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PROTEOLYTIC POTENTIAL OF ENZYMES PRODUCED BY CANDIDA PARAPSILOSIS AND RHODOTORULA. MUCILAGINOSA ISOLATED FROM LIQUID WHEY

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ABSTRACT

The search for yeast with proteolytic activity that can be explored in technology and product innovation was the objective of this study to isolate and identify strains present in liquid whey and evaluate the proteolytic activity of isolates. From the isolated strains were selected for molecular identification, those with proteolytic activity. Verification of proteolytic activity was performed on milk agar and visualized by a translucent halo. Four strains belonging to two yeast species were identified as protease producers. Whey is promising in microbial sources of biotechnological interest and *C. parapsilosis* (ES01) and *R. mucilaginosa* (ES04) strains were good protease producers in commercial agar-like milk agar. These results indicate the proteolytic potentiality of strains isolated from whey.

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INTRODUCTION

Whey is an industrial waste from cheese production, and it is a problem because it is a liquid that pollutes the effluents and the environment. However, whey has important nutritional qualities, and what was previously wasted is currently used for animal feed, ricotta, milk drink, dulce de leche and whey permeate for the production of yeast biomass (Barana *et al*., 2012). Therefore, an added value could be given to whey to be used as a source of microorganisms, especially yeast to be used as potential protease producers, and to provide knowledge and technologies in the generation of microbial sources to be used for purposes. Technologies in the market, in the development of new products such as enzymes. The biological catalysts of cells acting in the intracellular and extracellular spaces are classified according to their metabolic functions. Proteases synthesized by microorganism ribosomes, when released into the extracellular medium, transform proteins into peptides, which are transported into intracellular space. In cytoplasm, proteases break down these peptides into amino acids that are used for cell metabolismo (Lehninger,

2000). Proteases represent 60% of the total world market for biotechnological enzymes (Kumar and Jain, 2017), with annual revenues of US $ 3.0 billion for their manufacturers (Chen *et al*., 2016). To meet this demand, protease-producing microorganisms are isolated in various environments (Sangali and Brandelli, 2000; Lario *et al*., 2015; Souza *et al*., 2015), such as Antarctica (Lario *et al*., 2015) and the Amazon rainforest (Souza *et al*., 2019). They are also studied in foods, especially milk and dairy products (Roveda *et al*., 2010; Marques *et al*., 2014; Silva *et al*., 2018a; Silva *et al*., 2018b).
Proteases are added to pasteurized milk for cheese processing, resulting in the formation of rennet and the release of liquid whey (Alves *et al*., 2014). Whey is a source of proteins, lipids, carbohydrates and minerals, which retain approximately 55% of milk nutrients (Leite *et al*., 2012), so it favors the development of microorganisms (Guimarães *et al*., 2010). For this reason, whey is an interesting substrate for researching new protease-producing yeast strains remaining. Due to the multiplicity of industrial use, yeast proteases have technological potential that can be exploited economically, by biotechnological development in the preparation of diverse foods and reduction of pollution caused by residues of dairy industries released into the environment. Thus, the objective was to evaluate the proteolytic potential of enzymes produced by strains of yeast isolated and identified from liquid whey.

MATERIALS AND METHODS

Obtaining Whey Samples: Whey samples were obtained from a dairy factory in Teresina, Piaui, Brazil which produces 45,000 kg of cheese per month. Weekly, for two months, a sample of three liters of whey was collected in three sterile glass bottles, totaling eight samples. They were then transported to the Food Control, Microbiological Laboratory of the Food Studies, Research and Processing Center (NUEPPA) belonging to the Center for Agricultural Sciences, Federal University of Piaui in an isothermal container with recyclable ice.

Yeast cultivation and isolation: All serum samples were cultured for yeast growth as reported below. From each sample, a 1.0 mL aliquot was transferred to tubes containing Yeast Peptona Dextrose (YPD) broth supplemented with chloramphenicol (0.05 mg / mL) and incubated at 25 ° C for 48 hours at 150 rpm on a shaker table (SOLAB model SL-180 / DT). After this period, the tubes that presented microbial multiplication were selected for plaque sowing. From each culture, a 0.1mL aliquot was transferred to plates containing YPD agar and chloramphenicol (0.05 mg / mL) and spread with the sterile Drigalski handle until complete dispersion in the medium. The plates were then incubated at 25 ° C in a BOD oven for 48h. Then, colonies were isolated using YPD agar. Cultivation and isolation of yeasts were performed according to the methodology described in the Manual of Food Analysis (Brazil, 2017). All strains were picked every 20 days and preserved under freezing at –20 ° C in YPD plus 20% glycerin. The isolated strains followed the following nomenclature: E (enzyme), S (serum) and strain number.

Yeast protease production: For the submerged cultivation was used the methodology proposed by Porto (1996) with some modifications. From each isolated yeast a standard suspension of 107 cells / ml was prepared in a Neubauer chamber. Cell suspensions were inoculated into YPD broth in Erlenmeyer flasks supplemented with 10% Ultra High Temperature (UHT) skim milk incubated under shaking at 150 rpm for 48h and 25 ° C on a shaker table. After this time, the cultivation was conducted to a NOVA Instruments model NI 1801 bench top microcentrifuge, remaining for five minutes at 10,000 rpm at 4.0ºC. The supernatant was filtered on a 0.22 mm Millipore Sartorius Minisart filter. The filtrate was used to verify the presence of proteolytic activity.

Proteolytic activity of isolated yeasts: To verify the proteolytic activity of the isolated yeasts, 50.0 µL of the filtrate from each strain were transferred to a milk agar plate (meat peptone 5.0 g / L, yeast extract 3.0 g / L, skimmed UHT milk 10 1.0 g / L, 12g / L agar) containing 6.0 mm diameter wells. The plates were incubated in a BOD oven at 37 ° C for 24 hours. At the end of cultivation, the diameter of the translucent halo on the characteristic proteolysis agar was measured using a millimeter scale ruler. For qualitative measurement of strains proteolytic activity in milk agar, the Pz value (a / b) was used, which is expressed as the ratio between well diameter (a) and well diameter plus precipitation halo diameter around the area. of proteolysis (b). This activity was classified into four categories: Pz = 1,000 there was no enzymatic activity; Pz between 0.999 and 0.7000 low enzymatic activity; Pz between 0.699 to 0.400 moderate activity and Pz between 0.399 to 0.100 high enzymatic activity (RAMOS *et al*. 2015). For the positive control of the enzymatic production the commercial protease of Rhizopus sp, Sigma - Aldrich P0107-5g, from the SLB 2081V lot was used. For the negative control, a 50 µL aliquot of ultrapure water obtained from the sterile integral Milli-Q system was transferred. Proteolytic activity analyzes of the strains were performed in triplicate with five replicates.

Selection for yeast identification: Based on the protease yield evaluation, yeast strains isolated from whey that showed moderate proteolytic activity from milk agar culture were selected for species-level identification using molecular analysis.

Identification of yeast strain by molecular methods: The selected yeast strains were picked for Sabouraud broth kept overnight in a greenhouse at 35ºC to obtain cell mass. Genomic DNA from the yeast was extracted using the Gentra Puregene Yeast and Gram-positive Bacteria Kit (Qiagen, Germany) according to the manufacturer's instructions. DNA concentration was quantified on a Ultrospec 1100 Pro (Amersham Biosciences, England) and Gene QuantPro (Biochrom, England) spectrophotometer at µg / µL or ng / µL concentrations and their integrity was checked by 1.0 agarose gel electrophoresis. % in TBE buffer (0.1 M Tris-HCl pH 8.4; 0.09 M boric acid; 0.001 M EDTA) for approximately one hour under a 90 V voltage current. Then the gel was submerged. in ethidium bromide solution at 0.5 µg / mL for 30 min. The bands were visualized under ultraviolet light in a transilluminator (Abi-Chacra *et al*., 2013).

ITS1-ITS4 gene amplification and sequencing: The polymerase chain reaction (PCR) technique was performed using a total volume of 50 µL containing 100 ng of strain DNA, PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM MgCl2, Invitrogen, Brazil), 0.2 mM dATP, dCTP, dGTP and dTTP (each), 10 pmol of ITS1 primer (5'-TCCGTAGGTGAACCTGCGG-3 '), 10 pmol of primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3 ') (Invitrogen, Brazil) and 2.5 U of the recombinant enzyme Taq DNA polymerase (Invitrogen, Brazil).

Table 1. Bruker Daltonik MALDI Biotyper scores used to identify yeast species isolated from whey

Score	Parameter when compared to standard microorganism	Symbol	Color	Category
2.300 - 3.000	Secure species identification	(+++)	Green	A
2.000 - 2.299	Secure gender identification and likely species identification	(++)	Green	A
1.700 - 1.999	Likely gender identification	(+)	Yellow	B
0.000 - 1.699	Non-identification of microorganism	(-)	Red	C

Table 2. Isolates of yeast strains in whey samples, indicating protease producers by collection

Number of collections	Number of Yeast Isolates	Strains with proteolytic activity
1st collection	2	1
2nd collection	2	1
3rd collection	0	-
4th collection	3	1
5th collection	1	-
6th collection	0	-
7th collection	0	-
8th collection	4	1
Total Isolates	12	4

PCR was performed on the Veriti thermal cycler (Applied Biosystems, USA) according to the following program: initial denaturation, one cycle of 95 ° C for seven minutes, followed by 40 cycles of denaturation of 95 ° C for one minute, annealing of 54 ° C for two minutes. and at 72 ° C for one minute; and a final extension of 72 ° C for 10 min. Amplification products were purified using QIAquick PCR Purification Kit (Qiagen, Germany) and sequenced in both directions. Sequencing was performed on the DNA Sequencing Platform (ABI-3730; Applied Biosystems) (PDTIS / FIOCRUZ - Rio de Janeiro, Brazil). The sequences obtained were edited using the SeqMan program (DNASTAR software package, DNASTAR Inc., Madison, WI, USA). Finally, the isolate sequences were compared to the reference sequences available from GenBank using the Basic Local Alignment Search Tool (BLAST) from (U.S. National Library of Medicine, 2019).

MALDI-TOF MS Identification: Yeasts were sown on the Sabouraud Dextrose agar surface at 37ºC for 48h. Proteins were extracted with the formic acid protocol described below: a single colony from each yeast strain was transferred to a microcentrifuge tube containing 50 µl of 70% formic acid. Subsequently, the microtubes were vortexed for 20 seconds. Then 50 µl of 100% acetonitrile was added to the microtubes and vortexed again for 20 seconds. After this step the microtubes were again centrifuged for two minutes at 12,000 rpm. Subsequently, 1.0 µL of the supernatant was transferred to a reading plate (Bruker Daltonics, USA) and after evaporation, 1.0 µL of a matrix solution (α-cyano-4-hydroxycinnamic acid) was added and the step Crystallization occurred at room temperature. Protein readings were performed on Bruker Daltonik MALDI Biotyper (Bruker Daltonics, USA), Microflex Biotyper LT / SH version 3.1, database 7980 Spec (BRUKER 2011).

The results were interpreted using Bruker Daltonik MALDI Biotyper, Classification Results, with mean scores:

The corresponding standards of the Applied MSP Library: (BDAL, Filamentous Fungi Library 1.0, Mycobacteria Library 1.0) were used for the probable species surveyed: a) Candida parapsilosis (NCBI 5480): CBS 2197 CBS; CBS 2211 CBS; CBS 6318 CBS and CBS 7154 CBS; and b) Mucilaginous Rhodotorula (NCBI 5537): DSM 13621 DSM and CBS 316T CBS.

Statistical analysis: The experiment was evaluated by Kruskal Wallis nonparametric test, with pairwise comparison and Bonferroni correction, with a significance level of 5.0% using the SPSS package (IBM Corp., 2011).

RESULTS

Obtaining Whey Samples: From the eight samples of whey samples 12 yeast isolates were obtained in the YPD medium (Table 1). The occurrence of serum yeast strains was variable, there were three collections without yeast and four with more than one. This variation may be associated with improper handling of food by employees, poor hygiene of equipment and facilities, as well as longer exposure time to serum.

Identification of strains by molecular methods: After molecular identification it was found that the yeast isolates were: three from Candida parapsilosis (strains 1, 2 and 3) and one from Rhodotorula mucilaginosa (strain 4). The PCR products presented sizes of 500 bp for C. parapsilosis and 600 bp for R. mucilaginosa.

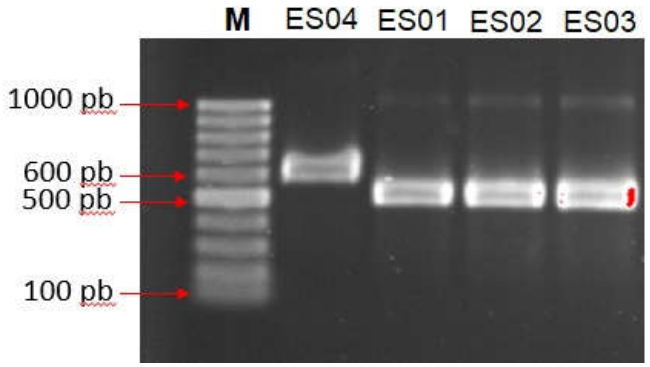

Figure 2. Band profile obtained from PCR reaction using ITS 1 and ITS 4 primers from yeast strains isolated in whey: Rhodotorula mucilaginosa ES04; Candida parapsilosis = ES01; ES02 and ES03 and M = molecular weight marker (base pairs)

Proteolytic activity: The four strains isolated presented moderate category for extracellular protease production (table 3) with varied halo size produced ($P < 0.05$). The strains ES04 and ES02 had Pz indices similar to the control performed with commercial Rhizopus sp. Protease (10mg / mL), indicating that the strains had the possibility of industrial use for protease production.

Table 3.0 Identification of strains of yeast isolated from whey by the MALD-TOF / MS method

Sample Code	Identification	Score	Symbol	Color	Category
ES01	*Candida parapsilosis*	2.016	(++)	Green	A
ES02	*Candida parapsilosis*	2.149	(++)	Green	A
ES03	*Candida parapsilosis*	2.099	(++)	Green	A
ES04	*Rhodotorula mucilaginosa*	2.012	(++)	Green	A

Table 4. Protease production, measured by enzymatic production (PZ), of Candida parapsilosis and Rhodotorula mucilaginosa strains isolated in whey

Number of Strains / Yeast Species Isolated	Enzyme Production Value (Pz)	Pz Categories
Negative control	1,000*	Negative
ES01 C. parapsilosis	0,540*	Moderate
ES02 C. parapsilosis	0,531	Moderate
ES03 C. parapsilosis	0,600*	Moderate
ES04 R. mucilaginosa	0,513	Moderate
Positive control	0,500	Moderate

* Statistical difference in relation to the positive control ($p < 0.05$).
Pz = value of enzyme production

DISCUSSION

The DNA results found in this research were close to those of Andreola *et al*. (2016) who also identified *C. parapsilosis* in the human oral cavity between 510 and 541 pbs. characterizing that these strains may present variation in the pbs sizes related to the environment in which they were isolated. These results were confirmed by the MALD-TOF / MS method when an "A" score was obtained with scores ranging from 2,000 to 2,299. In addition, these isolates can be found in environments opportunistically. Which may influence the production of different metabolites by the same species. *C. parapsilosis* has been considered as an emerging pathogen for humans due to its ability to adhere to host cells and produce biofilms and proteolytic enzymes (Trofa *et al*., 2008). In animal health this yeast is associated with clinical and subclinical mastitis of dairy cows (Costa *et al*., 2008; Dworecka-Kaszak *et al*., 2012; Sartori *et al*., 2014). In dairy farms, rapid tests are performed to evaluate if the cows have mastitis, but subclinical mastitis is not always diagnosed. If cows are symptomatic, they are treated and their milk is not used for consumption. In general, milk is supplied to dairy plants by small and medium producers, who have varying amounts of dairy cows with different zootechnical characteristics and diversified production potential. Milking is usually performed twice a day, in the morning and in the afternoon, and can be performed manually received in buckets or mechanized with milk taken to cooling plants.

After manual milking, the milk is transported to the factory in metal cans collected by isothermal trucks. In mechanical milking, milk is piped to the cooling tank where it remains at 4.0 ° C until it is piped to tank trucks. At the factory, the milk is stored refrigerated until pasteurized for use directly after filling or for the preparation of derivatives. In this way, products from a dairy factory are prepared to provide microbiological safety to the consumer. Dairy factories receive milk from several producers whose microbiological quality may vary. This was verified by the absence of yeast in three of the serum samples collected at the same company at different weeks. The efficiency of pasteurization of a food should be calculated by controlling the time and temperature of the process in order to reduce six potencies of 10 (10^6) pathogenic microorganisms in their vegetative form (Morais, 2012). Milk from mastic cows has somatic cell counts greater than 4.0 x 105 per milliliter and counts of mesophilic microorganisms in amounts that may be greater than pasteurization efficiency. The serum samples used to obtain the yeast isolates were provided by a dairy factory, which was probably derived from milk from cows with subclinical mastitis caused by *C. parapsilosis*. These cows were milked for use in the factory and their milks had higher microbial counts that favored the efficiency of heat treatment by rapid pasteurization (75 ° C / 15 seconds). Another possibility for isolation of the four strains of this yeast in liquid whey may be related to the probable thermal resistance of the isolated microorganism. The presence of *C. parapsilosis* in whey may also indicate contamination during processing due to failures in good manufacturing practice (GMP). In general, the strains of *C. parapsilosis* used in protease production testing come from human clinical cases (Yamamoto *et al*., 1992; Andreola *et al*., 2016; Shirkhania *et al*., 2016). Isolated strains of yeast in whey samples showed protease production capable of degrading milk proteins. The enzymatic production of strain ES02 had similar production performance to that obtained by commercial *Rhizopus sp.* that was used as a positive control. Although the protease production surveyed showed moderate performance according to the Pz index (Ramos *et al*., 2015), it was observed that individual productive variation of strains of this species may occur, from whey samples collected in the same establishment in days. many different. Another possibility would be the effect of cheese baking temperature interfering in the yeast enzyme production system. *Rhodotorula rubra* (Akdouche *et al*., 2018), *R. glutinis* (Spanamberg *et al*., 2008; Akdouche *et al*., 2018), *R. minuta* and *R. mucilaginosa* (Spanamberg *et al*., 2008) are considered to cause mycotic mastitis in dairy cows. *R. mucilaginosa* was another species of yeast isolated in liquid whey probably produced with mastic milk. *R. mucilaginosa* was considered by authors to have lipolytic activity (Silva *et al*., 2018 b) and elevated proteolytic activity (Neves *et al*. 2006; Lario *et al*., 2015; Yu *et al*., 2015; Chaud *et al*., 2016). The strain of this isolated yeast in liquid whey was a moderate protease producer, at Pz values similar to one of the *R. mucilaginosa* strains and the *Rhizopus sp* enzyme used as a control. Thus, strains of yeast isolated in whey have the potential to produce industrial proteases.

Conclusion

Proteases secreted by the *R. mucilaginosa* and *C. parapsilosis* yeasts that have been isolated from liquid whey have moderate

proteolytic capacity. Future studies should be performed using *R. mucilaginosa* (ES04) to optimize protease production, taking into consideration yeast growth variables, to be used in technological research for the development of food products, hydrolyzed protein supplements and reduction. of industrial waste. In addition, taking into account the problems caused by whey waste that pollutes effluents and the environment, value could be increased by using it as a carbon source for the production of *R. mucilaginosa* biomass (ES04). This strategy will allow to recover the environmental conditions altered by the pollution, adding to the agroindustrial residue (whey) and to develop new products (enzymes), besides providing knowledge and technologies in the generation of bio-inputs to be used for biotechnological purposes in the market.

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