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RESEARCH ARTICLE

SCREENING FOR THE PRODUCTION OF MULTIPLES ENZYMES HYDROLYTIC BY BACTERIA FROM POLLUTED URBAN STREAM IN RECIFE/PERNAMBUCO, BRAZIL

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ABSTRACT

The bacteria constitute a group of highly diverse microorganisms that play important roles in the environment, having a versatility in the production from multiple molecules, among them the enzymes that have wide use in biotechnology. Hydrolytic enzymes are widely used in different processes in industries as pharmaceuticals, textiles, food and biofuels, such as proteases, pectinases, lipases, asparaginases, cellulases, among others. The microorganisms isolated from impacted environments appear as potential sources for new molecules of employment in the industry due to the metabolic versatility and stability in extreme environmental conditions. This study investigated the hydrolytic enzyme production capacity and biotechnological potential of 25 isolates belonging to seven bacterial genera (*Bacillus*, *Enterobacter*, *Escherichia*, *Exiguobacterium*, *Klebsiella*, *Pseudomonas* and *Proteus*) collected from polluted urban stream in Recife/Pernambuco, Brazil. The production of L-asparaginase, protease, lipase, cellulase, amylaseand xylanasewas verified in 72%, 44%, 32%, 24%, 20% and 12% of bacteria, respectively. Bacteria have demonstrated a high hydrolases production capacity and can be applied at crucial stages in the bioprocess of biofuels, pharmaceuticals and food, associated with the intrinsic characteristics that can be used in recombinant DNA technology.

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INTRODUCTION

Enzymes are biological catalysts with great specificity; they participate in many biochemical reactions and their main function is metabolic control of the reactions. Enzymes have been used by humans since ancient times and currently offer diverse applications for the biotechnology industry. Enzymes afford several advantages compared with chemical catalysts, as they are relatively simple, easy to control, energetically efficient, require low-cost investment, and reduce environmental and toxicological problems (Albuquerque *et al*., 2014; Joshi *et al*., 2016; Dos Santos *et al*., 2018). Nearly 3.000 enzymes are identified in the market and recognized by the International Enzyme Commission. However, only approximately 60 enzymes have industrial applications and are used in considerable quantities, being, 75% of these are hydrolases (Coelho *et al*., 2008). Hydrolytic enzymes, are the most used enzymes in industrial process, catalyzing the breakdown of large-size polymers into smaller units. Hydrolysis activity involves water in the cleavage of covalent bonds within the substrate.

The most important hydrolases, amylases, cellulases, L-asparaginases, lipases, proteases, and xylanases, are widely used in diverse industries, such as textile, food, hygiene and cleaning products, paper, and leather, as well as for treating diseases (Kaushik *et al*., 2014, De Veras *et al*., 2018). Proteases are the most studied group of hydrolytic enzymes. These enzymes catalyze the cleavage of peptide bonds and are of great importance to some industries; for instance, biocatalyzers, which reduce the use of chemical products, such as solvents and caustic substances, decrease the environmental toxicity of detergents, as well as water and energy consumption (Khusro, 2016). L-asparagine amid hydrolases (L-asparaginases) catalyze the hydrolysis of L-asparagine into aspartic acid and L-ammonia. These enzymes are generally utilized in the healthcare field, specifically in the treatment of diseases; they have been established as the basis for treating acute lymphoblastic leukemia (Shrivastava *et al*., 2012). Amylases are the enzymes capable of hydrolyzing the starch at different points in its linear or branched structure being used in industry processes. The amylases, 1,4-D-glycanhydrolase, are enzymes that break the starch or glycogen into oligosaccharides of different sizes. Members of the family of

amylase are classified according to the type of hydrolysis of the molecules of starch. Alpha-amylase (EC 3.2.1.1), Beta-amylase (E.C 3.2.1.2), and Glycoamylase (E.C.3.2.1.3), have been widely used in detergents, food, paper and industrial textile (Nwagu, 2011; Selvam *et al*., 2016). Cellulases hydrolyze the β-1,4 glycosidic bonds in the cellulose chain, the main structural compound of plants, which provides osmotic protection and mechanical resistance to plant cells. This group is comprised of three types of enzymes: endoglucanases, exoglucanases, and beta-glucosidases, which possess considerable hydrolytic capacity with lignocellulosic materials (Idris *et al*., 2017, Dos Santos *et al*., 2018). The endo β 1,4 xylanases are extracellular enzymes, mainly produced by bacteria and fungi, having as hydrolysis the β 1,4-bonds in the xylan, being the β-xylopyranose residues, bound by β 1,4, with varying degrees of substitution in its side chains. The products of the hydrolysis of xylanases are constituted by the monomers D-xylose and xylo-oligosaccharides of different sizes, with outstanding industrial work and the sector of paper processing (Arantes and Saddler, 2010, De Veras *et al*., 2018). Lipases belong to a class of hydrolases that catalyze the hydrolysis of triglyceride glycerol and free fatty acids via an oil/water interface. In addition, these enzymes catalyze the hydrolysis and transesterification of other esters and are widely applied in biotechnological and industrial processes (Colla *et al*. 2010, Rios *et al*., 2018). This study evaluated the enzymatic production capacity of bacteria isolated from polluted aquatic environments with biotechnology relevance, aiming at industrial scale application.

MATERIALS AND METHODS

Isolates of Bacteria: The isolates of bacteria analyzed were obtained from the Cavouco - UFPE stream library, were isolated from the Cavouco stream, located at latitude 8 ° 2'52.05 "S and longitude 34 ° 57'10.33" W, in the state of Pernambuco (UFPE), Brazil, according to the methodology of Koneman and Winn (2006) and identified based on the *16S rRNA* gene sequence by Purificação-Junior *et al*. (2017).

Screening of L-Asparaginase: The detection of L-asparaginase-producing bacteria was performed according to the modified methodology of Mahajan *et al*. (2013). L-asparaginase activity was measured in terms of the rate of hydrolysis of L-asparagine by measuring the amount of ammonia released in the reaction, by the method of Wriston and Yellin (1973), utilizing Nesslerization, the most commonly used L-asparaginase assay. One unit of enzyme activity is defined as the amount of enzyme that catalyses the release of 1µmol of ammonia at 37 °C.

Screening of Protease: Protease production could be directly identified, through the presence of a translucent halo in the solid media, containing gelatin 1.0%. The protease activity was assayed containing 1.0 % gelatin as the substrate. One unit of protease activity was defined as that amount of enzyme causing an increase in absorbance of 0.01 at 280 nm of the TCA-soluble reaction products (Fry, 1994).

Screening of Lipase: Lipase screened using techniques on agar plates containing for on olive oil/rhodamine B. The halo formation around the bacteria indicates the production of lipase (Rowe *et al*., 2002). Lipase activity was determined by the spectrophotometric method with p-nitrophenyl palmitate (p-NPP) (Sigma, USA) as the substrate. One unit (U/mL) of lipase was defined as the amount of lipase that releases 1 mmol p-nitrophenol per minute at 30ºC, pH 9.0.

Screening of Cellulase: For assessing the cellulase production of bacteria, the solid media was prepared with 1.8% (v/w) agar, 1.0 % (v/w) carboxymethylcellulose, and 0.1 M sodium acetate buffer solution pH 5.0. For protease assay a solid media containing 1.8% (v/w) agar, 1.0 % (v/w) gelatin, 1.0 % (v/w) skin milk, and 0.1 M citrate phosphate buffer solution pH 5.0 was prepared. The cellulase enzyme activity was done according to the method of Ghose (1987) with carboxymethylcellulose (Sigma, USA) as the substrate. One unit of activity is defined as the amount of enzyme required to liberate 1 µmol of reducing sugars in 1 minute.

Screening of Amylase: Amylase production was verified using a solid media containing 1.8% (v/w) agar, 1.0 % (v/w) corn starch and 0.1 M citrate-phosphate buffer solution pH 5.0 (Silva *et al*., 2005). Estimation of amylase activity was carried out according to the DNSA (3, 5 dinitro salicylic acid) method. One unit of enzyme activity is defined as the amount of enzyme, which releases 1µmole of reducing sugar as glucose per minute, under the assay conditions (U/ml/min).

Screening of Xylanase: Xylanase production was evaluated on a solid media prepared with1.8% (v/w) agar, 1.0 % (v/w) birchwood xylan, and 0.1 M sodium acetate buffer solution pH 6.0 (Pradhan *et al*., 2013). L-asparaginase activity was modified M9 solid medium supplemented with 0.0054% (v/w) the phenol red and the pH was adjusted to 6.0 using 1 mol/L NaOH. Xylanase activity was determined at 50 ºC using 1 % (M/V) birchwood xylan in 50 mmol/L sodium acetate buffer (pH 5); the amount of released reducing sugar was determined. The activity is expressed in the amount of enzyme that releases 1 mol xylose-equivalent reducing sugar pers.

Protein quantification: The protein content of the sample was determined according to the method Bradford (1976), standard BSA curve was used to calculate protein concentration.

RESULTS AND DISCUSSION

This study identified inter and intra-specific variability in bacterial hydrolytic enzyme production, most likely due to biological or physicochemical factors that may have influenced both the frequency and enzymatic activity of these microorganisms (Zhang and Kim, 2010; Taprig *et al*., 2013). Among the isolates, eighteen produced L-asparaginases, eleven produced proteases, eight produced lipases, six produced cellulases, five produced amylases, and only three produced xylanases (Table 1 and Figure 1, 2). We observed variability in the enzyme production of isolates belonging to the same species (*Escherichia coli*, *Klebsiella pneumonia* and *Proteus mirabilis*), collected from the same location and from different locations in the stream (*E. coli* and *K. pneumoniae*). Additional studies using molecular typing techniques are required to clarify the dissemination of the bacterial populations in the environment under study (Table 1). Among the species analyzed examined, *K. pneumonia* produced nearly all types of enzymes. Most isolates produced four of the six enzyme types under study. The remaining species produced two to three enzyme types, while the *E. coli*, *Exiguobacterium* spp., and *P. mirabilis* isolates produced only one type of enzyme. Only one *P. mirabilis* isolate ($CP_3$5S) did not exhibit enzyme production potential (Table 1).

Table 1. Production of hydrolytic enzymes by bacteria isolated from an urban creek impacted in Recife/Pernambuco, Brazil

Isolated	Species	L-Asp(U/mL)	Pro(U/mL)	Lip(U/mL)	Cel(U/mL)	Amy(U/mL)	Xil(U/mL)
$CP_1$1S	*Bacillus cereus*	-	-	-	+ (0,056)	+ (*)	-
$CP_2$2P	*Klebsiella pneumoniae*	+ (0,5133)	+ (7,00)	+ (0,056)	+ (0,032)	-	-
$CP_2$3P	*Escherichia coli*	-	-	+ (0,059)	-	-	-
$CP_2$4P	*Escherichia coli*	+ (0,501)	+ (7,00)	-	-	-	-
$CP_3$5S	*Proteus mirabilis*	-	-	-	-	-	-
$CP_3$6S	*Pseudomonas spp.*	+ (0,613)	+ (25,00)	+ (0,055)	-	-	-
$CP_3$7S	*Escherichia coli*	+ (0,498)	-	-	+ (0,045)	-	-
$CP_3$8S	*Klebsiella pneumoniae*	+ (0,577)	+ (10,00)	+ (0,056)	-	-	+ (0,207)
$CP_3$9P	*Klebsiella pneumoniae*	-	+ (4,00)	+ (0,053)	-	-	+ (0,276)
$CP_3$10P	*Escherichia coli*	+ (0,474)	+ (6,00)	-	+ (0,030)	-	-
$CP_3$11P	*Escherichia coli*	+ (0,311)	-	-	-	+ (*)	-
$CP_3$13P	*Klebsiella pneumoniae*	+ (0,411)	+ (26,00)	+ (0,056)	+ (0,057)	-	-
$CP_4$14S	*Escherichia coli*	+ (0,626)	-	-	-	-	-
$CP_4$15S	*Enterobacter cloacae*	-	-	+ (0,054)	-	+ (*)	-
$CP_4$16P	*Klebsiella pneumoniae*	+ (0,655)	-	+ (0,056)	-	+ (*)	+(0,210)
$CP_4$18S	*Bacillus licheniformis*	-	+ (10,00)	-	+ (0,024)	-	-
$CP_4$19S	*Bacillus pumilus*	+ (1,001)	+ (21,00)	-	-	-	-
$CP_4$20P	*Exiquobacterium*spp.	-	-	-	-	+ (*)	-
$CP_5$22S	*Proteus mirabilis*	+ (0,429)	-	-	-	-	-
$CP_5$23S	*Proteus mirabilis*	+ (0,659)	-	-	-	-	-
$CP_5$25S	*Proteus mirabilis*	+ (0,910)	-	-	-	-	-
$CP_5$26P	*Proteus mirabilis*	+ (0,600)	-	-	-	-	-
$CP_5$27P	*Proteus mirabilis*	+ (0,630)	-	-	-	-	-
$CP_5$28P	*Proteus mirabilis*	+ (0,800)	+ (25,00)	-	-	-	-
$CP_5$29P	*Proteus mirabilis*	+ (0,836)	+ (23,00)	-	-	-	-

Legend: C (Cavouco); P (point of collection); number to classify the isolate; P (Depth), S (Surface). Cel = Cellulase; Pro = Proteinase; Xyl = Xylanase; Lip = Lipase; Amy = Amylase; L-Asp = L-asparaginase. * non-significant values.

Figure 1. Percentual distribution of bacteria isolates obtained from urban creek impacted according to its hydrolytic activity

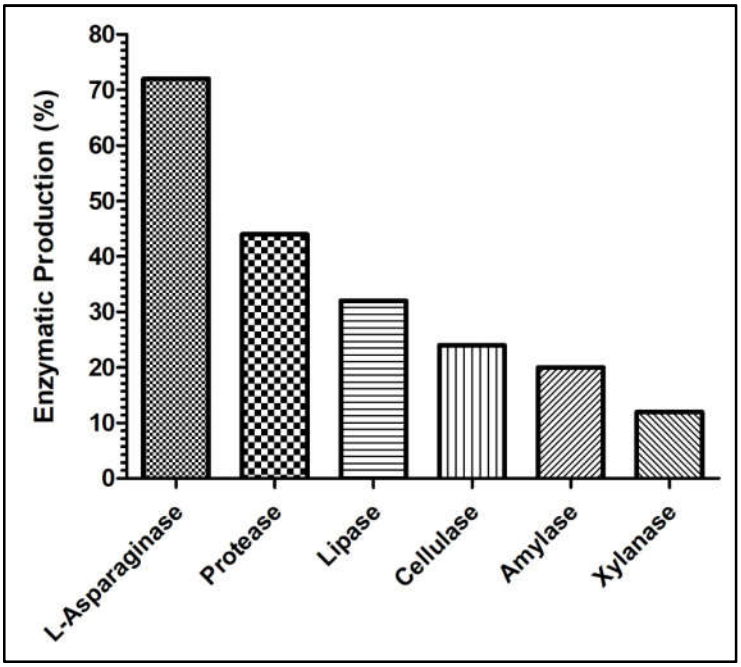
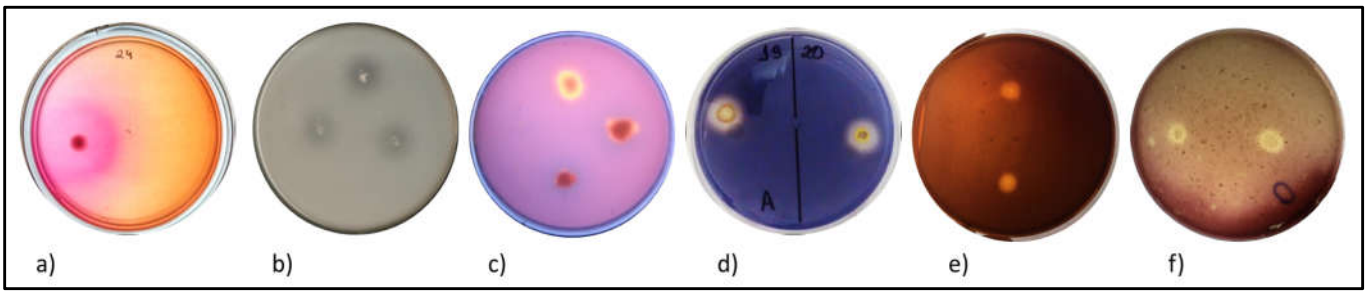


Figure 1. Percentual distribution of bacteria isolates obtained from urban creek impacted according to its hydrolytic activity

Quantitative analysis was used to evaluate only the enzyme-producing isolates. Of the samples examined, the best results were obtained for the proteolytic and L-asparaginase enzyme tests (Table 1). The highest protease production rate (26 U/mL) was observed in a *K. pneumoniae* isolate ($CP_3$13P) and this isolate was shown to be the most versatile among the investigated isolates. The lowest production rate (4 U/mL) was detected in a *K. pneumonia* isolate ($CP_3$9P). As these enzymes play an important role in organisms, they are found in a wide range of organisms from animals to microorganisms; however, microorganisms are the main source of these enzymes. Zhang and Kim (2010) affirm that proteases participate in homeostasis maintenance and inflammatory processes and are frequently used for removing devitalized tissues in wounds. Sivaprakasam *et al*. (2011) have reported the production of a protease from *Pseudomonas aeruginosa* BC1 and its application in tannery saline wastewater treatment. The diverse studies indicate that the production of proteins can occur by several species of bacteria with atypical and biochemical characteristics, however, these characteristics present an enormous biotechnological potential based on the final application. The highest L-asparaginase rate (1.001 U/mL) was exhibited by a *Bacillus pumilus* isolate ($CP_4$19S), followed by a *P. mirabilis* isolate ($CP_5$25S) with a production rate of 0.910 U/mL. The lowest rate was observed in an *E. coli* isolate ($CP_3$11P) with a production rate of 0.3117 U/mL.



Legend: Halos around bacterial colonies are indicative activity:(A)L-asparaginase, (B)protease, (C) lipase, (D) amylase, (E) cellulase and (F)xylanase.

Figure 2. Screening of hydrolytic enzymes by bacteria isolated from an urban creek impacted in Recife/Pernambuco, Brazil

The most common application of L-asparaginase enzymes is in the healthcare field, specifically in the treatment of diseases. L-asparaginase is an important enzyme for the treatment of patients with acute lymphoid leukemia (Mahajan *et al*., 2013, Lacerda *et al*., 2017). Immediately following injection into an individual presenting the disease, L-asparagine reserves, the energy source of cancerous cells, are completely converted into aspartic acid and ammonia by L-asparaginase, demonstrating its applicability in the medical field (Turki, 2013).To overcome the negative side effects of LA using the enzyme that shows toxicity and immunosuppression, new research is being carried out in the hope of finding new enzymes with new biochemical characteristics differentiated for application in antitumor therapy (Mahajan *et al*., 2013; Alqasim *et al*., 2018). Lipases are used in the treatment of industrial effluents, as well as the textile industry and cleaning products (Lima *et al*., 2005). The highest production for lipolytic enzymes was observed in an *E. coli* isolate ($CP_2$3P), with a production rate of 0.059 U/mL, followed by *K. pneumoniae* isolates $CP_2$2P, $CP_3$8S, $CP_3$13P, and $CP_4$16P (0.056 U/mL, each). The lowest rate was observed in a *K. pneumoniae* isolate ($CP_3$9P), with a production rate of 0.053 U/mL. Dong *et al*. (1999) isolated *Pseudomona s*sp. with lipotic activity in a contaminated soil sample. Musa and Tayo (2012) isolated strains of *Arthrobacter* sp., *Pseudomonas* sp., *Staphylococcus* sp., *Streptococcus* sp., *Bifidobacterium* sp., *Yersinia* sp., *Acinetobacter* sp., *Acetobacter* sp. and *Lactobacillus* sp., of different environments. Patel *et al*. (2018) isolated several bacteria from several impacted areas, obtaining several bacterial isolates producing lipases with high activity in the presence of organic solvents, and these can be used in technological bioprocesses that involve these components.

These studies demonstrate the immense production capacity of this enzymatic type by bacteria from impacted environments, as well as the results obtained in this work. According to Lima (2005) few bacteria can produce cellulases in quantities sufficient to solubilize cellulose. Cellulases are used for bleaching in the textile industry, detergent production, vegetable oil extraction, and animal food industry (Becker *et al*., 2009). Industries of biofuels that use lignocellulose, as the first raw material pre-treatment process for the release of cellulose, making it more accessible to the enzymatic action. During the processing of lignocellulose, once ionic, composed of soluble salts, are used as solvents of high efficiency in the treatment of biomass. The higher concentration of lignocellulose conversion processes in cellulose became incompatible with the enzymatic activity (Dos Santos, 2018; De Veras *et al*. 2018). The highest production rate for cellulases was detected in an *E. coli* isolate ($CP_3$13P), with a production rate of 0.057 U/mL; the lowest rate was observed in a *Bacillus licheniformis* isolate ($CP_4$18S), with a production rate of 0.024 U/mL (Table 1). Xylanase was the enzyme least produced by isolates; only three isolates produced this enzyme. Isolate $CP_3$9P was the highest producer (0.276 U/mL), while isolate $CP_3$8S was the lowest producer (0.207 U/mL). No significant variations were observed between the three isolates producing this enzyme. This was the only enzyme exclusively produced by isolates of the same bacterial species (*K. pneumoniae*). This species is ubiquitous in the environment and found in the hospital environment and it demonstrated the greatest diversity in hydrolytic enzyme production. Xylanolytic enzymes produced by microorganisms have been attracting increasing interest because of their biotechnological potential for various industrial processes such as the cellulose pulp and paper, food, solvent production, and liquid and gas fuels (Bergqvist *et al*., 1989, Vishwakarma and Banerjee, 2016). No significant amylase dosage values were observed in the quantitative tests. This result supports the use of qualitative analysis and demonstrates that the microorganisms in this aquatic environment produce few extracellular amylolytic enzymes. This may be due to the lack of this substrate, which is the basis for the production of this enzyme, in the aquatic environment under study. The use of microorganisms to produce enzymes has been widely investigated; however, only a few reports have examined the production of enzymes by microorganisms from aquatic environments (Mudrykand Podgorska, 2006). Joshi *et al*. (2016) emphasize that the biochemical and genetic diversity of these microorganisms is not yet fully known and that the examination of hydrolytic enzyme production potential has been limited.

Conclusion

The present study demonstrates that aquatic environments, although impacted by pollution, contain bacteria that produce hydrolytic enzymes with biotechnological potential, which may be used in the food and pharmaceutical industries.

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