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Protease Production by *Streptomyces* sp. Isolated from Brazilian Cerrado Soil

Optimization of Culture Medium Employing Statistical Experimental Design

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Abstract

Streptomyces are important microorganisms because of their capacity to produce numerous bioactive molecules. In the present work protease production, by *Streptomyces* sp. 594 isolated from a Brazilian Cerrado soil, was maximized by optimizing a low-cost culture medium composition (casitone and sugarcane molasses) using statistical experimental design. The final protease activity (56 U/mL) was 2.8-fold and 58-fold higher than that obtained in the beginning of this study, and in a previous work, using an actinomycete selection medium, respectively. Protease production, not growth associated, appeared to be modulated by an inducer system, whereby the C/N ratio seemed to play a significant role.

Index Entries: *Streptomyces*; sugarcane molasses; protease production; experimental design.

Introduction

The streptomycetes, abundantly found in soils, are a well-known bacterial group that are especially important because of their capacity to produce antibiotics and several classes of enzymes and enzyme inhibitors *(1)*. Brazilian soils under cerrado vegetation are very peculiar tropical soils that are not sufficiently explored and probably rich in new actinomycete species

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(2). These soils may constitute an excellent source for the discovery of new enzymes, including proteases, which are of considerable commercial value for use in food, pharmaceutical, detergent, and tanning industries *(3)*. Sugarcane molasses has not yet been reported in the literature as an adequate substrate for protease production, but it is a very abundant byproduct of the sugar industry in Brazil, and therefore is an attractive substrate.

The aim of the present work was to maximize the production of proteases by an actinomycete, *Streptomyces* sp. strain 594, isolated from a Brazilian cerrado soil. The optimization of casitone and sugarcane molasses concentrations in the culture medium was performed using a central composite experimental design. The kinetics of microbial growth and protease production were also investigated.

Materials and Methods

Actinomycete Strain

Streptomyces sp. 594 was isolated from a red-yellow latosol (oxisol) under cerrado vegetation cover, located on the Central Plateau, Brasilia, Federal District, Brazil. The screening and identification of this proteolytic strain are described elsewhere *(4)*.

Preparation and Maintenance of Inoculum

Agar-grown mycelia were inoculated in a 1-L Erlenmeyer flask containing 300 mL of malt extract–yeast extract (MY) medium, pH 7.0. Incubation was carried out under agitation, using a stirring bar, for 72 h at 28°C, to obtain fragmented biomass. For quantification, several decimal dilutions of the fragmented biomass suspension were prepared and inocula spreaded over MY agar medium. After 10 d of incubation at 28°C, colonies were quantified as the number of colony forming units (CFU) of *Streptomyces* sp. per milliliter.

Experimental Design and Optimization of Culture Medium

The medium's components, sugarcane molasses (1.01% N and 29.6% C) and casitone (11.3% N and 45.4% C), were selected previously, using a factorial experimental design (data not shown). In the present work, their concentrations were optimized by employing a central composite experimental design *(5)*, with two factors and triplicate of the central point. The concentrations (% [w/v]) of molasses and casitone were normalized and tested at –1, –0.71, 0, +0.71, and +1 levels, as shown in Table 1. The software Essential Regression and Experimental Design *(6)* was employed to generate the experimental design and to analyze the experimental results. Experiments were carried out by seeding standard cell suspensions (10^5 CFU/mL) in the different culture media and incubating for 96 h at 30°C under agitation (200 rpm). Enzyme production and overall yield coefficient ($Y_{P/X}$) (units of protease produced per milligram of cell dry weight) were evaluated. An empirical model describing the proteolytic activity as

Table 1
Optimization of Sugarcane Molasses and Casitone Concentration (X_1 and X_2, respectively) for Protease Production by *Streptomyces* sp. 594[a]

Run	X_1 (% w/v)	Normalized X_1	X_2 (% w/v)	Normalized X_2	Protease activity (U/mL)
1	0.30	0	0.30	0	15.3
2	0.30	0	0.50	+1	12.2
3	0.10	–1	0.30	0	5.51
4	0.44	+0.71	0.44	+0.71	18.2
5	0.16	–0.71	0.44	+0.71	6.53
6	0.30	0	0.30	0	15.6
7	0.16	–0.71	0.16	–0.71	6.89
8	0.30	0	0.10	–1	6.27
9	0.44	+0.71	0.16	–0.71	14.5
10	0.5	+1	0.30	0	21.1
11	0.30	0	0.30	0	16.4

[a]A central composite design was used, and results are from fermentation experiments carried out at 30°C for 96 h.

a function of molasses and casitone concentrations was obtained through statistical analysis of the experimental results.

Analytical Methods

Determination of Biomass

Culture samples were withdrawn daily. Samples were filtered using 0.45-µm membranes, and microbial growth was measured by dry weight determination. Biomass concentration was expressed as milligrams of dry cells per milliliter of medium.

Reducing Sugar and Total Nitrogen

Reducing sugar and total nitrogen data were determined from filtered samples according to the methods of Somogyi *(7)* and Bremner *(8)*, respectively.

Proteolytic Activity

Extracellular protease activity in filtered samples was determined by azocasein (2%) assay in 50 m*M* phosphate buffer, pH 7.0 at 60°C *(9)*. One unit of proteolytic activity was defined as the amount of protease required to produce an absorbance increase of 0.01 at 440 nm.

Results and Discussion

Experimental Design and Optimization of Culture Medium

As a consequence of an increase in molasses and casitone concentration from 0.1 to 0.5% (w/v), protease production by *Streptomyces* sp. 594 varied from 5.51 to 21.1 U/mL (Table 1). Through statistical analysis of the

results, an empirical quadratic model was obtained, as a function of molasses (M) and casitone (C) normalized concentrations (Eq. 1). To guarantee that only statistically significant terms were included in the correlation, only those parameters with a critical significance (p value) < 0.05 were considered. The regression coefficient of the polynomial model (R) was 0.972 and $R^2_{adjusted}$ was 0.920, indicating a satisfactory model adequacy:

$$\text{Protease (U/mL)} = 14.67 + 7.305 \times M + 2.073 \times C - 5.716 \times C^2 \quad (1)$$

From Eq. 1, it can be seen that in the concentration range covered by this correlation (0.1–0.5% [w/v]), molasses concentration has a linear positive effect on protease activity, while the casitone effect is a more complex, quadratic one. Through differentiation of the empirical model with respect to casitone concentration, and by calculating the casitone value that would equate this first derivative to zero, it was possible to calculate the optimum normalized casitone concentration as 0.181, corresponding to a real casitone concentration of 0.336% (w/v). This behavior can be confirmed in Fig. 1, where the response surface plot corresponding to Eq. 1 is shown. Therefore, further experiments were carried out using a casitone level of 0.3% (w/v).

Casitone concentration commonly cited in current literature for the production of different enzymes by distinct microorganisms are between 0.2 and 0.5% *(10)*. However, the present results show that casitone concentrations >0.3% may exert a negative effect on protease production by *Streptomyces* sp., probably owing to a feedback inhibition of enzyme synthesis *(11)*.

Effect of Higher Sugarcane Molasses Concentrations on Protease Production and Cell Growth

Since the previous experiments showed that molasses had a linear positive effect on protease production in the whole range of 0.1 to 0.5% (w/v), a univaried experiment was performed to investigate the effect of higher molasses concentrations on protease production and cell growth. Casitone was used at its optimized level (0.3% [w/v]), and sugarcane molasses concentration was varied from 0.5 to 3.0% (w/v). Figure 2 compares the biomass concentration and protease activity obtained after 96 h of cultivation. There is a clear positive effect of the increase in molasses concentration on cell growth, indicating that the media tested previously were carbon limited. In response to the increase in molasses availability from 0.5 to 3.0% (media C/N ratio from 7.3 to 15.95), biomass varied steadily from 1.01 to 2.32 mg/mL (Fig. 2A). The overall positive effect of molasses concentration on cell growth was not equally observed on protease production, since a maximum protease activity (31 U/mL) was obtained at 1.0% molasses, decreasing afterward (Fig. 2B). In conclusion, a threshold molasses availability, related to the medium C/N ratio, apparently triggers a metabolic shift in *Streptomyces* sp. 594 toward cell growth, to the detriment of enzyme production. Similar results were reported for different enzymes (e.g., lipases) produced by other microorganisms *(12)*.

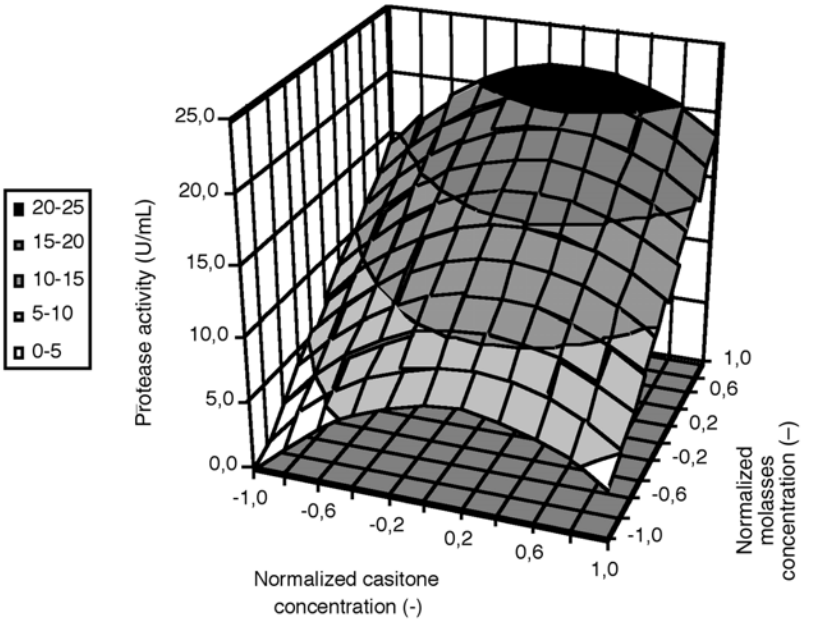

Fig. 1. Theoretical response surface plot obtained from central composite design for protease production by *Streptomyces* sp. 594 as a function of normalized sugarcane molasses and casitone levels. Experiments were carried out at 30°C for 96 h.

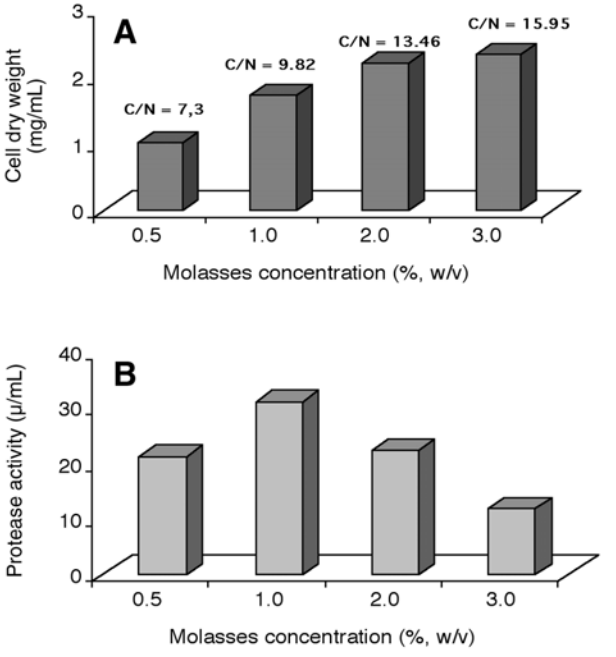

Fig. 2. Effect of higher sugarcane molasses concentrations on (**A**) cell dry weight and (**B**) protease production by *Streptomyces* sp. 594 using 0.3% (w/v) casitone in shake-flask fermentations (170 rpm) carried out for 96 h at 30(C and pH 7.0.

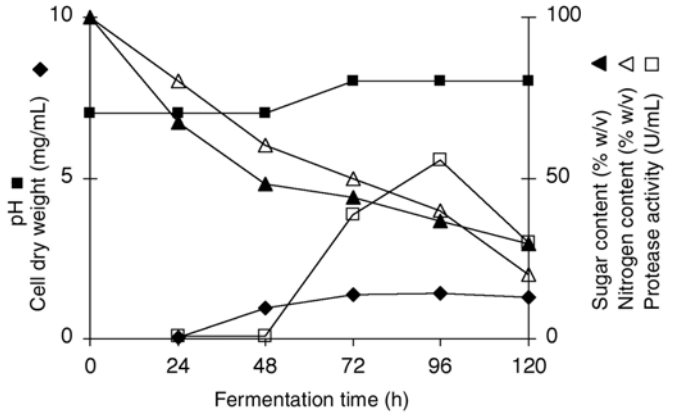

Fig. 3. Typical time course for cell growth, protease production, pH, and sugar content in *Streptomyces* sp. 594 shake-flask fermentations (200 rpm, 30°C) using the optimized medium (0.3% casitone and 1% molasses, pH 7.0) and an inoculum of 10^5 CFU/mL.

Fermentation Kinetics

The fermentation time course for protease production by *Streptomyces* sp. 594 at 200 rpm is shown in Fig. 3. The maximum proteolytic activity (56 U/mL) was observed after 96 h of cultivation, and its production was shown to be not growth associated, occurring at the middle of the stationary phase (Fig. 3). Báscaran et al. *(13)* and Porto et al. *(14)* observed that maximum protease production by *S. clavuligerus* occurred at the end of exponential and beginning of stationary growth phase. Different results were obtained by Kim and Lee *(15)*, who observed protease production by a strain of *S. exfoliates* in batch submerged fermentations as being associated with mycelium growth.

According to Fig. 3, sugar and nitrogen contents in the medium decreased slowly but were not completely consumed along all growth phases. Similar results for protease production by *S. clavuligerus* were observed after nutritional shiftdown, indicating that the beginning of protease production decreased with nutrient availability *(13)*. By contrast, Aretz et al. *(16)* and Brabban and Edwards *(17)*, working with some *Streptomyces* species, observed that sugars were rapidly consumed from lag to exponential phase, and then fell to undetectable levels. The mechanism by which control of protease production is achieved in many prokaryotes systems is not known yet *(16)*.

Employing the optimized medium (0.3% [w/v] casitone and 1.0% [w/v] molasses), the maximum protease activity obtained (56 U/mL; $Y_{p/x}$ = 40 U/mg) was 2.8-fold higher, when compared to previous experiments (data not shown) employing molasses and casitone at a 0.5% (w/v) concentration. When compared to protease activities obtained in a previous work, using starch casein salt medium *(18)*, a common medium for actinomycetes, a 58-fold increase in protease activity was obtained. Lower levels

of protease were produced by *S. viridosporus* (0.38 U/mL) *(19)* when corn oil was used in shake-flask fermentations.

In conclusion, the present results indicate that sugarcane molasses and casitone are good carbon and nitrogen sources for protease production by *Streptomyces* sp. 594. The kinetics of fermentation showed that protease synthesis is not associated with biomass growth. Optimization of the concentration of medium components, using response surface experimental design and statistical analysis, enabled a significant increase in proteolytic activity. The good results obtained confirm this strain to be promising for protease production using a low-cost carbon source.

Acknowledgments

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References

1. Gilbert, M., Morosoli, R., Shareck, F., and Kluepfel, D. (1995), *Crit. Rev. Biotechnol.* **15,** 13–39.
2. Sêmedo, L. T. A. S., Linhares, A. A., Gomes, R. C., Manfio, G. P., Alviano, C. S., Linhares, L. F., and Coelho, R. R. R. (2001), *Microbiol. Res.* **155,** 291–299.
3. Peczynska-Czoch, N. and Mordarski, M. (1988), in *Actinomycete Enzymes*, Goodfellow, M., Williams, S. T., and Mordarski, M., eds., Academic, New York, NY, pp. 219–283.
4. De Azeredo, L. A. I., Leite, S. G. F., Freire, D. M. G., Benchetrit, L. C., and Coelho, R. R. R. (2001), *J. Microbiol. Methods* **45,** 207–212.
5. Neto, B. B., Scarminio, I. S., and Bruns, R. E. (1995), in *Planejamento e Otimização de Experimentos*, Neto, B. B., Scarminio, I. S., and Bruns, R. E., eds., Editora da Unicamp, São Paulo, Brazil, pp. 61–97.
6. Steppan, D. D., Werner, J., and Yeater, R. P. (1998), *Essential Regression and Experimental Design* (book and software downloadable, *see* Website: www.geocities.com/SiliconValley/network/1032).
7. Somogyi, M. (1952), *J. Biol. Chem.* **195,** 19–23.
8. Bremner, J. M. (1965), in *Methods of Soil Analysis*, Black, C. A., Evans, D. D., White, J. L., Ensminger, L. E., and Clark, F. E., eds., American Society of Agronomy, Madison, WI, pp. 1149–1178.
9. Sarath, G., Motte, R., and Wagner, F. W. (1989), in *Proteolytic Enzymes*, Beynon, R. J. and Bond, J. S., eds., IRL, Oxford, England, UK, pp. 22–25.
10. Lee, D., Lee, E. L., and Lee, K. M. (2000), *J. Microbiol. Biotechnol.* **10,** 208–214.
11. Malathi, S. and Chakraborty, R. (1991), *Appl. Environ. Microbiol.* **57,** 712–716.
12. Baillargeon, M. W., Bistline, R. Jr., and Sonnet, P. E. (1989), *Appl. Microbiol. Biotechnol.* **30,** 92–96.
13. Báscaran, V., Hardisson, C., and Brana, A.F. (1990), *Appl. Microbiol. Biotechnol.* **34,** 208–213.
14. Porto, A. L. F., Campos-Takaki, G. M., and Filho, J. L. (1996), *Appl. Biochem. Biotechnol.* **60,** 115–122.
15. Kim, I. S. and Lee, K. J. (1996), *Microbiology* **142,** 1797–1806.
16. Aretz, W., Koller, K. P. and Riess, G. (1989), *FEMS Microbiol. Lett.* **65,** 31–36.
17. Brabban, A. D. and Edwards, C. (1996), *J. Appl. Bacteriol.* **80,** 651–658.
18. Mackay, S. J. (1977), *Appl. Environ. Microbiol.* **33,** 227–230.
19. Macedo, J. M. B., Gottschalk, L. M., and Bon, E. P. (1999), *Appl. Biochem. Biotechnol.* **77–79,** 735–744.