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Production and Characterization of Collagenase by *Penicillium* sp. UCP 1286 Isolated From Caatinga Soil

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ABSTRACT

A new *Penicillium* sp. strain isolated from the soil of Caatinga, a Brazilian Biome (UCP 1286) was selected for collagenase production. Fermentation system allowing obtention of collagenolytic activity about 2.7 times higher than existing data, with the highest values of collagenolytic and specific activity (379.80 U/mL, 1460.77 U/mg, respectively), after 126 hours. Applying a factorial design, enzyme production was increased by about 65% compared to the preliminary results. The factorial design demonstrated the existence of two factors with statistical significance on the production of the enzyme: pH and temperature, both with negative effects. Enzyme was found to be more active at pH 9.0 and 37 °C, and also to be very stable in comparison with the collagenase produced by other microorganisms. The enzyme seems to belong to collagenolytic serine proteases family. Concerning the substrate specificity, it was observed that the highest enzyme activity corresponds to azocoll, there was no relevant activity on azocasein and the enzyme showed to be more specific to type V collagen and gelatin than the commercial colagenase produced by *Clostridium histolyticum*. Major band observed at electrophoresis was approximately 37 kDa. Zymogram analysis confirmed the collagenolytic activity. All data indicates this enzyme as promising biotechnology product.

1. INTRODUCTION

Collagen is the major fibrous element of skin, bones, tendons, cartilage, blood vessels and teeth found in all animals [1,2]. Collagen is found in connective tissues, making up approximately 30% of the protein in human body [3,4]. Because of the rigid structure of collagen (three helically wound polypeptide fibrils) its degradation is restricted to a few proteases [2]. Collagenases are specific enzymes that can hydrolyze both native and denatured collagens [5,6]. These enzymes can degrade native triple helix of collagen to small fragments and play an important role in connective tissue metabolism [7,8]. Collagenolytic enzymes are a kind of proteases that are related to various physiological and pathological processes and have several applications in industry, medicine and biotechnology [6,9–11]. With biotechnology accelerated growth, applications of proteases have expanded to new areas such as clinical, medicinal and analytical chemistry [12]. Among various sources of proteases, those produced by microorganisms play an important role in biotechnological processes and are used with increasing frequency, as large amounts of these enzymes can be produced quickly and at low cost [13]. Search for microbial collagenases has been increasing due to their wide application, as they are able to cleave collagen helix at multiple sites, while mammalian collagenases cleave at a single site [1,14].

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Microorganisms are preferred as source of collagenolytic enzymes due to their biochemical diversity and susceptibility to genetic manipulation [15]. Furthermore, microbial collagenases are quite versatile, possess broad substrate specificities and are able to hydrolyze both water-insoluble native collagens and water-soluble denatured collagens [16–18].

The extracellular production of fungi collagenases makes particularly easy its recovery after the end of fermentation [19]. Studies have reported the biosynthesis of collagenases by fungi belonging to the different genera, such as *Aspergillus*, *Cladosporium, Alternaria, Penicillium* [8,20–22], *Candida* [23], *Microsporum* [24] and *Rhizoctonia* [14]. Species of *Penicillium* genus have a higher biotechnological potential compared to other genera cited, both for production of proteases and other enzymes, as they have the capacity of growth in different culture conditions, using a wide variety of substrates as nutrients [25].

Caatinga is an exclusively Brazilian Biome, located in Northeast Brazil, with about one million square kilometers, mostly overlapping with semiarid region, inhabited by approximately 23 million people [26,27]. Soils are a rich environment for microorganisms and their characteristics affect directly microbiota [28]. The microbial diversity of Caatinga soils is still poorly studied, but this biome has severe climate conditions, such as high temperature, high UV exposure and long periods of drought which promote the presence of microorganisms with distinct taxonomic and functional composition in relation to other biomes [26,28].

The aim of this work was to report a new producer strain of collagenase, isolated from the soil Caatinga (Pernambuco – Brazil), and determine the best conditions for the production of the enzyme using an inexpensive culture medium. Furthermore, collagenase was characterized under the most favorable conditions and a variety of substrates was tested, to study its specificity to azocasein and different forms of collagen.

2. MATERIAL AND METHODS

2.1. Microorganism

The *Penicillium* sp. strain (UCP 1286) isolated from Serra Talhada city soil, in Caatinga biome (Pernambuco – Brazil), were obtained from UCP - the Catholic University of Pernambuco Collection, UNICAP.

2.2. Culture medium

The maintenance medium used was malt extract agar containing: malt extract (0.5%), peptone (0.1%), glucose (2%) agar 1.5 (%). The culture medium used for enzyme production is composed of: gelatin (0.5% w/v), $MgSO_4 \cdot 7H_2O$ (0.025 w/v), K_2HPO_4 (1.5 w/v), $FeSO_4 \cdot 7H_2O$ (0.015 w/v), $CaCl_2$ (0.025 w/v) and mineral solution (1% v/v), according Lima *et al*. (2011b) modified. The mineral solution was prepared by adding, per 100 mL of distilled water, 100 mg $FeSO_4 \cdot 7H_2O$, 100 mg $MnCl_2 \cdot 4H_2O$, 100 mg $ZnSO_4 \cdot H_2O$, and 100 mg $CaCl_2 \cdot H_2O$, and it was used 1% from total volume of fermentation. Both medium were sterilized in autoclave at 121 °C for 15 min.

2.3. Kinetic of growth and collagenolytic enzyme production

Inoculum spores were produced in maintenance medium plates containing a cell culture grown for 5 days at 28 °C, and then suspended in 3 mL of a 0.9% (w/v) NaCl and 0.01% (v/v) Tween 80 solution, previously sterilized at 121 °C for 20 min. After inoculation with a 150 µL spores suspension (10^6 spores/mL), fermentation was carried out for 8 days at 28 ºC and 150 rpm in 1 L Erlenmeyer flasks containing 250mL of the culture medium. At 6-hour intervals, 2 mL samples were taken for protein content and collagenolytic activity determination. Cotton caps were used to minimize water evaporation. The broth obtained at the end of fermentation was vacuum filtered through 0.45 µm-pore diameter nitrocellulose membranes to remove the mycelium.

2.4. Screening of significant variables for collagenolytic enzyme production

To evaluate the influence of the initial pH, initial concentration of gelatin, temperature and agitation on extracellular collagenolytic enzyme production by *Penicillium* sp. UCP 1286 isolated from Caatinga soil, a 2^4 factorial design was carried out at all combinations of levels given in Table 1.

Table 1: Factors levels used in 2^4 design to investigate the production of collagenolytic enzyme by *Penicillium* sp. isolated from Caatinga soil

Factors	Level		
	Lowest (– 1)	Central (0)	Highest (+1)
pH	6.0	7.0	8.0
Gelatin concentration (%, w/v)	0.25	0.50	0.75
Temperature (°C)	24	28	32
Orbital agitation speed (rpm)	100	150	200

The center point was performed in quadruplicate, to provide a variance estimate of pure experimental error responses. From this, it was predicated the effects of experimental errors and used to assess the significance of the effects and interactions of the independent variables - pH, concentration of carbon and nitrogen source (gelatin), temperature and orbital agitation speed - on the production of collagenolytic enzyme. All statistical and graphical analyzes were performed with 95 % confidence using the Statistica 8.0 software (StatSoft Inc., Tulsa, OK, USA).

2.5. Azocoll assay for collagenolytic enzyme activity determination

The Azo dye-impregnated collagen (Azocoll; Sigma Chemical Co., St Louis, MO) assay was carried out according to a modified version of the method developed by Chavira *et al*. [29]. Azocoll was washed and suspended in 0.05 M Tris-HCl buffer (pH 7.2) containing 1 mM $CaCl_2$ up to a final concentration of 0.5% (w/v). Subsequently, 150 µL of cell-free filtrate and 150 µL of buffer were mixed with 270 µL of azocoll suspension in a 2.0-mL reaction tube. The reaction tubes were incubated at 37 °C in a water bath under agitation. After 18 h of incubation, the samples were chilled in ice for 5 min to stop the reaction and centrifuged at 10,000 × *g* and 4 °C for 20 min (model KR-20000T; Kubota Seisakusho, Tokyo, Japan). The absorbance of the supernatant was

measured at 520 nm by a UV-Vis spectrophotometer (model B582; Micronal, São Paulo, Brazil). One unit of enzyme activity (U) was defined as the amount of enzyme, per millilitre, that led, after 18 h of incubation, to an absorbance increase of 0.1 at 520 nm, because of the formation of azo dye-linked soluble peptides.

2.6. Saline precipitation with ammonium sulfate

Crude extract was placed in an ice bath and $(NH_4)_2SO_4$ was added, at different concentrations of saturation (0 – 20%, 20 – 40%, 40 – 60%, 60 – 80% and 80 – 90%), with constant stirring for 2 hours. Precipitated protein was removed by centrifugation at 10.000 rpm, for 15 min at 4 °C and the supernatant was discarded. The precipitated protein was dissolved in 4 mL of 0.05M Tris-HCl buffer of pH 9.0. The 60 – 80% was selected based on its specific collagenolytic activity. Ammonium sulfate was removed using dialysis.

2.7. Protein and Biomass determination

Protein concentration was determined by the method of Bradford [30] modified using the dye as "Coomassie Blue Bright G-250" to detect protein in enzymatic samples. The calibration curve was obtained from stock solutions of bovine serum albumin (BSA).

Total volume of fermentation flasks was filtered for biomass determination. Biomass was determined by the dry weight method using pre-weighted nitrocellulose membranes with 0.45 µm-pore diameter, after drying at 80 ºC for 24 h.

2.8. Effects of pH and temperature on collagenolytic enzyme activity and stability

To evaluate pH effect on collagenolytic enzyme activity, the pH of the reaction mixture containing 0.5% (w/v) of azocoll was varied over the range 3.0 ~ 11.0. The buffers used were 0.05 M citrate (pH 3.0 ~ 6.0), 0.05 M Tris-HCl (pH 7.0 ~ 9.0), and 0.05 M carbonate-bicarbonate (pH 10.0 ~ 11.0). For stability tests, the culture filtrated was incubated at 4 ºC (selected as a cold storage reference temperature at which the enzyme thermo inactivation is expected to be very low) in the above buffers at different pH values. The incubation time of samples varied from 1 to 24 h.

An analogous study was done for the effect of temperature in the same azocoll-buffer solution. To determine optimum temperature for enzyme activity, the reaction mixture was incubated at the selected temperature (from 25 to 70 ºC), while for thermo stability tests the enzyme was pre-incubated for 15 ~ 180 min at the same temperatures.

The residual activity was calculated as the ratio between the enzymatic activity, observed at the end of each incubation run, and that at the beginning, and expressed as percentage (%).

2.9. Substrate specificity

To test substrate specificity, the proteolytic activity of extracellular collagenolytic enzyme produced by *Penicillium* sp. UCP 1286 and commercial collagenase was also assayed on insoluble collagen (type I and type V), gelatin and azocasein. Azocasein was used as comparison substrate to check on enzyme specificity.

The activity was assayed on insoluble collagen according to Endo *et al*. [31]. The standard reaction mixture, containing 25 mg collagen (type I and V, from bovine Achilles tendon) in 5 mL of 0.05 M Tris-HCl buffer (pH 7.0), was incubated with 1 mL enzyme samples at 37ºC. The amount of free amino groups released was measured by the ninhydrin method of Rosen (1975). One activity unit (U) was defined as the number of µmol of L-leucine released as a result of the action of 1 mL culture filtrate containing collagenolytic enzyme, after 18 h at 37ºC.

Collagenolytic enzyme activity on gelatin was assayed by the method of Moore and Stein [33], slightly modified. Reaction was carried out at 37ºC for 18 h after the addition of 0.1 mL of the enzyme solution to 1.0 mL of a solution containing 2 mg gelatin in 0.05 M Tris-HCl buffer (pH 7.5). The reaction was stopped by the addition of 0.1 mL of 10% (w/v) trichloroacetic acid. The medium was centrifuged at 10,000 x g for 10 min. The supernatant (0.2 mL) was mixed with 0.5 mL of ninhydrin solution, heated at 100ºC for 10 min, cooled in ice water for 5 min, and the mixture was then diluted with 2.5 mL of 50% (v/v) 1-propanol. After centrifugation at 12,000 x g for 10 min, the absorbance of the mixture was measured at 570 nm. One unit (U) of enzyme activity was expressed as µmol of L-leucine equivalents released per min.

The enzyme activity on azocasein was determined according to Leighton *et al*. (1973), with 1% (w/v) azocasein in a 0.1 M Tris-HCl buffer (pH 7.2). One unit (U) of protease activity was defined as the amount of enzyme required to raise the optical density at 440 nm by one unit after 1 h.

2.10. Effect of inhibitors

Protease inhibitors effect was investigated following the procedures of the manufacturer's guide of inhibitors. The inhibitors tested were: phenylmethylsulphonyl fluoride (PMSF) for serine proteases, ethylenediaminetetraacetic acid (EDTA) for metalloproteases, and iodoacetic acid (IAA) for cysteine proteases, at the concentration of 10 mM. For sensitivity determination, the enzyme was pre-incubated for 30 min at 37ºC with the inhibitors. The residual activity was determined as the percentage of the proteolytic activity in an inhibitor-free control sample. After all, proceeded collagenolytic activity using the azocoll method.

2.11. Polyacrylamide gel electrophoresis (SDS-PAGE)

Samples were analyzed by SDS-PAGE in a 15% polyacrylamide gel, according to Laemmli [34]. The protein molecular markers were phosphorylase b (97.0 kDa), bovine serum albumin (66.0 kDa), ovalbumin (54.0 kDa), carbonic anhydrase (30.0 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa). The gel was loaded with 20 µL of concentrated enzyme by lyophilization, and subject to electrophoresis at a constant current of 100 V. Gel was stained with 0.25% (w/v) Comassie Brilliant Blue R-250 in methanol-

acetic acid- water (45:10:45) and distained in the same solution without dye.

2.12. Zymogram

Proteolytic activity of enzyme bands was confirmed by zymogram analysis. To prepare a zymogram, concentrated enzyme was mixed under non-reducing conditions with SDS-PAGE sample buffer and electrophoresed in a 15% polyacrylamide with 0.1% (w/v) gelatin as substrate incorporated in the gel. Gel was loaded with 20 μL of concentrated supernatant, subject to electrophoresis at a constant current of 100 V at 4°C and incubated for 1 h at room temperature with 2.5% (v/v) Triton X-100 and for 18 h at 37 °C in 50 mM Tris-HCl buffer, pH 9.0. Gel was stained and distained as described in the previous section.

3. RESULTS AND DISCUSSION

3.1. Enzyme production kinetics

Table 2 reports data from earlier work related to the production of collagenolytic enzyme by different microorganisms. Among some fungi and bacteria, it can be seen that the *Penicillium* genus provides the highest values. Thus, this genus was selected for enzyme production. Moreover, there are few reports in the literature describing the production of collagenase by *Penicillium*. The values for collagenolytic activity reported on this work are about 2.7 times higher than existing data.

The composition of the culture medium is another aspect that must noticed. It is known that the costs of culture media and substrates are key factors on the production of industrial enzymes being thus necessary to develop processes that make use of inexpensive and easy to prepare substrates [8]. The use, as in the present work, of a low cost and readily available substrate, as is the case of gelatin and trace elements, may be an important step in the advancement of these processes. Figure 1 shows the results of enzyme kinetics production. At 96 hours it can be observed a sharp increase in the enzyme activity. Collagenolytic enzyme reached its maximum activity after 126 hours of fermentation (379.80 U/mL). Based on results, the incubation time used for further study was 126 hours. It can be observed a comparison between total protein in the culture broth and collagenolytic activity over fermentation time at Figure 1. The specific activity of collagenolytic enzyme was calculated as 1460.77 U/mg after 126 hours.

At Figure 1, after 126 hours it can be observed a gradual decrease in the biosynthesis of the enzyme, probably due to a period of microorganism adaptation to the medium containing only salts and gelatin or due to depletion of nutrients. The production effectiveness was evaluated by volumetric collagenolytic activity due to the industrial relevance of this parameter [8].

In addition, the enzyme concentration remained constant (a slight increase was observed) for the first 84 hours, most likely caused by the fungus need to get adapted to the poor culture medium, as compared to the other media listed in Table 4.

The maximum specific collagenolytic activity was obtained also at 126 hours of fermentation (1460.77 U/mg) (Figure 1). This value is much higher than other published results. Baehaki *et al*. [35] achieved a specific activity of 0.546 U/mg, with *Bacillus licheniformis*; Jain and Jain [1] found 15.66 U/mg of specific activity by *Streptomyces exfoliates*, Wu *et al*. [6] obtained 2.77 U/mg using *Bacillus pumilus*, Mahmoud *et al*. [36], 97.12 U/mg with *Aspergillus flavus* and Ok and Hashinaga [37], 70.4 U/mg with *Zygosaccharomyces rouxii*, all of them with more than 15 times lower activity than the produced by *Penicillium* sp. UCP 1286. The literature describes the importance of defining parameters that have a significant influence on the extracellular enzyme production by microorganisms - not only the composition of the culture medium as a carbon and nitrogen sources and trace elements should be considered but also the culture conditions such as pH, temperature and stirring speed [23].

Table 2: Description of work reported in the literature relating to the production of collagenolytic enzymes.

Microorganism	Culture Medium	Time (h)	A_c (U/mL)	References
Aspergillus flavus	Collagen-dependent-medium	144	82.95	Mahmoud *et al*. (2007)
Bacillus cereus	Glycerol and gelatin	24	23.07	Suphatharaprateep *et al*. (2011)
Bacillus licheniformis	Yeast extract and collagen	N.I.	3.10	Baehaki *et al*. (2012)
Bacillus licheniformis	Luria Broth and collagen	35	0.546*	Baehaki *et al*. (2014)
Bacillus pumilus	Gelatin and Peptone	24	35.97	Wu *et al*. (2010)
Bacillus subtilis	Yeast extract and gelatin	14	3.07	Tran and Nagano (2002)
Candida albicans	Malt extract and gelatin	72	5.00	Lima *et al*. (2009)
Klebsiella pneumoniae	Glycerol and gelatin	24	10.53	Suphatharaprateep *et al*. (2011)
Penicillium aurantiogriseum	Soybean flour	72	164.00	Lima *et al*. (2011a)
Penicillium aurantiogriseum	Soybean flour	72	231.00	Lima *et al*. (2011b)
Rhizoctonia solani	Sabouraud, glucose and collagen	108	212.33	Hamdy (2008)
Streptomyces exfoliatus	Soybean flour	120	43.50	Jain and Jain (2010)
Zygosaccharomyces rouxii	Yeast extract, peptone and glucose	50	70.4	Ok and Hashinaga (1996)
Penicillium sp.	Gelatin	126	632.70	Present work

A_c = collagenolytic activity (U/mL), *Specific activity (U/mg), N.I. = not informed.

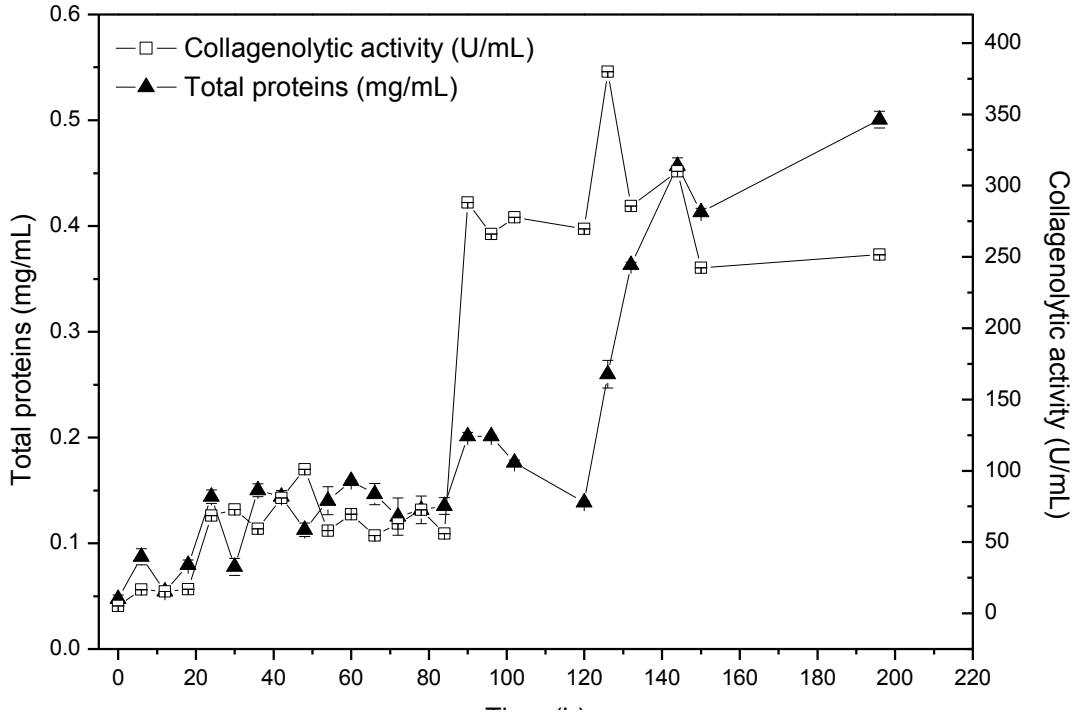

Fig 1: Collagenolytic activity and total protein content produced by *Penicillium* UCP 1286 isolated from Caatinga in gelatin culture medium.

Table 3: Conditions and results of fermentations conducted according to the 2^4 factorial design.

Run	pH	S_o (% w/v)	T (°C)	Agitation (rpm)	X (g/L)	TP (mg/mL)	A_c (U/mL)	a_c (U/mg)
1	6	0.25	24	100	0.48	0.15	531.55	3603.73
2	8	0.25	24	100	0.36	0.18	481.95	2744.20
3	6	0.75	24	100	0.80	0.25	447.65	1786.13
4	8	0.75	24	100	0.92	0.24	481.70	1996.68
5	6	0.25	32	100	0.53	0.13	560.20	4351.07
6	8	0.25	32	100	0.17	0.24	177.70	746.25
7	6	0.75	32	100	0.62	0.26	396.65	1544.14
8	8	0.75	32	100	0.72	0.19	349.50	1798.07
9	**6**	**0.25**	**24**	**200**	**0.45**	**0.16**	**632.70**	**3954.38**
10	8	0.25	24	200	0.30	0.16	471.20	2888.58
11	6	0.75	24	200	0.98	0.36	475.10	1319.72
12	8	0.75	24	200	0.40	0.36	424.15	1188.51
13	6	0.25	32	200	0.48	0.12	468.05	3920.84
14	8	0.25	32	200	0.51	0.15	424.10	2815.60
15	6	0.75	32	200	0.53	0.32	405.95	1271.08
16	8	0.75	32	200	0.38	0.20	338.65	1662.09
17	7	0.5	28	150	0.77	0.22	427.95	1923.37
18	7	0.5	28	150	0.75	0.23	424.85	1882.99
19	7	0.5	28	150	0.72	0.24	432.90	1842.13
20	7	0.5	28	150	0.74	0.23	428.80	1874.54

Results related to 126 hours of fermentation. pH = initial pH of the medium; S_o = initial concentration of gelatin; T = temperature; X = biomass concentration; TP = total protein; A_c = volumetric collagenolytic activity; a_c = specific collagenolytic activity.

3.2. 2^4 Factorial Design

Table 3 shows fermentations conditions and results after 126 hours of production. The highest values of collagenolytic activity (632.70 U/mL) and specific activity (3954.38 U/mg) were obtained in run 9, conducted using 0.25% (w/v) gelatin, 200 rpm, pH 6.0 and 24°C.

The factorial design increased the enzyme production by about 65% compared to the preliminary results achieved on the kinetics experiments, obtaining a collagenolytic activity of 632.70 U/mL. According to Jain and Jain [1], the maximum production obtained for *Streptomyces exfoliates* (43.5 U/mL) was observed after 5 days of culture at 30 °C and 150 rpm. Lima *et al*. [23] conducted a 2^3 full factorial for the production of collagenase with *Candida albicans* and found the highest value (7.6 U/mL) with a 2% substrate concentration, agitation of 160 rpm and pH 7.0. For *Penicillium aurantiogriseum*, Lima *et al*. [9] reported that the

highest values of collagenolytic activity (164 U/mL) and biomass concentration (1.8 g/L) were obtained with 0.75% substrate concentration, 200 rpm, pH 8.0 and 28°C. The results described in this paper show that the concentration of substrate (0.25%) used for the maximum production of collagenolytic enzyme (632.70 U/mL) was lower than those reported in the literature, which can represent an economy in production. With respect to the agitation, the results were similar to those related by Lima *et al*. [9] (200 rpm). Temperature found in present work was lower than others papers (24 °C), and pH was slightly higher (9.0) showing that maximum enzyme production conditions are milder than those reported.

A full factorial model was designed to compare the activity data. This model included four main effects, six two-factor, four three-factor and one four-factor interactions. The statistically significant estimates of the effects (at the 95% confidence level) are listed of Table 4. The values of the significant effects indicate that, on average, higher values of collagenolytic activities were obtained when factors 1 (pH) and 3 (temperature) were selected at their lowest levels, both with negative effects. With respect to the effect of the factors on the biomass concentration, the gelatin concentration was the only one that presented positive significant effect.

Table 4: Statistically significant main effects and interactions estimated from the collagenolytic activity and biomass concentration values listed in Table 2

Factors	Effects on collagenolytic activity	Effects on biomass concentration
(1) pH	-96.11*	-0.14
(2) Gelatin concentration	-53.51	0.25*
(3) Temperature	-103.15*	-0.09
(4) Agitation	26.62	-0.07
1 by 2	63.27	0.01
1 by 3	-39.11	0.04
1 by 4	15.19	-0.07
2 by 3	18.70	-0.12
2 by 4	-34.54	-0.12
3 by 4	11.55	0.04

*Significant

Temperature is an important factor in regulating enzymatic synthesis [38]. In a study reporting the production of collagenase by *Penicillium aurantiogriseum*, the temperature also had a negative effect [9]. Since the initial pH of the culture medium influences many enzymatic processes, enzyme production, cell transport across membranes and expression of extracellular proteases, we observed, a gradual decrease in collagenase production when the external pH was raised from 6 to 8. Suphatharaprateep *et al*. [2] reported that the optimal initial pH for collagenase production from *Klebsiella pneumonia* and *Bacillus cereus* were 7.5 and 6.0, respectively.

Regarding the effect of the different factors on biomass concentration, only the concentration of gelatin (2) proved to be statistically significant. Jain and Jain [1] showed that the maximum collagenase production (43.50 U/mL) was observed with higher biomass concentration (5.60 cm^3 of cells). The initial pH of the medium was shown by Lima *et al*. [9] to be the factor that exhibited a significantly higher effect on the biomass, with an increase in pH causing a decrease in cell growth.

With a 2^4 experimental design, it was possible to establish fermenting conditions that allowed for a 66% increase in the enzyme activity as compared to the initially obtained values. It is observed that maximum collagenolytic production occurred when lowest concentration of substrate and temperature were utilized, which facilitates industrial process, reducing costs.

3.3. Effect of pH on collagenolytic activity and stability

Figure 2(A) shows the pH dependence of collagenolytic activity in azocoll. The enzyme was found to be very active between pH 7.0 and 9.0. At pH 6.0, only 20% of the activity remained, and the results were even worse for the most acidic conditions (pH 3.0 to 5.0) and more basic (pH 10 and 11). Figure 2(B) shows the stability at pH between 3.0 and 11.0, during 24 hours of incubation at 4 °C, the selected storage temperature. The enzyme was quite stable between pH 7.0 and 9.0, keeping about 85 to 90% of stability, after 24 hours. With the pH 5.0 and 6.0, the residual activity decreased to 70%. In others values of pH, the activity was below 30%.

Concerning pH effect on collagenolytic activity, Jain and Jain [1] determined the collagenase activity from *Streptomyces exfoliatus* at different pH (4-10) and found that the enzyme retained 65% residual activity at pH 10 as compared to its optimum activity at pH 7. The collagenase from *Klebsiella pneumoniae* had an optimum pH range from 6.0 to 8.0, with maximal activity at 7.0 in Tris-HCl buffer while *Bacillus cereus* collagenase activity was recorded as the highest in the pH range of 5.4-8.2 [2]. The collagenase from *Bacillus pumilus* [6] was evaluated in a range from 3 to 10 being the highest activity at pH 7.5. Baehaki *et al.* [35] used a pH range between 2 and 12 and observed the maximum activity at pH 9.0. Lima *et al*. [9] showed that the enzyme produced by *P. aurantiogriseum* was very active at the pH range 8 to 10, and the highest activity occurred at pH 9.0, as in the present work. Ok and Hashinaga [37] related that optimum pH to collagenase produced by *Zygosaccharomyces rouxii* was 8.2. Only the enzyme produced by *Rhizoctonia solani* was produced under acid pH (5.0) [14]. The results described at present work are in agreement with those reported in literature that show that collagenases exhibit optimum activity values under neutral or alkaline conditions [23,39]. These results indicate that this collagenolytic enzyme belongs to the group of alkaline proteases.

Lima *et al*. [9] evaluated collagenase stability at pH range 3.0 to 11.0. The enzyme was stable between pH 6.0 and 10.0 during the first 8 h, but after 28 h its stability was restricted to a pH range from 7.0 (50.7%) to 9.0 (75.1%). In accordance with Wu *et al*. [6], collagenase from *B. pumilus* is stable between pH 6.5 and 8, the enzyme retained above 84% of full activity after 30 min. By retaining more than 80% of its stability at pH 8 and 9, the enzyme described in the present work proved to be very stable in comparison with the collagenase produced by other microorganisms.

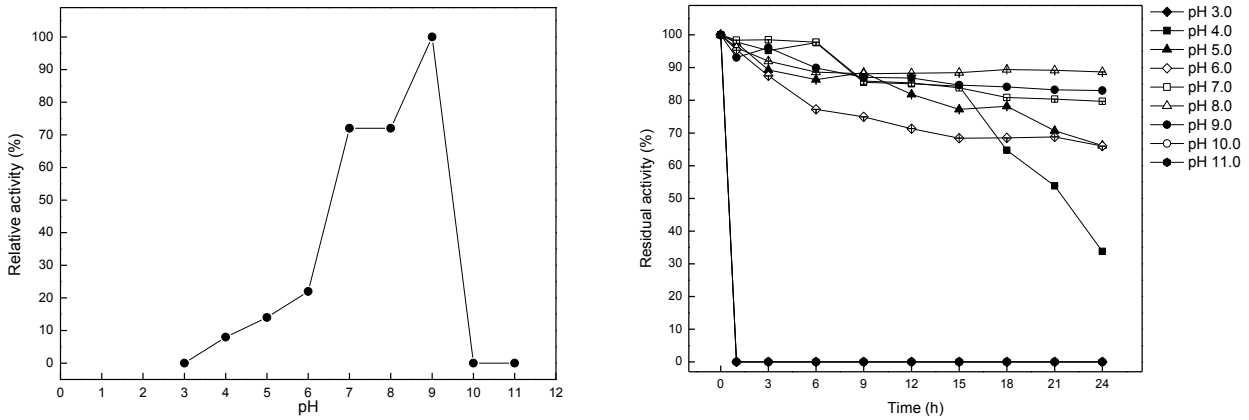

Fig. 2: (A) Effect of pH on the activity of extracellular collagenase produced by *Penicillium* sp. UCP 1286 isolated from Caatinga, expressed as percentage of the maximum one obtained in 0.05 M Tris-HCl buffer (pH 9.0). (B) Effect of pH on the stability of extracellular collagenase produced by *Penicillium* sp. UCP 1286 isolated from Caatinga, expressed as the residual activity with respect to that at the beginning. Each value is the average of results of three experiments, and the error bars show the standard deviations.

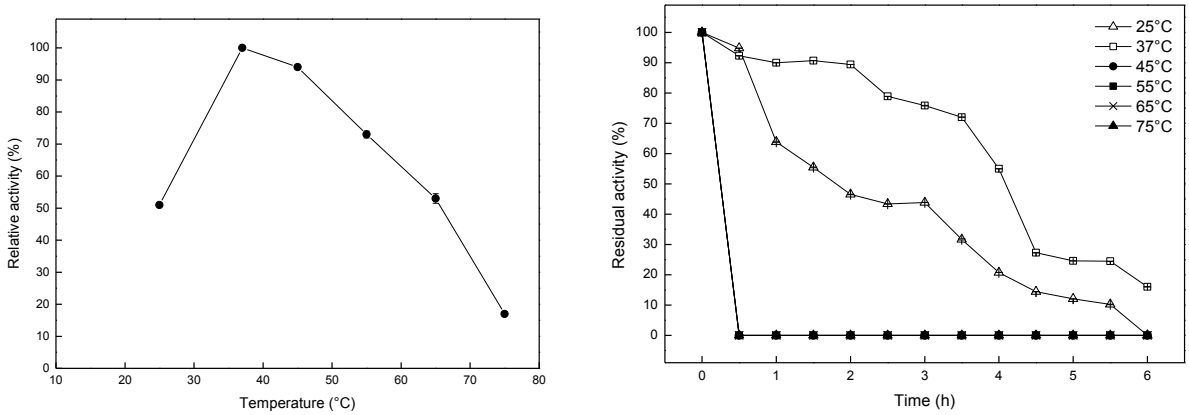

Fig. 3: (A) Effect of temperature on the activity of extracellular collagenase produced by *Penicillium* sp. UCP 1286 isolated from Caatinga, expressed as percentage of the maximum one obtained at 37°C. (B) Effect of temperature on the stability of extracellular collagenase produced by *Penicillium* sp. UCP 1286 isolated from Caatinga, expressed as the residual activity with respect to that at the beginning. Each value is the average of the results of three experiments, and the error bars show the standard deviations.

3.4. Effect of temperature on collagenolytic activity & stability

Figure 3(A) shows that the highest value is observed at 37 °C. At 45°C, approximately 85% of the enzymatic activity still remained, and then a gradual decrease occurs until almost all activity is lost at 75 °C. Figure 3(B) shows the results of enzyme stability over 6 hours at the different temperatures evaluated. A gradual decrease on enzyme stability was observed for 25 and 37 °C, while for the other temperatures considered, after the first 30 minutes, no collagenolytic activity was observed.

Comparing results of temperature assays, Wu *et al.* [6] found for *B. pumilus* collagenase an optimum temperature of 45 °C, and with heating, the collagenase retained above 50% activity at 70 °C. Baehaki *et al.* [35] assayed the collagenase activity between 30 and 90 °C, being observed an increase in enzymatic activity between 30 and 50 °C, with a strong reduction occurring above 60 °C. *S. exfoliatus* showed the maximum collagenase activity at 70 °C [1]. In the study of Suphatharaprateep *et al.* [2], both *K. pneumoniae* and *B. cereus* were shown to produce collagenases with the optimal temperature of 37 °C. Hamdy [14] related optimal temperature for *R. solani* collagenase at 40 °C. For *P. aurantiogriseum* collagenase, the optimal temperature was also 37 °C; at temperature lower or higher than 37 °C, the collagenase production decreased, as well as in the present work [9].

Figure 3B shows collagenase stability to temperature. At 25 and 37 °C, the enzyme retained about 90% and 60% of its enzymatic activity after 1.5 hours of incubation. After this period, the values were gradually decreased until reaching 15% at 37 °C after 6 hours and about 10%, at 25 °C after 5.5 hours. Regarding the others temperatures, may have occurred protein denaturation. The enzyme from *P. aurantiogriseum* was stable after 1.5 h incubation in the temperature range 25 to 45 ºC, retaining 96.2, 96.3, and 81.6% of its initial activity at 25, 37, and 45 ºC, respectively while at 70 ºC its activity was completely lost after only 15 min [9].

The results for a collagenase from *B. licheniformis* showed that the enzyme remained relatively stable and retained above 50% activity under 20 min incubation at 50 and 70 °C. However, the enzyme activity decreased gradually after 20 min incubation [35].

3.5. Substrate specificity

Five protein substrates were tested (azocoll, type I collagen, type V collagen, gelatin and azocasein). Besides azocasein, the culture filtrate showed activity on all of the substrates. Higher enzyme activity was observed with azocoll as substrate (692.65 U/mL and 3463.25 U/mg). The enzyme´s activity towards azocoll was assumed as 100% (Figure 4). The relative activity on other substrates was lower, but it can be observed that the enzyme produced by *Penicillium* sp. UCP 1286 has much higher affinity for collagen-derived substrates when compared to noncollagen-derived substrate azocasein.

Commercial enzyme produced by *Clostridium hystolyticum* exhibits highest affinity for collagen type I (32.22% relative activity) when compared to the collagenolytic enzyme produced in this work, that presented higher relative values for type V collagen (46.90%) and gelatin (22.41%) (Figure 4).

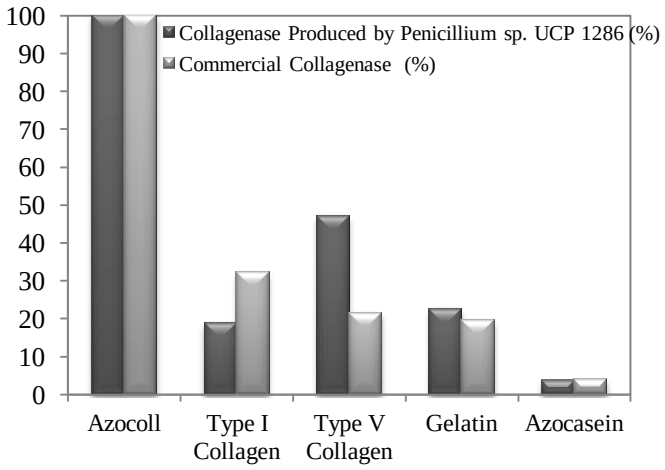

Fig. 4: Substrate specificity of Collagenase from *Penicillium* sp. UCP 1286 and Commercial Collagenase (produced by *Clostridium hystolyticum*). The enzyme´s activity towards azocoll was assumed as 100%.

The action of collagenase is very specific, acting only on collagen and gelatin and not on any of the other usual protein substrates [15]. Lima *et al*. [9] showed that *P. aurantiogriseum* collagenase highest activity was also obtained with azocoll (164.00 U/mL and 393.00 U/mg). Hamdy [14] related specificity of collagenase produced by *R. solani* to gelatin and collagen (33.23 U/mg and 120 U/mg, respectively). Ok and Hashinaga [37] tested collagenase activity using soluble (27.1 U/mL) and insoluble collagen (5.6 U/mL), besides synthetic peptides as Cbz-GPLGP (21.1 U/mL) and FALGPA (0.41 U/mL). The collagenase produced by *B. licheniformis* exhibited the highest activity on casein, being also able to hydrolyze collagen, gelatin and fibrin [35]. For *B. pumilus* collagenase, in addition to hydrolyzing the native collagen from bovine Achilles tendon, it was also able to act on gelatin, with a cleavage rate of 50.72% and 62.56%, respectively [6]. Among the collagen types, type V is classified as a member of the family of fibrillar collagens, based on their primary structure and their potential to form fibrils in the interstice [40,41]. This type of collagen has been described as involved in maintenance processes of vascular injury in patients with certain diseases, such as systemic lupus erythematosus and vasculitis [42]. More specific studies are required to better understand the action of the enzyme produced by *Penicillium* sp. UCP 1286 against type V collagen, but already indicates a potential biotechnological application for this protease (Figure 4).

3.6. Effects of inhibitors

The culture filtrates obtained from *Penicillium* sp. UCP 1286 was subjected to inhibition by phenylmethylsulfonyl fluoride (PMSF), which is a method to establish whether or not one enzyme is a serine protease. After incubation with PMSF, the enzyme activity was reduced to 0, whereas in the presence of iodoacetic acid (that inhibits cysteine proteases) there was maintenance of a residual activity of 88.31%. The collagenolytic enzyme was slightly inhibited by EDTA, known to inhibit metalloproteinases (a loss of only about 6% of activity was observed). Enzyme inhibitors are molecules that interact with the enzyme or compounds that chelate metal ions required by the enzyme to maintain its conformation [16]. In particular, the conditions selected for tests with PMSF were consistent with the observations of James [43], and those for tests with EDTA according Hamdy [14]. Although more tests for a complete characterization of the enzyme are required, the preliminary results suggest that the enzyme belongs to the family of collagenolytic serine proteases, since it was completely inhibited by their reversible inhibitor of serine proteases. The collagenolytic enzyme from *P. aurantiogriseum* kept only 24% on its activity in presence of PMSF, and retained 100 and 93.6% of its activity in the presence of iodoacetic acid and EDTA [9]. The enzyme produced by *Bacillus pumilus* was strongly inhibited by EDTA [6]. As in the present work, Jain and Jain [1] showed that the collagenase produced by *S. exfoliates* completely lost its activity in the presence of PMSF and retained 65.91% in the presence of EDTA. Mahmoud *et al*. [36] tested inhibition of collagenase produced by *A. flavus* using cetrimide, a reversible competitive inhibitor against collagenase, and collagenolytic activity was 0 with 1 mg/mL of cetrimide. The results of Hamdy [14] showed that collagenase by *R. solani* was inhibited by EDTA (31% of relative activity), iodoacetate (18%) and sodium arsenite (13%).

3.7. Electrophoresis and Zymogram

Electrophoresis was used for collagenolytic enzyme characterization, estimating its molecular weight. Reported molecular weights vary significantly based on the enzyme type (serine or metallocollagenase) and the source (microbial or animal tissue) [16]. Electrophoresis and zymogram of the culture filtrate and 60-80% fraction from saline precipitation are presented in Figure 5A and 5B, respectively. The electrophoresis presented a major band corresponding to a molecular mass (MM) of approximately 37 kDa, while others proteins can be observed at 28 and 29 kDa (Figure 5A). The zymogram analysis (Figure 5B) confirmed the bands collagenolytic activity. It can be observed that

the 60-80% fraction showed 4 bands at the lowest protein volume concentration applied (10 μg), with approximately 140 kDa, 120 kDa, 100 kDa and 37 kDa with activity towards gelatin. Multiple collagenases have been reported at literature by zymography [35,44]. Roy *et al*. [45] reported that serine collagenases have, typically, molecular weights in the range of 24 and 36 kDa. Baehaki *et al*. [35] found several protein bands and zymography analysis indicated that the molecular mass of collagenase fractions were approximately 124, 35, 31 and 26 kDa from *B. licheniformis*. Baehaki *et al*. [44] reported multiple collagenases in range 14.5 – 210 kDa produced by *B. licheniformis*. Sakurai *et al*. [39] produced a purified collagenase from *S. parvulus* with a relative molecular mass of 52 kDa. Matsushita *et al*. [46] reported that collagenases isolated from related species of *C. perfringens* had molecular weights ranging from 80 to 120 kDa. Mahmoud *et al*. [36] related a collagenase produced from *A. flavus* with molecular weights between 72 and 92 kDa. Hamdy [14] produced a purified collagenase from *R. solani* that showed a molecular weight at 66 kDa. Some researchers isolated serine collagenases from digestive glands of marine organism with molecular weights < 60 kDa [47,48]. The wide range of molecular weight is to be expected for an enzyme such as collagenase that does not have a single structure [16].

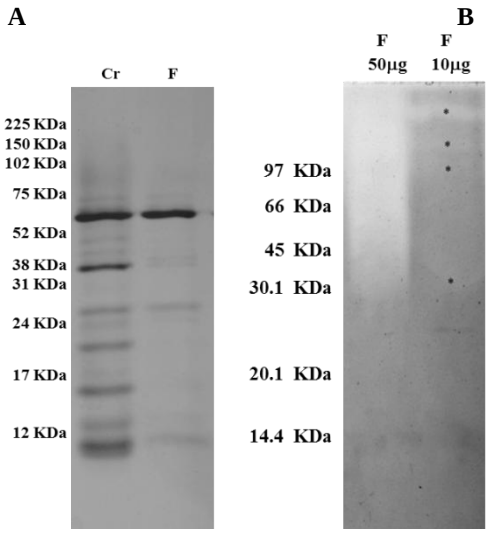

Fig. 5: (A) SDS-PAGE patterns of *Penicillium* UCP 1286 crude extract (Cr) and 60-80% fraction obtained from precipitation with ammonium sulfate (F). MM: molecular mass. (B) Zymogram analysis of collagenase.

4. CONCLUSIONS

The *Penicillium* sp. UCP 1286 fungus isolated from Caatinga was shown to produce large amounts of extracellular collagenase, using only gelatin as carbon and nitrogen source. Moreover, the activity of produced collagenolytic enzyme was much higher that other microbial production systems reported in the literature. This makes this production system as a very promising alternative for collagenase production as it associates a high producer microbial with the use of an inexpensive and readily available substrate. Complementary, optimal enzyme production conditions were established, with the temperature showing the greatest effect. The enzymes was seems to be a serine alkaline protease, having the optimal collagenolytic activity at 37°C and pH 9.0. Concerning specificity, the produced enzyme hydrolyses different types of collagen, including azocoll, type I, type V and gelatin. The data indicates that the produced enzyme presents a higher affinity to type V collagen and gelatin, when compared to commercial colagenase. Also, low azocasein activity indicates a collagen specificity of this produced enzyme, desirable property for many applications.

5. ACKNOWLEDGMENTS

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