FACTORIAL DESIGN FOR COLLAGENASE PRODUCTION BY *Penicillium* sp. SELECTED FROM THE CAATINGA SOIL

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ABSTRACT

Collagenolytic proteases can hydrolyze both denatured and native collagen and are becoming increasingly important commercially. The aim of this work was to select a new strain of Penicillium sp. isolated from the soil of Caatinga for collagenase production. A factorial design 2^4 was performed to determine the best conditions of enzyme production. Collagenolytic activity reported on this work is about 2 times larger than existing data. According to the growth kinetics, after 126 hours of production, were obtained the highest values of collagenolytic activity and specific activity. The highest values of collagenolytic activity and specific activity were obtained on a culture medium containing 0.25% (w/v) gelatin, 200 rpm, pH 8.0 and 24 °C. Only two factors were statistically significant: pH and temperature, both with negative effects. The experimental design made possible to define fermentation culture conditions able to increase by 66% the value of the initial enzyme activity.

1. INTRODUCTION

Collagen is the major fibrous element of skin, intestines, bones, tendons, cartilage, blood vessels and teeth found in all animals, corresponding to 30% of the total protein and 6% of the human body weight (Jain and Jain, 2010; Suphatharaprateep et al., 2011).

Collagenolytic proteases can hydrolyze both native and denatured collagen and are becoming increasingly important commercially (Lima et al., 2009). The collagenase produced by microorganisms is preferable because of its biochemical diversity and susceptibility to genetic manipulation (Lima et al., 2011a; Rosso et al., 2012).

Among the collagenolytic enzyme producing microorganisms, filamentous fungi have great advantages such as high productivity and low production costs and the resulting enzyme can be easily modified (Lima et al., 2011a). The enzyme production is extracellular which makes it particularly easy to recovery at the end of fermentation (Sandhya et. al., 2005). Studies report collagenase biosynthesis by fungi belonging to genera *Aspergillus, Cladosporium, Penicillium* and *Alternaria* (Yakovleva et al., 2006).

The *Penicillium* species have a greater biotechnological potential compared to the other genera mentioned, both for the production of proteases and other enzymes. They can grow in different conditions as well as to use a wide variety of substrates (Ikram-ul-Haq and Mukhtar, 2007).

The aim of this work was to select a new strain of the *Penicillium* genus, isolated from the soil of Caatinga (Pernambuco – Brazil), as a collagenase producer and to determine the best conditions for the production in an inexpensive culture medium containing only gelatin as substrate, evaluating the effect of temperature, pH, agitation and initial concentration of substrate.

2. MATERIAL AND METHODS

2.1. Microorganisms

The strains of *Penicillium* sp. (SIS 20, SIS 21, SIS 23, SIS 24, SIS 26 and SIS 27) isolated from the soil of Serra Talhada and São José do Belmonte cities, present in Caatinga biome (Pernambuco – Brazil), were obtained from UCP - the Collection of the Catholic University of Pernambuco, UNICAP.

2.2. Culture media

The culture media used for enzyme production was gelatin medium, consisting 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% w/v). This medium was sterilized in an autoclave at 121 °C for 15 min.

For the experiments aiming at defined the optimal composition of the culture medium, gelatin concentration values were (0.25% w/v), (0.50% w/v) and (0.75% w/v).

2.3. Screening of *Penicillium* Strains as Collagenase Producers

Collagenase of *Penicillium* sp. strains was obtained from the culture supernatant. Strains were sub-cultured on malt extract agar plates, incubated at 30 °C for 72 h and a calibrated cellular suspension (corresponding to an average cell concentration of 10^6 cells/mL) was used to inoculated a 125 mL Erlenmeyer flask containing 25 mL of gelatin culture medium (0.50 w/v) supplemented with 1.0% (v/v) of a mineral solution, and grown for 72 h at 28 °C in an orbital incubator shaker with 150 rpm. After this time, cultures were filtered and the supernatants analyzed for collagenase production.

2.4. Kinetics of Growth and Collagenase Production

Inoculum spores were produced in agar 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 °C and 150 rpm in 1 L Erlenmeyer flasks containing 250 mL of the culture medium. At 6-hour intervals, samples were taken for determination of biomass, protein content and collagenolytic activity.
Cotton caps were used to minimize water evaporation. The broth obtained at the end of fermentation (126 h) was vacuum filtered through 0.45 µm-pore diameter nitrocellulose membranes to remove the mycelium.

2.5. Screening of Significant Variables for Collagenase Production

A 2^4 full design was carried out at all combinations of the levels given in Table 1. The center point was performed in quadruplicate, to provide an estimate of the variance of the pure experimental error responses. All statistical and graphical analyzes were performed with 95 % confidence using the Statistica 8.0 software (StatSoft Inc., Tulsa, OK, USA).
Table 1. Factors and corresponding levels used in a 2^4 design to investigate the production of collagenase by *Penicillium* sp. isolated from caatinga

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

2.6. Analytical methods

The Azo dye-impregnated collagen assay was carried out according to a modified version of the method developed by Chavira et al. (1984). Azocoll was washed and suspended in 0.05 M Tris-HCl buffer 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, 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. The absorbance of the supernatant was measured at 520 nm by a UV-Vis spectrophotometer. 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. Protein concentration was determined by the method of Bradford modified using "Coomassie Blue Bright" to detect minute amounts of protein in biological fluids. The calibration curve was made from stocks of bovine serum albumin (BSA) solutions. Biomass was determined by the dry weight method using pre-weighted nitrocellulose membranes with 0.45 µm-pore diameter, after drying at 80 °C for 24 h.

3. RESULTS AND DISCUSSION

The strain selected among the six species was *Penicillium* SIS 21, which had the best collagenolytic activity. The results for growth and collagenase production kinetics are shown in Figure 1. Enzyme synthesis is shown to begin began during the microbial growth phase, but the highest production occurs in the stationary phase. An increase in the enzyme activity is observed at 96 hours.

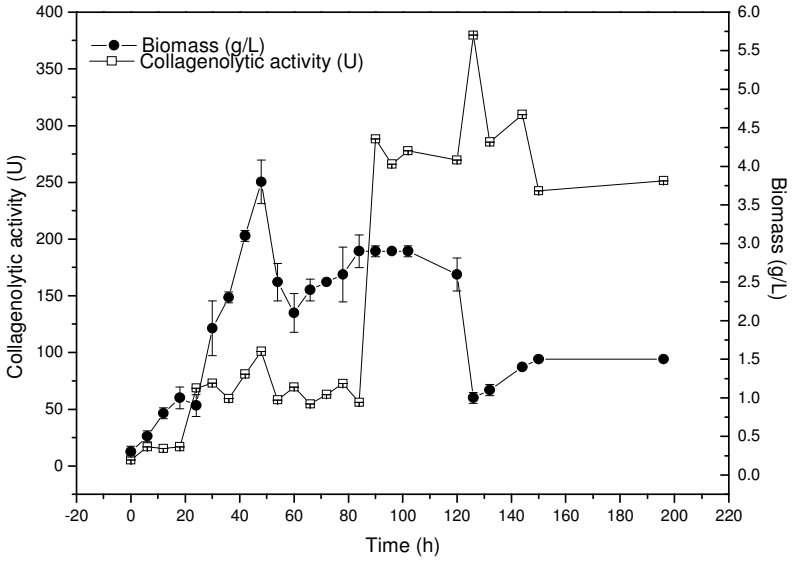

Figure 1. Kinetics of growth and collagenase production by Penicillium SIS 21 isolated from caatinga in gelatin culture medium

As the culture medium contained only gelatin and salts, it is likely that the fungus required an adaptive period. This agrees with results reported by other authors that described the production of collagenase after the cell growth period (Lima et al., 2011b; Petrova et al., 2006; Suphatharaprateep et al., 2011). The highest collagenase activity was obtained after 126 hours, reaching 379.80 units. Based on these results, the incubation time for further optimization study was 126 hours.

Table 2 shows the conditions and results of the fermentations (after 126 hours) conducted according to the established experimental design. The highest values of collagenolytic activity (632.70 U/mL) and specific activity (3954.38 U/mg) were obtained with the run 9, conducted with 0.25% (w/v) gelatin, 200 rpm, pH 8.0 and 24 °C.

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) indicates that, on average, higher activities were obtained when factors 1 (pH) and 3 (temperature) were selected at their lowest levels. With respect to the effect of the factors on the biomass concentration, the substrate concentration was the only one that presented positive significant effect.

Overall, the application of the factorial design allowed increasing the enzyme production by about 66%. The obtained values for collagenolytic activity values were substantially higher than those reported in the literature for *Bacillus sp.*: 43.5 U/mL (Jain and Jain, 2010), *Candida albicans*: 7.6 U/mL (Lima et al., 2009) and *Penicillium aurantiogriseum*: 164 U/mL (Lima et al., 2011a).

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

Run	pH	S_o (%)	T (°C)	Agitation (rpm)	X (g/L)	TP (mg/mL)	A_c (U)	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

The values of the significant effects indicate that, on average, higher activities were obtained when factors 1 (pH) and 3 (temperature) were selected at their lowest levels. With respect to the effect of the factors on the biomass concentration, the substrate concentration was the only one that presented a positive significant effect.

4. CONCLUSIONS

The fungus Penicillium SIS 21 isolated from soil of the caatinga proved to be a promising producer of the collagenase enzyme. The micro-organism was able to produce significantly higher values than reported in the literature using a simple and inexpensive substrate as culture medium.

5. ACKNOWLEDGMENTS

This work was supported by Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco (FACEPE) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

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