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GENETIC APPROACHES TO SELECT L-ASPARAGINASE PRODUCING *Bacillus* STRAINS

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Abstract

An important enzyme for both the pharmaceutical and food industries is L-asparaginase (EC 3.5.1.1). This enzyme is produced by a wide variety of microorganisms. However, their potential use as sources of L-asparaginase at industrial scale is limited if glutaminase and urease are also produced. This is mainly due to the complexity and expenses of the purification process required to obtain l-asparaginase which make the production system inefficient. In order to select L-asparaginase highly producing strains, lacking detectable glutaminase and urease activity different isolation steps were established and rapid tests are the first recommended. However such studies need to be completed with quantitative analysis. Moreover, additional molecular studies can also confer useful information regarding the biotechnological potential of the selected strains. The aim of this paper is to correlate the microbiologic and biochemical tests with genetic approaches in order to improve the selection process of biotechnologically relevant Bacillus strains.

Key words*: L-asparaginase, ansA and ansZ genes, Bacillus.*

INTRODUCTION

Enzymes are proteins that have the ability to catalyse various reactions in which the substrate is transformed into reaction products. (El-Hadi et al., 2019). The L-asparaginase enzyme catalyses L-asparagine hydrolysis into aspartic acid and ammonia (Batool et al., 2016). This reaction is highly important in the medical field, as well as in the food industry.

In medicine L-asparaginase is used as antineoplastic chemotherapy drug especially for patients with acute lymphocytic leukemia (Egler et al., 2016). Although L-asparagine aminoacid is essential for the cells to synthesis important proteins to stay alive (Starkova et al., 2018), it should be mentioned that only normal cells are able to make this aminoacid, while many cancer cells cannot, due to the lack of L-asparagine synthetase (Salzer et al., 2018). Therefore, tumour cells will need this aminoacid to stay alive (Chiu et al., 2020). In the case of such cancer patients L-asparaginase treatment is administrated as chemotherapeutic drug with cytotoxic activity (Kato & Manabe, 2018). L-asparaginase plays a key role in acute lymphoblastic leukemia treatment especially, but has a potential role also in preventing metastases from solid tumours (Brumano et al., 2019). However L-asparaginase is not limited only as an antitumor agent, but it could also be used as antimicrobial agent, in the treatment of infectious diseases, autoimmune diseases, as well as in feline and canine cancer (Vimal & Kumar, 2017).

L-asparaginase used in clinical treatments is mainly obtained from *Escherichia coli* and *Erwinia chrysanthemi*, but new sources of prokaryotic, eukaryotic and even genetic mutations are still being sought to improve the function of this enzyme (Beckett & Gervais, 2019).

The use of L-asparaginase in medicine has some limitations, because it has been shown to cause some side effects to those with history of pancreatitis, thrombosis or allergies. These are mainly due to the secondary activity of glutaminase (Ramya et al., 2012).

In addition to the medical significance, this enzyme is also used extensively in the food industry to mitigate acrylamide formation, a carcinogenic compound that is formed in

carbohydrate-containing foods that are cooked at high temperatures (Muneer et al., 2020).

The importance of L-asparaginase and the applications it may have, maintained a continuing interest over time in order to detect sustainable and safe sources of enzyme production and easier extraction. Studies revealed a variety of microorganisms able to produce L-asparaginase (Ashok et al., 2019), although before, this enzyme was extracted from guinea-pig serum (Lee & Bridges, 1968). Microbial enzyme production is conditioned by kinetic parameters (El-Gendy et al., 2021). Biotechnology was able to improve industrial production of L-asparaginase using selected strains of *E. coli* and *E. chrysanthemi* bacteria. But trends are targeting the use of genetically improved strains (Onishi et al., 2011), or the use eukaryotic microorganisms due to the presupposition of a better compatibility with the human body (Cachumba et al., 2016).

In bacteria there have been seen two types of L-asparaginase: one found in the bacterial cytoplasm (AnsI) and the second (AnsII) found in the bacterial periplasm. The AnsII was shown to have higher affinity for L-asparagine, being the only one used in clinical treatments (Brumano et al., 2019). If AnsI can hydrolase both L-asparagine and L-glutamine, the AnsII is more specific to the substrate, being the one to have anti-cancer activity (Maggi & Scotti, 2019). The AnsII from *E. coli* (named EcAII) is more often used in medical treatments, probably preferred due to the biotechnological plasticity of this bacterial specie; while the AnsII from *E. chrysanthemi* (named ErAII) is used as an alternative if immune reactions occur to EcAII (Maggi et al., 2017).

The AnsI enzyme is encoded by *ansA* gene in various bacterial species, such as *Bacillus subtilis* (Hegazy et al., 2012), *B. tequilensis* (Shakambari et al., 2018), *E. coli* (Maggi et al., 2017), *Corynebacterium glutamicum* (Kalinowski et al., 2003), *Pseudomonas* spp. (Kishore et al., 2015), *Rhizobium etli* (Moreno-Enríquez et al., 2012), *Streptomyces griseus* (Meena et al., 2015), and many others. The AnsII is expresses only in certain conditions such as limited nitrogen levelsss and oxygen stress (Maggi & Scotti, 2019). The encoding genes of L-asparaginase type II could be *ansB* gene, as it was shown in *E. coli* (Jennings & Beacham, 1990), or *ansZ* gene as in *B. subtilis* (Fisher & Wray, 2002). The AnsAB operon, containing the *ansA* and *ansB* genes, can be repressed by *ansR* activity (Sun & Setlow, 1993); while *ansZ* gene expression is activated by *TnrA* or *GlnR* transcription factors. The *trnA* trigger the expression of *ansZ* gene in nitrogen-limited growth, while *GlnR* represses *ansZ* transcription at excess nitrogen exposure (Fisher & Wray, 2002).

In our study we aimed to correlate qualitative *L-asparaginase* tests with genetic approaches in order to improve the selection of AnsII producing *Bacillus* strains, lacking detectable glutaminase, usease and nitrate reductase enzymes.

MATERIALS AND METHODS

Biological material and culture media

Seven strains of rhizobacteria and three endophytes, all belonging to *Bacillus* spp. were analysed for their L-asparaginase potential (Table 1). All the strains are maintained in collection as frozen in 25% glycerol. Conventionally they are grown on Luria Bertani (LB), at 28°C.

Table 1. Microbial strains and culture media

Strains		Microbial Collection
Rhizobacteria	*Bacillus subtilis* ATCC6633	Reference strain from the American Type Culture Collection
	Bacillus subtilis B5	Faculty of Biotechnology, USAMV Bucharest
	Bacillus subtilis B6	
	Bacillus sp. BPA	
	Bacillus sp. BIR	
	Bacillus amyloliquefaciens BW	
	Bacillus sp. OS15	Research-Development Institute for Plant Protection, Bucharest
Endophytic bacteria	*Bacillus* sp. LT MYM1	Plant Biotechnology Laboratory – Faculty of Biotechnology, USAMV Bucharest
	Bacillus sp. LFF MYM1	
	Bacillus sp. LFF MYM5	

DNA extraction

The DNA isolation and purification was performed from fresh bacterial cultures

obtained overnight in LB broth. For extraction a HP PCR Template Preparation kit (Roche LifeScience) was used, starting from 1ml of culture, following the manufacturer's instructions with some modifications. To improve *Bacillus* sp. cell lysis, an additional step of mechanical disruption was added, in which samples were subjected 2 times at bead-beating, for 30 seconds in Mini-Beadbeater-8 (BioSpec). To increase DNA purity, a final RNase A (10 mg/mL) treatment was applied for 15 min at 37°C.

The DNA quantification was performed in 0.5 µl of volume sample using a SpectraMax QuickDrop spectrophotometer. To equalize the DNA concentration among samples dilutions were needed to obtain 100 ng/µl.

Amplification of L-asparaginase genes

The *ansA* and *ansZ* genes are encoding for two L-asparaginase isoenzymes, L-asparagine amidohydrolase I and II, respectively. To detect *ansA* gene the primers pair Bs_ansAf (5′ - CCC AAG GAA GTC TTT TTC CA - 3′) and Bs_ansAr (5′ - AGT GAA GAG GTG CAT GGT ATG A - 3′) that amplify a 1100bp fragment of *B. subtilis* chromosomal DNA containing *ansA* gene was used (Hegazy et al., 2012). The DNA fragment containing the *ansZ* gene was obtained by PCR amplification with the set of primers mentioned by Fisher & Wray (2002).

The PCR reaction was performed in a total volume of 25 µL containing 14 ng/µL of template DNA, 1X Mango*Taq*™ Colored Reaction Buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs mix (Bioline), 0.5 µM of upstream and downstream primers (ThermoScientific LSG), 0.5 U Mango*Taq*™ DNA Polymerase (Bioline) and 12.65 µL nuclease-free water.

The thermal cycling profile for genes amplification involved an initial denaturation at 94°C for 5 min, followed by a number of 35 cycles of 1 min denaturation at 94°C, 1 min annealing at 55°C and 2 min extension at 72°C, completed with a final extension cycle of 7 min at 72°C (Fisher & Wray, 2002). The amplification program was performed in Eppendorf 5331 Mastercycler Gradient equipment.

The PCR products were migrated at 100V for 1 h, in 1% (w/v) agarose gel and 0.5X TBE, after staining with ethidium bromide. The length of DNA fragments was compared to 1KB Ladder (ThermoScientific LSG).

Rapid screening method for L-asparaginase production

The qualitative enzyme assay for L-asparaginase production was carried out on L-asparagine monohydrate (Chinoin Budapest) substrate. The screening was performed on a 96-well plate. The bacteria were inoculated in 200 µL medium/well, in 8 replicates per trial. The medium used was M9 broth supplemented with 0.5% L-asparagine as substrate. The M9 medium contained 0.2% glucose, 0.6% Na_2HPO_4, 0.3% KH_2PO_4, 0.05% NaCl, 0.05% $MgSO4 \cdot 7H_2O$, 0.015% $CaCl_2 \cdot 2H_2O$, with a pH of 7.0±0.5 and was enriched with 0.009% phenol red as indicator dye.

Comparative screening assay of L-asparaginase, glutaminase, urease and nitrat reductase

Comparative screening for the production of other relevant enzymes was evaluated is similar matter using three different substrates: L-glutamine (CellPure®, Carl Roth), urea (Carl Roth) and sodium nitrate (Reactivul București). L-glutaminase test was performed in 8 replicates per trial for each bacterial strain; while urease and nitrate reductase were performed in 4 replicates per trial for each strain. Qualitative enzymes assays were repeated two times.

RESULTS AND DISCUSSIONS

Molecular determination of *ansA* gene

The gene encoding for L-asparaginase type I in *B. subtilis* was amplified by PCR, using the primers designed by Hegazy et al. (2012) based on the published *ansA* gene sequences from *B. subtilis* 168 strain.

In our study the primers used were able to amplify a 1100bp DNA fragment corresponding to *ansA* gene. The reference strain, *B. subtilis* ATCC6633, revealed a strong specific band of PCR amplification product, while in four of the tested strains (BIR, BPA, OS15, and LT MYM1) beside the specific DNA of *ansA* gene, various non-specific PCR

amplification products were also obtained (Figure 1).

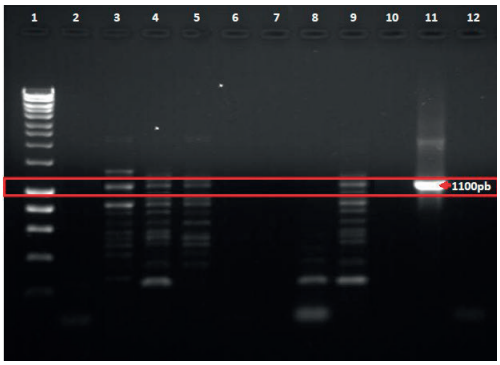

Figure 1. Agarose gel electrophoresis of the PCR products obtained by amplifying the bacterial DNA with the primers set designed for *ansA* gene

Legend: Line1 = 1kb DNA Ladder, Line 2 = BW, Line 3 = BIR, Line 4 = BPA, Line 5 = OS15, Line 6 = B5, Line 7 = B6, Line 8 = LT MYM1, Line 9 = LFF MYM1, Line 10 = LFF MYM5, Line 11 = ATCC6633 reference strain, Line 12 = Negative Control.

The PCR reaction for *ansA* gene performed for *B. amyloliquefaciens* BW, *B.subtilis* B5 and B6 rhizobacteria, as well as *Bacillus* spp. LT MYM1 and LFF MYM5 endophyte bacterial strains did not reveal the presence of targeted DNA fragment. These can be explained as lack of AnsI enzyme production.

Molecular determination of *ansZ* gene

The gene encoding for AnsII enzyme in *B. subtilis* was amplified by PCR. According to Ameen et al (2020), the gene in harbouring a 1128bp DNA fragment, which can be accessed in NCBI database as MN566442 number.

Our results revealed similar amplification products in 7 strains (BW, BIR, BPA, B6, LT MYM1, LFF MYM1 and LFF MYM5) among the ten studied bacteria (Figure 2).

Figure 2. Detail of the agarose gel electrophoresis for DNA fragment of *ansZ* gene amplified by PCR.

Legend: Line1 = 1kb DNA Ladder, Line 2 = BW, Line 3 = BIR, Line 4 = BPA, Line 5 = OS15, Line 6 = B5, Line 7 = B6, Line 8 = LT MYM1, Line 9 = LFF MYM1, Line 10 = LFF MYM5, Line 11 = ATCC6633 reference strain, Line 12 = Negative Control.

Taking into consideration that L-asparaginase I type II enzyme is the only one used in medicine due to its affinity for the substrate (Brumano et al., 2019), we can consider that the *Bacillus* strains revealing *ansZ* gene and lacking *ansA* could be highly valuable for biotechnological processes.

Qualitative determination of L-asparaginase production and other relevant enzymes

The qualitative enzyme assays were able to differentiate the bacterial strains able to produce L-asparaginase, without showing glutamine, urea or sodium nitrate notable digestion.

Enzymes production was observed following the rapid colour change test of the growth medium. According to Shakambari et al. (2019), the phenol red indicator dye is showing the positive reaction due to the bright red colour change of the media.

In our study positive reactions were seen only in L-asparagine containing medium after 3 days of incubation at 28°C (Figure 3a). No activity of glutaminase, urease and nitrate reductase enzymes were visual noticed in the colour dependent reactions (Figure 3b, c).

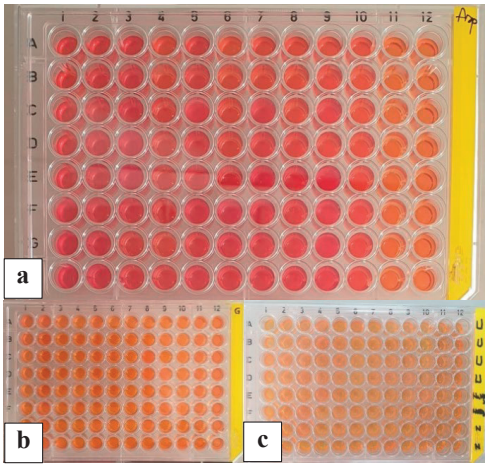

Figure 3. Microplate qualitative assay of enzymes production in various *Bacillus* spp. strains:
a. L-asparaginase; b. glutaminase; c. urease (A, B, C, D rows) and nitrate reductase (E, F, G, H rows) production

Legend: Line1 = BW, Line 2 = BIR, Line 3 = BPA, Line 4 = OS15, Line 5 = B5, Line 6 = B6, Line 7 = LT MYM1, Line 8 = LFF MYM1, Line 9 = LFF MYM5, Line 10 = ATCC6633 reference strain, Line 12 and 13 = Negative Control.

Although *B. subtilis* B5 and B6 strains did not revealed *ansA* and *ansZ* genes they were able to synthesize L-asparaginase (Figure 3).
Two explanations for these results could be identified: either the results are due to the presence of other L-asparaginase encoding genes that have not been subjected to or study, or the affinity between the primers pairs used and the bacterial strains tested (two rhizobacterial strains) is lacking.

CONCLUSIONS

Molecular analysis of *ansA* and *ansZ* genes can provide useful information regarding the biosynthesis of valuable biotechnological products by the selected *Bacillus* strains.
Based on the production of L-asparaginase type II encoded by the *ansZ* gene, which has high affinity for substrate, possible applications in medicine could be developed.
Qualitative enzyme micro-assays were able to confirm the PCR results and differentiate L-asparaginase producing strains lacking detectable glutaminase and urease activity.

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