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SCREENING OF HALOTOLERANT BACTERIA PRODUCING HYDROLYTIC ENZYMES WITH BIOTECHNOLOGICAL APPLICATIONS

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Abstract

Hydrolytic enzymes produced by halotolerant/halophilic microorganisms display polyextremophilic properties, which determines particular industrial interest. The goal of this work was to detect at least one halotolerant bacterial strain for the ability to produce multiple enzymatic activities (amylases, proteases, lipases, cellulases) with biotechnological importance. Eighteen halotolerant bacterial strains, isolated from hypersaline water located in Lopătari, Romania, have been used in this study. Hydrolytic enzymes production was assayed by plate screening on basal media supplemented with following sources: 1% soluble starch (for amylase activity); 1% carboxymethylcellulose (for cellulase activity); skimmed milk (for protease activity); 1% Tween 80 and $CaCl_2$ (for lipase activity). Positive reactions have been shown by the presence of a clear halo or precipitates around the bacterial isolates. All strains had at least one enzymatic activity. The lipases and amylases activities were the predominant hydrolytic activities detected. Our results revealed the ability of different halotolerant bacterial strains to present a combination of two or three hydrolytic enzymes: 10 isolates have produced amylases and proteases enzymes; 2 isolates - lipases and proteases enzymes; 1 isolate - amylases, proteases and cellulases enzymes and 3 isolates - proteases, lipases and cellulases enzymes, respectively. Potential enzymes production by isolate 11.5 also was evaluated in submerged cultivation on different substrates. Among the substrates tested, the production of hydrolytic enzymes by isolate 11.5 was enhanced when grounded cereals mixture 1% was used as sole substrate as carbon and nitrogen source: 312 U/ml (amylases), 0.22 U/ml (cellulases) and 0.09 U/ml (proteases).

Key words: *halotolerant bacterial isolates, hydrolytic enzymes, plates screening.*

INTRODUCTION

Microbial communities of hypersaline environments represent valuable sources with industrial applications such as food/feed, chemical, pharmaceutical, detergents, environmental, bioremediation and biosynthetic processes, being one of the main reasons for studying them (Oreon, 2010; Kamekura and Enache, 2010; Patel and Saraf, 2015; Yin et al., 2015; Flores-Gallegos et al., 2019; Liu et al., 2019). Ventosa et al. (1998) classified halophiles into slight halophiles growing optimally at 1-5% sodium chloride (NaCl); moderate halophiles growing optimally at 5-20% NaCl; and extreme halophiles growing optimally at 20-30% NaCl. Halophiles (especially bacteria and Archaea) have been isolated from few Romanian saline areas: Lake Telega (Enache et al., 2007), Salina Unirea (Cojoc et al., 2009), Balta Alba (Neagu et al., 2014) Techirghiol (Enache et al., 2009) and Lopătari (Proca et al., 2017). In a recent study, Proca et al. (2017) reported that of a total of 44 bacterial strains isolated from a hypersaline water in Lopătari, România, 4.5% demonstrated a salt large tolerance ranging from 0% to 12.5%; 7% ranging from 2.5% to 30%; 41% ranging from 5 to 25%, respectively.

In relatively recent years, an interesting research topic was considered the selection of the best halophiles microorganisms producing a wide range of extracellular hydrolytic enzymes (Sánchez-Porro et al., 2003; Cojoc et al., 2009; De Lourdes Moreno et al., 2013; Suganthi et al., 2013; Babavalian et al., 2014; Enache et al. 2014; Neagu et al., 2014; Karray et al., 2018). These enzymes are good candidates for the use in industrial processes, because they are active under extreme conditions such as high salt concentration, low water activity (aw), at extreme pH and temperature (Kamekura and

Enache, 2010; Oreon, 2010; De Lourdes Moreno et al., 2013; Patel and Saraf, 2015; Flores-Gallegos et al., 2019).

Bacterial strains isolated from different saline environments have been investigated as hydrolytic enzymes producers, such as proteases (Sánchez-Porro et al., 2003; De Lourdes Moreno et al., 2013; Suganthi et al., 2013; Babavalian et al., 2014; Karray et al., 2018), amylases (Amoozegar et al., 2003, Enache et al., 2009; De Lourdes Moreno et al., 2013; Babavalian et al., 2014; Karray et al., 2018), cellulases (Babavalian et al., 2014; Karray et al., 2018), lipases (De Lourdes Moreno et al., 2013; Babavalian et al., 2014; Moreno et al., 2016; Karray et al., 2018) or xylanases (Babavalian et al., 2014; Karray et al., 2018).

There are few Romanian researches reporting the screening of the extremely halophilic microorganisms producing hydrolytic enzymes (Enache et al., 2009; Cojoc et al., 2009; Enache et al. 2014; Neagu et al., 2014). Enache et al. (2014) reported the ability of halophilic bacteria isolated from salted lakes and salt crystals from the salt mine, to produce a wide range of extracellular hydrolytic enzymes able to degrade several macromolecular substrates, such as sugar based polymers or proteins. In another study, Neagu et al. (2014) reported the capacity of halophilic bacterial strains isolated from Balta Albă salty lake to synthesize two or three different enzymes.

The aim of this work was to select the halotolerant bacteria isolated from a hypersaline water in Lopătari, România as producers of hydrolytic enzymes (amylases, cellulases, lipases and proteases). After the initial screening, a halotolerant strain exhibiting relevant and divers hydrolitic activities (isolate 11.5) has been selected for in depth characterisation of its enzymatic activity.

MATERIALS AND METHODS

Microorganisms and growth conditions

Eighteen bacterial isolates originating from a hypersaline water in Lopătari, România (Proca et al. 2017) have been used in this study. The bacterial strains were grown and maintained on Nutrient Agar (Merck, Germany) containing 5% NaCl.

Plate screening of enzymes producing bacteria

All halotolerant bacterial isolates were streaked in line on the surface of culture media distributed in Petri dish.

Amylase activity was evaluated on nutrient agar medium with 5% NaCl supplemented with 1% of soluble starch. After incubation at 37°C for 2 days, the cultures were flooded with 0.3% I_2-0.6% KI solution. Positive reaction was indicated by a clear zone surrounding a halotolerant bacterial isolate.

Cellulase activity was tested on nutrient agar medium with 5% NaCl supplemented with 1% carboxymethylcellulose (CMC). After incubation at 37°C for 2 days, the hydrolysis zone was visualized after staining with 0.1% Congo red solution and washed the plate with 1 M NaCl.

Protease activity was detected on milk agar medium containing skim milk (0.1% fat): water (v/v) (1:2) and 2% agar. After incubation at 37°C for 2 days, the halotolerant bacterial isolates showing clear zone of the degradation of casein were read as positive for protease production.

Lipase activity was determined on nutrient agar medium containing 1% Tween 80 as a lipid substrate and 0.01% $CaCl_2xH_2O$. Positive reactions were directly observed by a precipitation zone around the inoculated halotolerant isolates.

Production of hydrolytic enzymes by halotolerant isolate 11.5 in submerged cultivation

The bacterial isolate 11.5 was cultured in nutrient broth medium with 5% NaCl supplemented with soluble starch (0.5% and 1%), carboxymethylcellulose (0.5% and 1%) and respectively skim milk (diluted 1: 2) as substrates. Also, we used grounded cereals mixture 1% (maize, wheat, sunflower, soybean, in unknown quantities) as sole substrate for carbon and nitrogen source. Fermentation carried out in orbital incubator shaker operating at 120 rpm, at 37°C for 72 hours. At every 24 h, the cultures were harvested by centrifugation at 4000 g for 10 min at 4°C. The cell-free culture supernatant was used as preparation of crude enzyme to measure the activities of amylase, cellulase, and protease.

Enzymatic activity

Amylase activity. 0.5 ml sample was added with 0.5 ml of 0.2 M phosphate buffer and 1 ml of 1% (w/v) starch disolved in 0.2 M phosphate buffer and 20% (w/v) NaCl. The reaction mixture was incubated for 10 min at 30°C. The reaction was stopped by 2 ml of 3,5-dinitrosalicylic acid and the mixture was heated at 100°C for 5 min in boiling water bath. 8 ml of water was added and the absorbance was determined at 546 nm. One unit of enzyme activity (U) was defined as the amount of enzyme liberating 1 μg of maltose per minute under the assay conditions. The amount of enzyme was determined by dinitrosalicylic acid (DNS) method of reducing sugar described by Miller (1959) and maltose was used as a standard.

Cellulase activity. The reaction mixture consisting of 2 ml of a 1% (w/v) CMC in 50 mM citrate buffer pH 6.4 as a substrate and 0.2 ml of crude enzyme solution was incubated for 10 min at 50°C. The reaction was stopped by 3 ml of 3,5-dinitrosalicylic acid and the mixture was heated at 100°C for 15 min in boiling water bath. One unit of enzyme activity was defined as the amount of enzyme capable of releasing 1 mg of reducing sugar per minute under the assay conditions, according to the standard method (Miller, 1959), and glucose was used as a standard.

Protease activity. Protease activity was determined using casein as a substrate. The reaction mixture containing 5 ml of 0.65% (w/v) casein solution, prepared in 50 mM potassium phosphate buffer was mixed with 1 ml of protein as crude cell-free enzyme source and incubated at 37°C for 10 minutes. The reaction was stopped by adding 5 ml of 110 mM trichloroacetic acid (TCA), followed by and incubation at 37°C for 10 minutes. After filtration using a 0.45 µm polyethersulfone syringe filter, 2 ml samples filtrate were mixed with 5 ml of sodium carbonate and 1 ml of Folin's reagent and incubated at 37ºC for 30 minutes. The absorbance was determined at 660 nm and tyrosine was used as a standard. One unit of protease activity (U) was defined the amount in micromoles of tyrosine equivalents released from casein per minute under the assay conditions (Folin and Ciocalteu, 1927; Anson, 1938).

RESULTS AND DISCUSSIONS

Screening of halotolerant bacterial isolates for extracellular enzymes

On-plate screening has been used to evaluate eighteen halotolerant bacteria for their ability to produce extracellular enzymes.

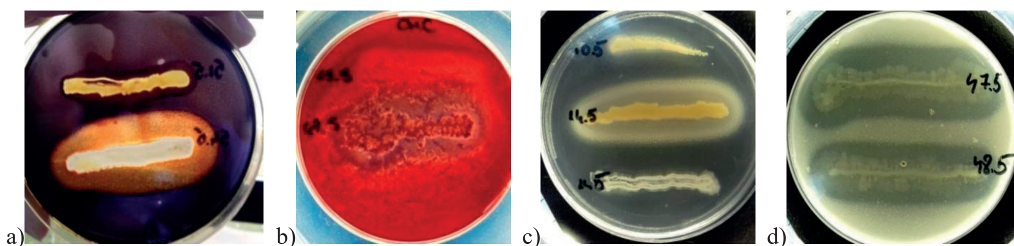

Figure 1. Plate screening aspects of enzyme assays for halotolerant bacterial isolates: amylase (a), cellulase (b), lipases (c), proteases (d)

Aspects of the of positive results for the hydrolytic enzymes screening have been visualised by the ability to produce halo or precipitate zones around the microorganisms, as seen in Figure 1; as procedure, the diameters of clear zones were measured in millimeters (mm). Lipase and amylase were the most abundant activities shown by 16 and 12 strains, respectively (Table 1). The minimum amylase activity was recorded on strain coded 51.5 and lipase activity on strain coded 57.5. Six strains were able to produce proteases. The isolates 11.5, 47.5, 48.5 presented the highest clear zone formed around the inoculation wells on milk agar plates and followed by the isolates 46.5 and 63.5, and the 46.5 showed the lowest. Cellulase activity was detected only in the case of four strains coded 11.5, 47.5, 48.5, and, respectively 63.5 (Table 1). In another study, Enache et al. (2014) reported the ability of halophilic bacteria

isolated from salted lakes and salt crystals from the salt mine, to produce a wide range of extracellular hydrolytic enzymes able to degrade several macromolecular substrates, such as starch, casein, Tween 80, and carboxymethylcellulose.

Our results revealed the ability of several halotolerant bacterial strains to produce a combination of two or three hydrolytic enzymes: 10 isolates have produced amylases and lipases enzymes; 2 isolates - lipases and proteases enzymes; 1 isolate - amylases, proteases and cellulases enzymes; 3 isolates - cellulases, lipases and proteases enzymes, respectively (Table 1).

Table 1. Plate screening of enzymatic activity from the halotolerant bacterial isolates

No.	Isolate code	Amylases	Cellulases	Lipases	Proteases
1.	10.5	+		+	-
2.	11.5	+++	+++	-	+++
3.	13.5	++		+++	-
4.	14.5	++		++	-
5.	45.5	-	-	++	++
6.	46.5	-	-	+	+
7.	47.5	-	+++	++	+++
8.	48.5	-	+++	++	+++
9.	50.5	++	-	+++	-
10.	51.5	+	-	-	-
11.	54.5	+++	-	+++	-
12.	55.5	++	-	++	-
13.	57.5	-	-	+	-
14.	58.5	+++	-	++	-
15.	59.5	++	-	+++	-
16.	60.5	++	-	++	-
17.	62.5	+	-	++	-
18.	63.5	-	+++	++	++

+: 0.1-0.9 mm; ++1.0-1.9 mm;: +++ : > 2 mm; −: no enzymatic activity

Neagu et al. (2014) also reported the capacity of halophilic bacterial strain isolated from Balta Albă salty lake to synthesize two or three enzymes: one halophilic bacterial strain combined cellulase, esterase and gelatinase activities and other seven strains combined two hydrolytic activities, either esterase and gelatinase or cellulase and gelatinase.

Karray et al. (2016) isolated halophilic strains from Chott El Jerid, a hypersaline lake in the south of Tunisia and identified as members of the genera: *Salicola, Bacillus, Halorubrum, Natrinema* and *Haloterrigena*. Most of these isolates were able to produce hydrolytic enzymes such as amylase, protease, lipase, cellulase, xylanase, pectinase and some of them showed combined activities.

In our study, the isolate 11.5 registered high enzymatic activity in the case of three hydrolytic enzymes, respectively amylases, cellulases, proteases. Data not published (personal data) indicate that the isolate 11.5 exerted also a broad-spectrum antimicrobial activity. Due to these facts, the isolate was kept for further investigations as a halotolerant bacteria of biotechnological potential.

Preliminary studies on extracellular hydrolytic enzymes of halotolerant isolate 11.5

Initially, the hydrolytic enzymes production of the isolate 11.5 was evaluated by submerged cultivation during 72 hours using different substrates: 1% starch, CMC 1% and skim milk: water (1:2). The enzymatic activity was determined at different time intervals: 24, 48 and 72 hours.

The amylase and cellulase activities increased 10-fold (Figure 2a and b) and 2-fold for the protease activity (Figure 2c) after 48 h.

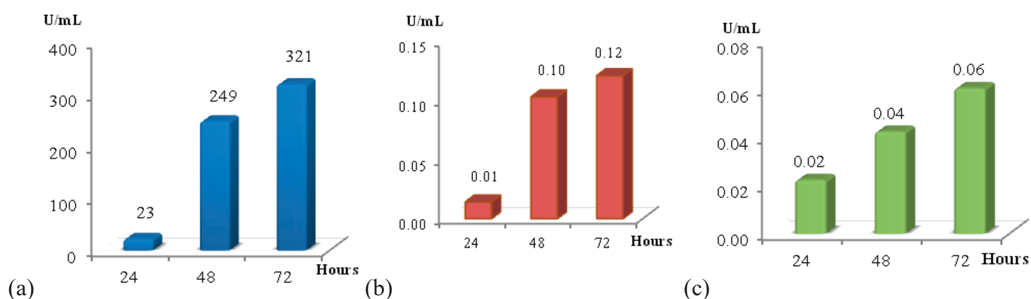

Figure 2. Enzymes production of 11.5 isolate on different substrates during 72 hours: amylases (a), cellulases (b), proteases (c)

Also, the 11.5 potential for enzymes production was evaluated in submerged cultivation on different substrates and in different concentrations (Figure 3).
The amylase activity increased 2.39-fold after increasing the starch concentration from 0.5% to 1%. A similar result of the amylase activity was also obtained when were used grounded cereals mixture as sole carbon and nitrogen source under submerged cultivation (312 U/ml) (Figure 3a).

The cellulase activity of isolate 11.5 increased 1.33-fold when increasing the concentration of CMC from 0.5% to 1%. A similar result of the cellulase activity was also obtained when grounded cereals mixture was used as sole carbon and nitrogen source under submerged cultivation (0.22 U/ml) (Figure 3b).
Regarding the protease activity, it increased 1.29-fold under submerged cultivation on grounded cereals mixture comparing to skim milk medium (Figure 3c).

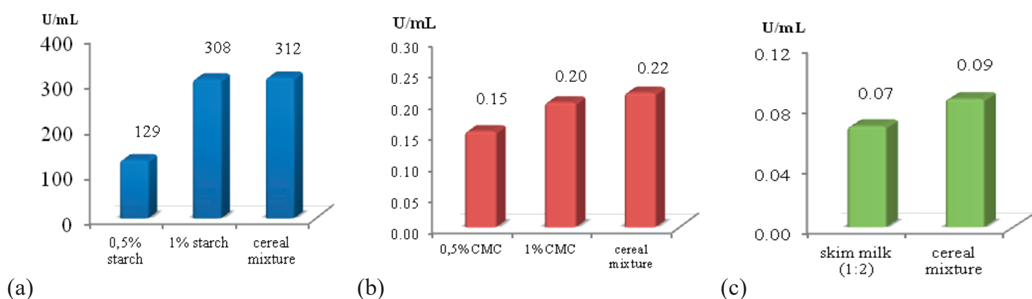

Figure 3. Effect of different concentrations of substrates on enzymes production of the isolate 11.5: amylase (a), cellulase (b), protease (c)

CONCLUSIONS

The results of the present study show that the halotolerant bacterial strains isolated from a hypersaline water in Lopătari, România could be a potential source of hydrolytic enzymes production. From the total halotolerant bacterial isolates (eighteen) 66.7% produce two enzymes types, 22.2% produce a combination of three different hydrolytic enzymes and only 11.1% produced one hydrolytic enzyme.
The present study showed that the production of hydrolytic enzymes by the isolate 11.5 was enhanced when grounded cereals mixture was used as sole carbon and nitrogen source under submerged cultivation reaching the following values: 312 U/ml (amylases), 0.22 U/ml (cellulases) and 0.09 U/ml (proteases).
As a consequence, the eventually minimization of the use of nutrients, and chemicals sources could reduce considerable the production costs on an eventual industrial scale-up.

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