also revealed by Chergui et al., which revealed that in Actinomycete *CA01* culture the production of the enzyme started from second day of microbial incubation and increased exponentially until 6th day, at 1.73 IU/ml (Chergui et al., 2018). According to the same authors, after six day of culturing, L-asparaginase production significantly decreases. This aspect was also confirmed by our studies, as the amount of enzyme after 7 days was significantly decreased compared to the 3rd day (Table 3).

The enzymatic activity after three days of microbial growth showed that *B. subtilis* B5 produced the highest amount of L-asparaginase compared to the other tested strains (1.305 U/ml).

Table 3. L-asparaginase activity in various microbial cultures

Microbial strain	L-asparaginase activity (U/ml)		
	7 days	24 h	72 h
ATCC10231	0.039[e]	–	–
CBS604	0.198[d]	–	–
P1S7	N.D.	–	–
SS16	N.D.	–	–
B5	0.375[c]	N.D.	**1.305[a]**
B6	0.399[b]	0.042[b]	1.059[c]
OS15	**0.423[a]**	N.D.	1.053[c]
BPA	0.408[b]	0.013[c]	1.151[b]
LT MYM1	0.405[b]	0.047[b]	0.878[d]
LFF MYM1	0.381[c]	0.107[a]	0.948[d]

Legend: N.D. the enzyme activity was to low to be determined; – not tested.
Note: the values followed by the same letter are not significantly different.

Among the studied microorganisms best results were obtained with *Bacillus* spp. rhizobacteria.
In order to reveal the appropriate incubation time and other growth parameters of *B. subtilis* B5 and *Bacillus* sp. OS15 strains to optimize L-asparaginase production advanced studies should be performed.
Studies performed on *Bacillus* species producing L-asparaginase revealed they are able to produce both intracellular type I and extracellular type II bacterial L-asparaginases (Abdelrazek et al., 2019). Studies performed on a wild/natural strain of *Bacillus licheniformis* revealed a maximum L-asparaginase activity at 40°C, pH 8.6, on 40 mM L-asparagine, in the presence of 10 mM $ZnSO_4$.

CONCLUSIONS

Qualitative screening methods are highly valuable when selecting enzyme producing microorganisms. By using L-asparagine, glutamine, urea and ammonium nitrate as single nitrogen sources we were able to differentiate the microbial strains producing only L-asparaginase.
Quantitative analysis performed on ten selected strains of rhizobacteria, bacterial endophytes, streoptomycetes and yeasts revealed that *Bacilus* spp. strains are higher producers of L-asparaginase.
B. subtilis B5 revealed an L-asparaginase activity of 1.305 U/ml when grown for three days on M9 synthetic media with 0.5% L-asparagine.

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