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Fibrinolytic protease production by new *Streptomyces* sp. DPUA 1576 from Amazon lichens

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ABSTRACT

Background: *Streptomyces* sp. DPUA 1576 from Amazon lichens was studied to protease and fibrinolytic production. A 2^2 factorial experimental design was applied to optimize its protease enzyme production using two independent variables, namely soybean flour and glucose concentrations.
Results: The optimal conditions to obtain high protease production (83.42 U/mL) were 1.26% soybean flour and 1.23% glucose concentration. A polynomial model was fitted to correlate the relationship between the two variables and protease activity. In relation to fibrinolytic activity, the highest activity of 706.5 mm^2 was obtained at 1.7% soybean flour and 1.0% glucose concentration, which was 33% higher than plasmin. Fibrinolytic production was not optimized in the studied conditions.
Conclusions: These results show that the optimization of the culture medium can enhance protease production, thus becoming a good process for further research. In addition, *Streptomyces* sp. DPUA 1576, isolated from Amazon lichens, might be a potential strain for fibrinolytic protease production.

1. Introduction

Among the different types of cardiovascular diseases, thrombosis is one of the most widely current diseases in modern life. The fundamental pathophysiological process related to this problem is the accumulation of fibrin when injury on blood vessels occurs. Fibrin is formed from fibrinogen by the action of thrombin (EC 3.4.21.5) and it is lysed by plasmin (EC 3.4.21.7), a secretory serine protease, which is generated from inactive precursor plasminogen via limited cleavage by plasminogen activator (PA) [1].

Urokinase (u-PA) and tissue-type plasminogen activator (t-PA) are still widely used as biological medicines for the treatment of cardiovascular disease, but these agents have some undesirable side effects such as gastrointestinal bleeding, toxicity and allergic reactions. Several studies have focused on researching of cheaper and safer resources [2,3].

Fibrinolytic proteases with potential thrombolytic effects have been purified from diverse sources such as fermented food, earthworms, mushrooms and microbial sources [4]. Microbial fibrinolytic proteases have attracted medical attention during decades [5]. Streptokinase produced by *Streptococcus hemolyticus* and Staphylokinase produced by *Staphylococcus aureus* were early proved to be effective for thrombolytic therapy [6,7].

There are few reports using *Streptomyces* as fibrinolytic agents [4]. Extracellular enzyme production by microorganism is greatly influenced by media components, especially carbon and nitrogen sources [5,6,7,8,9,10]. Statistical approaches are a well-known method applied in the optimization of variables responsible for the production of biomolecules [8]. However, to produce the fibrinolytic enzyme efficiently, the optimization of broth culture medium for fermentation is required. Response surface methodology (RSM), which is the most accepted statistical technique for bioprocess optimization, can be used to examine the relationship between a set of controllable experimental factors and observed results [11].

The aim of this work was to evaluate the influence of soybean flour (SF) and glucose (G) concentrations on protease and fibrinolytic enzyme production by *Streptomyces* sp. DPUA 1576 isolated from Amazon lichens.

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2. Material and methods

2.1. Microorganism and culture maintenance

Streptomyces sp. DPUA 1576 isolated from lichens Amazon was obtained from the Culture Collection of the Parasitology Department of the Federal University of Amazonas (DPUA), Brazil. The strain was maintained in ISP-2 culture medium at 25°C in Castellani method [12].

2.2. Culture medium, inoculum preparation and protease production

Inoculum preparation was done in ISP-2 agar plates containing a culture of cells grown on ISP-2 agar plate [13] for 7 d at 30°C. After, the inoculum was incubated in ISP-2 broth in orbital shaker 200 rpm for 48 h at 28°C. Cell concentration of 10^6 cells/mL was inoculated in 250 mL Erlenmeyer flask containing 50 mL of culture medium described by Porto et al. [14] with different SF and G concentrations (Table 1). After 72 h of cultivation, cell-free extract was obtained by centrifugation at 8000 × g, at 4°C for 10 min and submitted to protease and fibrinolytic activity determinations.

2.3. Evaluation of protease activity

Proteolytic activity was made according to Alencar et al. [15] using azocasein as substrate (Sigma Chemical Co., St Louis, MO). One unit (U) of enzymatic activity was defined as the amount of enzyme capable to produce a 0.001 change in absorbance at 440 nm per minute.

2.4. Evaluation of fibrinolytic activity

The crude extract was submitted to fibrinolytic activity on agar plate according to the method described by Astrup and Mullertz [16], with minor modifications as follows. The fibrin agarose plate was made by 1% agarose, 0.1% human fibrinogen (Sigma Chemical Co., St Louis, MO), and 8 U/mL of human thrombin (Sigma Chemical Co., St Louis, MO). The clot was allowed to stand for 1 h at room temperature (25 ± 2°C). Then, 20 μL of sample solution was spotted directly onto the fibrin plate, and then incubated at 37°C for 18 h and the diameter of the lytic halo was measured. In the fibrin plate method, a clear transparent region is observed in which fibrin is hydrolyzed, and its diameter is directly proportional to the potency of the fibrinolytic activity. The lysed area is given in mm^2.

2.5. Experimental design and statistical analysis

Response surface method was utilized to determine the influence of the two independent variables SF and G concentration on the two responses variable selected for this study, namely protease and fibrinolytic production. To this purpose, multivariable regression analyses were done under the conditions preliminarily determined by the experimental design (Table 1). Such a design was based on the methodology called "star planning," proposed by Barros Neto et al. [17], which consists of two factors in five levels of independent variables. The central point was three fold repeated so as to check the reproducibility of results. The independent variables SF and G concentration and their corresponding ranges were selected on the basis of the results of Porto et al. [14].

For predicting the optimal point, a second order polynomial function was fitted to correlate relationship between independent variables and responses. For the two factors this equation is:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} {X_1}^2 + \beta_{22} {X_2}^2 + \beta_{12} X_1 X_2 \qquad \text{[Equation 1]}$$

where Y is the predicted response, β_0 is the model constant; X_1 and X_2 are the independent variables; β_1 and β_2 are the linear coefficients; β_{12} is the cross product coefficients and β_{11} and β_{22} are the quadratic coefficients. The quality of fit of the polynomial model equation was expressed by the coefficient of determination R^2. All data were treated with Statistica 8.0 software (StatSoft Inc., Tulsa, OK, USA).

Table 1
Experimental design and results of the central composite design for protease and fibrinolytic activity production by *Streptomyces* sp. DPUA 1576.

Run	Coded variable		Uncoded variable		Response		
	X_1	X_2	Soybean flour (%)	Glucose (%)	PA (U/mL) measured	PA (U/mL) predicted	FA (mm^2)
1	-1	-1	0.5	0.5	55.00	57.22	600.87
2	1	-1	1.5	0.5	60.00	52.51	615.44
3	-1	1	0.5	1.5	1.11	17.83	146.62
4	1	1	1.5	1.5	84.89	91.89	672.38
5	0	0	1	1	74.39	74.33	660.19
6	0	0	1	1	71.78	74.33	637.62
7	0	0	1	1	76.83	74.33	490.63
8	-1.414	0	0.293	1	45.00	33.51	397.41
9	1.414	0	1.707	1	80.28	82.54	706.5
10	0	-1.414	1	0.293	39.22	51.69	572.27
11	0	1.414	1	1.707	73.39	51.69	637.62

PA: protease activity (U/ml); FA: fibrinolytic activity (mm^2).

3. Results and discussion

3.1. Protease production

A Plackett–Burman design was used to evaluate the maximum protease production as a function of SF and G concentrations. Using a *P*-value lesser than 0.05, the analysis indicates that SF, G and their interaction were significant variables, as shown in the Pareto chart (Fig. 1).

Pareto's chart is a graphical representation of Student's *t*-test. The parameter SF linear showed higher effect than quadratic SF indicating that the increase of the concentration from 0.293 to 1.707% led to an increase in protease production of 45.00 to 80.28 U/mL. In earlier reports soybean meals were found effective ingredients for the protease production by *Nocardia* sp. [18]. Soybean flour is commonly used as a protein source in industrial fermentations to enzyme and antibiotic production. Their protein concentration ranged 51.2–53.2% depending on changes in growing conditions, but the overall amino acid composition was relatively constant. The synthesis and secretion of protease are induced by peptides or other protein substrates, such as soybean flour. Depending on the peptide nature and level, protease synthesis and secretion may be induced or repressed [18]. Thus, protease production can be employed across a wide range of SF.

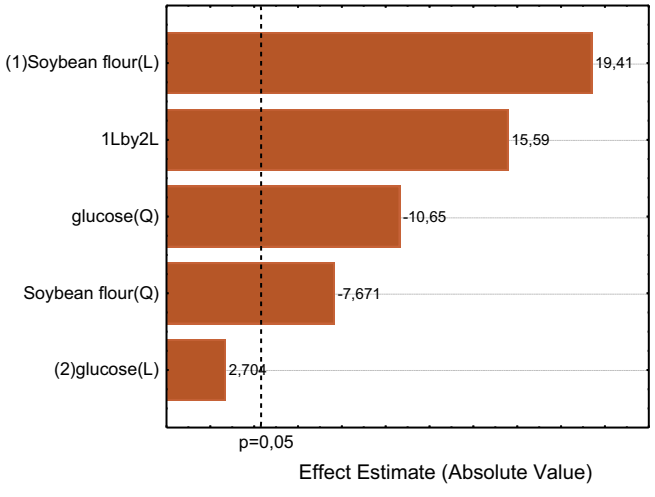

Fig. 1. Pareto chart for protease production by *Streptomyces* sp. DPUA1576.

Soybean medium may be considered a promising and inexpensive alternative for protease production. For Brazil, the utilization of soybean flour is especially important, as the country is the world's largest soybean producer [18]. The linear effect of G was not statistically significant (Table 2), but it remained in the model to improve the regression coefficient. The quadratic effect of G was negative and statistically significant indicating that the range of G evaluated was adequate to optimization G on protease production. Other authors are in agreement with these results. Mehta et al. [19] and Patel et al. [20], working respectively with alkaliphilic actinomycete was isolated from soil and *Bacillus* sp., noted that high concentrations of glucose inhibited enzyme production, and a 0.5% (w/v) concentration was optimal for protease production.

Interaction between SF and G had a positive effect for protease production, indicating that the variable influences each other; this may be caused by the SFB, although with high content of nitrogen from protein, it has a carbon skeleton which may be used as carbon source and which can interfere in glucose uptake by microorganisms.

In order to approach the optimum response region of the enzymes production, significant independent variables (soybean concentration, X_1 and glucose concentration, X_2) were further explored, each at five levels. Statistical optimization of medium components is a critical point for better protease production.

Regression coefficients of the fitted quadratic model [Equation 1] obtained for protease activity as the function of SF and G are presented in Table 1, and it was examined in terms of the appropriateness of fit. To improve the regression fit for protease activity, the linear coefficient of G was neglected in the adjustment of the mathematical model.

The first step for the determination of an empirical model, beyond response surface methodology, is approximating the function (f), in independent variable region. If the response can be modeled by a linear function, then the approximate function is a model of first order. If curvature exists in the system or in the optimum region, then a polynomial of superior degree, as a second order model, must be utilized to approach the response [21].

Statistical significance which was evaluated by ANOVA (Table 1) indicated that the second-order model generated for maximum protease activity (Y) was statistically significant and showed a satisfactory determination coefficient of $R^2 = 0.81$ (a value of $R^2 > 0.75$ indicates the aptness of the model) [22], which ensured a satisfactory adjustment of the quadratic model to the experimental data and independent variable.

The model shows all significant terms (P-value lesser than 0.05). Thus, the difference between the experimental values and the values predicted by the model could be elucidated only by experimental error. The residues explained the lasting 9%. Thus, the mathematical model that describes the production of protease can be represented by [Equation 2]:

$$Y = 74.33 + 17.33X_1 - 8.152X_1^2 - 11.32X_2^2 + 19.69X_1X_2 \quad \text{[Equation 2]}$$

where Y is the predicted response of protease activity, and X_1 and X_2 are the coded values of SF and G, respectively. This model was used to construct response surface plots, which show the experimental and predicted values of maximum protease activity as a response (Fig. 2).

Table 2
Statistical analysis of Plackett–Burman's design showing coefficient values and p-values for each variable for protease activity.

	Coefficient regression	P-value
Mean/intercept	74.33	0.000385
X_1	17.33	0.002643
X_1^1	-8.152	0.016573
X_2^2	-11.32	0.008699
X_1X_2	19.95	0.004086

Adjusted $R^2 = 0.81$; $P < 0.05$.

From the response surface presented in Fig. 3, an optimum region comprising the 1.26% of SF and 1.23% of G can be observed, which means the appropriate range for reaching the best results for protease activity. Fig. 2 also shows that there are maximum values of protease activity, indicating that this variable was optimized as a function of SF and G concentrations.

The ANOVA was conducted for the second order response surface model. The significance of each coefficient was determined by P-values, which were listed in Table 2.

Results still show that the optimization promoted an increase of 12% in protease values when compared to basal medium ISP-2. Indeed, it is a clear advantage of using RSM to estimate optimal SF and G concentrations for enzymatic activity, providing higher flexibility in the development of different bioprocesses.

The optimization of protease production by *Streptomyces* sp. DPUA 1576 of 83 U/mL achieved was greater than the optimized enzyme production achieved in other works which employed *Bacillus* sp. I-312 (28.630 U/mL) [23] and *Streptomyces* sp. 594 isolated from a Brazilian cerrado soil (56 U/mL) [24], demonstrating that SF and G represent a viable culture medium for protease production from *Streptomyces* sp. DPUA 1576.

3.2. Fibrinolytic enzyme production

In order to search for the optimal combination of some components of the fermentation process, to enhance the fibrinolytic production, experiments were performed according to a 2^2 factorial design (Table 1). Concerning the results, it can be observed that the lowest fibrinolytic activity (1.11 mm^2) was obtained by employing low levels of SF and high levels of G. Considering these observations, SF and G sharply influenced the fibrinolytic production.

Although the influence of the studied variables can be analyzed separately, the main effects and the existence of interaction effects among them could be better evaluated by employing the statistical analysis. Fig. 3 shows the Pareto's chart that represents the estimated effects of SF and G on fibrinolytic activity response. The measure of each bar is proportional to the estimate effect. The vertical line is used to evaluate which effects are statistically significant in a 90% of confidence level. SF and interaction between SF and glucose presented a positive significant effect on fibrinolytic activity ($P < 0.10$), which

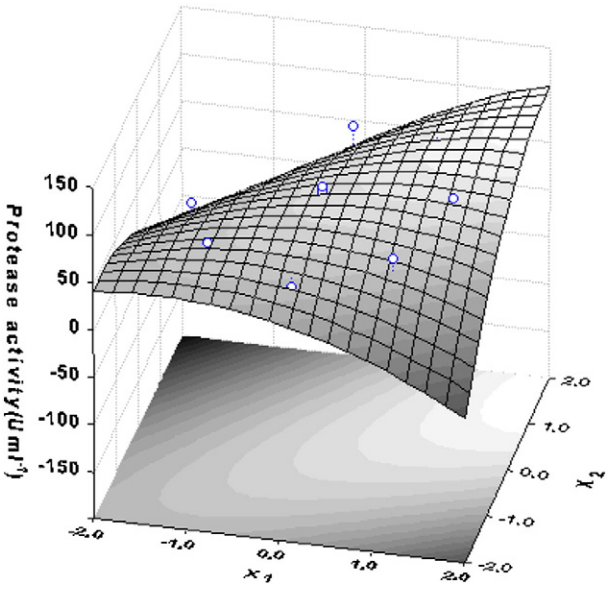

Fig. 2. Response surface showing the effects of soybean flour and glucose on protease production by *Streptomyces* sp. DPUA 1576.

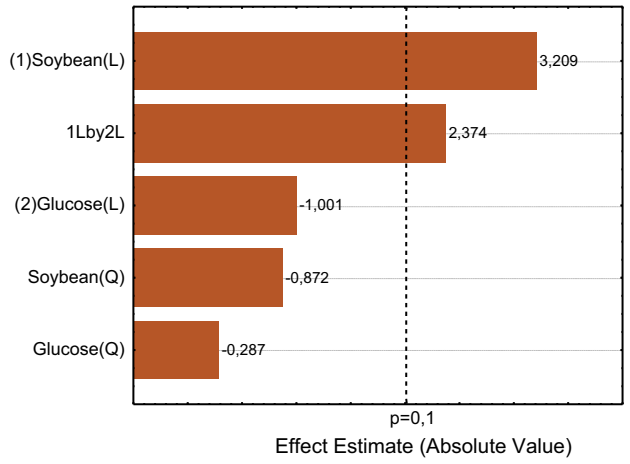

Fig. 3. Pareto chart for fibrinolytic enzyme production by *Streptomyces* sp. DPUA 1576.

means that the highest SF improves the fibrinolytic enzyme production (Table 1).

The highest main effect observed to the specific activity response was the variable SF that presented estimation of (3.21), followed by interaction between SF and glucose concentration, (+4.53). All other variables and their interactions had no significant effects on fibrinolytic production. The highest fibrinolytic activity was around 670 mm^2, values higher than those from 6 strains were isolated from *natto* fermented foods (475 mm^2) and similar to 6 strains isolated from *doufuru* fermented foods (655 mm^2) [25]. Soybean powder was reported as the best nitrogen organic source for fibrinolytic protease production from *Streptomyces* sp. NRC 411 [26]. Requirement for specific nitrogen source differs from organism to organism or even among the same species isolated from different sources [27].

There is no general defined medium for fibrinolytic enzyme production by different microorganisms. Every microorganism has its own peculiar nutritional requirements for enzyme production. In view of the commercial utility of the enzyme, devising a cost-effective media formulation becomes a primary concern.

4. Conclusions

Streptomyces sp. DPUA1576 is a suitable microorganism to protease and fibrinolytic production. The maximum predicted protease production (83.42 U/mL) could be achieved with the medium consisting of soybean flour 1.26% and glucose 1.23% concentration. To fibrinolytic production, the high activity of 706.50 mm^2 was obtained at 1.7% of soybean flour and 1.0% of glucose which was 33% higher than plasmin positive control (176.62 mm^2). Therefore, the fibrinolytic protease production by *Streptomyces* sp. DPUA1576 emerges as a good alternative for further therapeutical application using biotechnology process.

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Conflict of interest

The authors declared no potential conflict of interest with respect to the research, authorship, and/or publication of this article.

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