Optimization of Keratinolytic Protease Production by *Purpureocillium lilacinum* LPS # 876 as a Sustainable Management of Tannery Hair Waste

by

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Abstract

Hair waste is recognized as a solid waste generated after a hair-saving unhairing process in leather technology, and it is produced in large amounts in Buenos Aires province, Argentine. The present method for this solid waste is disposal, which is environmentally troublesome and costly for the tanneries. Biodegradation of hair waste by keratinolytic microorganisms represents an alternative for bioconversion of keratin waste. This paper reports the optimization of keratinases production by *Purpureocillium lilacinum* LPS # 876. Firstly, one factor-at-a-time method was used to investigate the effect of nitrogen-sources, metal ions concentration in the basal mineral medium, as well as, initial pH and temperature on enzyme production. Medium composition for the keratinases production by *P. lilacinum* was optimized using two statistical methods: Plackett-Burman design and central composite design -combined with response surface methodology- used to optimize three significant variables: initial pH, glucose and $CaCl_2$. The optimal conditions were determined as 7.10 g l^{-1} of glucose; 0.0065 mg l^{-1} of $CaCl_2$ and initial pH of 5.60; at this condition the maximum keratinase yield predicted was 26.7 U ml^{-1}. The model validation showed that it described adequately the influence of glucose and calcium concentration and initial pH on enzyme production. Production of keratinases by *P. lilacinum* is a less-exploited field that might represent a novel and promising biotechnological application for this microorganism.

Resumen

El residuo pelo es conocido como un residuo sólido que es generado luego de un proceso de pelambre con recupero de pelo en la tecnología del cuero, y es producido en grandes cantidades en la provincia de Buenos Aires, Argentina. El método actual de tratamiento de este residuo es la disposición del mismo, lo que es problemático para el medio ambiente y costoso para las curtiembres. Su biodegradación por microorganismos queratinolíticos representa una alternativa para el tratamiento del mismo. En el presente trabajo se presenta la optimización de la producción de queratinasas por *Purpureocillium lilacinum* LPS # 876. En primer lugar, se utilizó el método de un factor a la vez, para investigar el efecto de las fuentes de nitrógeno, la concentración de iones metálicos en el medio mineral basal, así como, el pH inicial y la temperatura en la producción de la enzima. La composición del medio de cultivo para la producción de queratinasas por *P. lilacinum* se optimizó por el uso de dos métodos estadísticos: el diseño Plackett-Burman y el diseño central compuesto -combinado con metodologia de superficies de respuesta- utilizada para optimizar las tres variables estadísticamente representativas: pH inicial, glucosa y $CaCl_2$. Se determinaron las condiciones óptimas como 7.10 g l^{-1} de glucosa; 0.0065 mg l^{-1} de $CaCl_2$ y pH inicial de 5,60; en esta condición, el rendimiento máximo predecible en actividad queratinolítica fue 26.7 U ml^{-1}. La validación del modelo mostró que el modelo propuesto describe adecuadamente la influencia de la concentración de glucosa y el calcio y el pH inicial en la producción de enzimas queratinoliticas. La producción de queratinasas por *P. lilacinum* es un campo poco explotado que podría representar una nueva y prometedora aplicación biotecnológica para este microorganismo.

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INTRODUCTION

Keratinous wastes increasingly accumulate in the environment mainly in the form of feathers and hair waste, as byproducts of agroindustrial activities such as poultry processing and leather industries. Nowadays, these wastes are becoming a part of solid waste management and wastage, being a rich protein reserve because these keratin wastes are tough to degrade by common proteolytic enzymes. A highly rigid structure rendered by extensive disulfide bonds and cross linkages contributes to this recalcitrant nature. Hence, there is an indispensable and urgent need for developing biotechnological alternatives for recycling such wastes.[1,2]

Despite the recalcitrant nature, keratin wastes can be efficiently degraded by a variety of bacteria, actinomycetes and fungi due to the elaboration of keratinolytic proteases. Keratinases are special proteases, which attack 'hard-to degrade', insoluble keratin substrates. They are robust enzymes displaying a great diversity in their biochemical characteristics. They stand apart from the conventional proteases due to their broad substrate specificity towards a variety of insoluble, keratin rich substrates including feather, wool, nail and hair. The most important property of keratinases, which separates them from other proteases, is their substrate specificity. Owing to this ability, keratinases find immense applications in various environmental and biotechnological sectors where conventional proteases lag behind. By virtue of their broad substrates specificity, robustness and diverse biochemical properties, keratinases are considered as promising biocatalysts for several industries. Besides replacing conventional proteases in industrial applications, keratinases find immense application in sectors such as feather recycling, leather, textile, feed, fertilizers and cosmetics.[1,2]

The fungal biotransformation of the "hair waste" implies considering it as a raw material instead of the present idea of disposability. Thus, hair waste would be the substrate, on which the fungi would act, giving rise to a (partially) hydrolyzed protein with different potential uses (i.e., as animal feed, fertilizer, etc.). In addition, the fungi would produce a proteolytic (keratinolytic) extract of biotechnological interest with a variety of potential applications (named above). The commercial application of bulk enzymes depends on its production in high levels, at low or moderate costs. Therefore, knowledge of culture factors affecting the protease/keratinase yield is of essential importance. The enzyme production by microorganisms is strongly influenced by medium composition, thus optimization of media components and cultural parameters are the primary task in a biological process. The classical optimization strategy used is one-variable-at-a-time optimization, where each parameter is optimized by changing it while maintaining the other factors at a constant level.[3,4] Single variable optimization methods are not only tedious, but also can lead to misinterpretation of results, especially because the interaction effects among different factors are overlooked.[5] Nevertheless, limitations of the single factor optimization can be replaced by employing factorial designs and response surface methodology (RSM). RSM is a useful model for studying the effects of several factors influencing the responses by varying them simultaneously and carrying out a limited number of experiments. The Plackett-Burman (PB) is a fractional factorial design that allows the screening of the main factors from a large number of process variables. This design is quite useful in preliminary studies in which the principal objective is to select variables that can be fixed or eliminated in further optimization processes. RSM may be summarized as a collection of experimental strategies, mathematical methods and statistical inferences for constructing and exploring an approximate functional relationship between a response variable and a set of design variables. A number of studies have reported satisfactory optimization for keratinase production using statistical approach, but none of them used this new waste as substrate.[6-9]

In this work, the production of a protease with keratinolytic activity by *Purpureocillium lilacinum* LPS # 876 using hair waste was investigated. In order to identify significant variables influencing the keratinolytic enzyme production, a Plackett-Burman design was used. The levels of the significant variables were further optimized using central composite design (CCD).

MATERIALS AND METHODS

Reagents

Azocasein was purchased from Sigma Chemical Co. (St Louis, MO, USA). Potato-dextrose agar was purchased from Britania and the others chemicals used in ***basal hair medium*** as well as the ones used in the study of the effect of the addition of some salts on keratinolytic enzymes production and in the effect of nitrogen sources were purchased from Biopack and were of analytical degree.

Microorganism and culture conditions

P. lilacinum (formerly *Paecilomyces lilacinus*) LPS #876 belonging to Spegazzini Institute fungal culture collection (La Plata National University, Argentina) was used. It was maintained in potato-dextrose agar slants under mineral oil at 4ºC. Cultures were performed in 500-ml Erlenmeyer flasks with 100 ml of *basal hair medium* containing (per liter) 10 g hair waste, 496 mg NaH_2PO_4, 2.486 g K_2HPO_4, 16 mg $FeCl_3.6H_2O$, 13 mg $ZnCl_2$, 10 mg $MgCl_2$, and 0.11 mg $CaCl_2$ (pH 7.0). Hair waste, obtained from a local tannery, was washed extensively with tap water; dried at 60ºC for 2 days and then ball-milled; and used as the source of carbon, nitrogen, and sulfur. The culture flasks were autoclaved at

121ºC for 15 min for sterilization and then, after cooling, inoculated with 2 x 10^7 conidia per ml. The cultures were incubated in an orbital shaker (New Brunswick Scientific Co, New Jersey, USA) at 200 rpm and 28ºC. At a regular time interval (every 6 h), samples of 5ml were withdrawn and centrifuged at 5,000 g and 4ºC for 20 min in order to precipitate the fungal biomass. The cell-free supernatant was used for pH determination and then kept at -20ºC until used for determination of enzyme activity.

Determination of proteolytic activity

Protease activity was measured as described by Liggieri et al.[10] with azocasein as substrate, but with some modifications. An aliquot of 0.1 ml of the crude enzyme, suitably diluted (dilution factor 1/10), was mixed with 0.250 ml of Tris-HCl buffer (100 mM, pH 9.0) containing 1% (w/v) azocasein, and incubated for 30 min at 37ºC. The reaction was stopped by the addition of 1.0 ml of trichloroacetic acid (10%, w/v). After a further 15 min at room temperature, the mixture was centrifuged at 5,000 g for 10 min. A reaction blank was performed with 0.1 ml of heat-inactivated enzyme (10 min at 100ºC). One ml of 1M NaOH was then added to 1 ml of the supernatant and the absorbance measured at 440 nm. One unit of protease activity was defined as an increase of 0.1 unit per minute in the absorbance at 440 nm under those experimental conditions.

Optimization of fermentation medium using one-variable-at-a-time method

Effect of environmental conditions on production of keratinases

Temperature (28 and 37°C) and initial pH (5.0, 6.0, 7.0 and 8.0) were evaluated using the hair basal media.

Effect of the concentration of the metal ions present in the basal mineral medium

Several experiments were done in order to study the effect of concentrations of four metal ions (Ca^{2+}, Fe^{3+}, Zn^{2+} and Mg^{2+}) present in hair basal medium on keratinase production. Concentrations equivalent to 1:2, 1:4, 1:10, 1:25 and 1:50 of the original medium were tested. The initial pH of the medium was set at 7.0.

Effect of the addition of some salts on keratinolytic enzymes production

Previously, Mizusawa et al.[12] and Ferreyra et al. [13] reported that the addition of Mn^{+2} and Co^{+2} improved enzyme production considerably by supplementing with cobalt ($CoCl_2.6H_2O$) and manganese ($MnCl_2.4H_2O$) at a concentration of 2mg/l and a mixture of these two metal ions to the *hair basal medium*. The initial pH of the medium was set at 7.0.

Effect of nitrogen sources

The influence of various nitrogen sources on the keratinase production was studied. Yeast extract (2.23 g l^{-1}), urea (0.40 g l^{-1}), $(NH_4)_2SO_4$ (1.10 g l^{-1}) and KNO_3 (1.69 g l^{-1}) were added to the hair basal medium. The initial pH of the medium was set at 7.0.

In order to validate the one-at-a-time approach, a culture was performed using the optimal condition of each variable.

Identification of the significant factors by Plackett-Burman (PB) design

The significant medium components with respect to their main effects were screened by PB design with a two-factorial design. It identified the main parameters required for maximal keratinases production by screening *n* variables in *n+1* experiments; each variable was examined at two levels.[14] Table I lists the factors under investigation as well as the level of each factor used in the experimental design with the actual code level of the variables. Results were analyzed by Design Expert® (Stat-Ease, Minneapolis, MN, USA) version 8.0.7.1 trial version software. The factors significant at 90% of confidence level ($p < 0.10$) were further optimized by a central composite design.

Central Composite Design and Response Surface Methodology

Response surface methodology (RSM) is a collection of mathematical and statistical techniques that are useful for the modeling and analysis of problems, in which a response of interest is influenced by several variables and the objective is to optimize this response [15]. After the selection of the significant parameters by PB design, the next stage was to determine the optimal levels of three variables, glucose and $CaCl_2$ concentration and initial pH on keratinase production. For this purpose, the RSM by using a set of experimental design (central composite design with five coded levels) was performed. For the three factors, the design was made up of a full 2^3 factorial design with its four points augmented, four points diminished, six replications of the central point (all factors at level zero) and the six star points, that is, points having one factor an axial distance to the center of $\pm\alpha$, whereas the other two factors are at level zero. The axial distance α was chosen to be 1.68 to make the design orthogonal. A set of 20 experiments was carried out. The range and levels of experimental variables investigated are presented in Table II. The central values (zero level) chosen for experimental design were: Glucose concentration 5g/l, $CaCl_2$ concentration 0.0110 mg/l and initial pH: 6.0. Although culture time was not considered as a variable for statistical design, the enzyme activity was evaluated after 94, 110 and 118 h of incubation, but the regression equation was made with enzyme activities obtained at 94 h of incubation (maximal values).

For statistical analysis, each independent variable X was coded as x_i according to the following equation:

$$x_i = (X_i - X_0)/ DX_i$$

TABLE I
Plackett-Burman experimental design matrix with protease enzyme production levels

	Variable							Protease activity (Uc/ml)		
Trial code	pH	$CaCl_2$ (mg/l)	$MgCl_2$ (mg/l)	$ZnCl_2$ (mg/l)	$FeCl_3.6H_2O$ (mg/l)	Glucose (g/l)	Yeast extract (g/l)	94 h	110 h	118 h
1	5.5	0.014	0.75	0.97	2.0	7.5	3.0	15.5	15.7	9.6
2	5.5	0.008	0.75	0.97	1.2	2.5	1.0	12.0	10.2	6.7
3	6.5	0.014	0.75	1.62	1.2	2.5	3.0	6.8	4.4	3.0
4	6.0	0.011	1.0	1.30	1.6	5.0	2.0	11.1	7.5	5.5
5	6.5	0.014	1.25	0.97	2.0	2.5	1.0	8.2	6.2	4.7
6	6.5	0.008	1.25	0.97	1.2	7.5	3.0	8.6	8.4	6.6
7	6.0	0.011	1.0	1.30	1.6	5.0	2.0	8.5	7.1	5.7
8	6.5	0.008	0.75	1.62	2.0	7.5	1.0	6.8	5.7	4.1
9	5.5	0.014	1.25	1.62	1.2	7.5	1.0	18.8	17.6	9.8
10	5.5	0.008	1.25	1.62	2.0	2.5	3.0	11.9	9.5	5.6
11	6.0	0.011	1.0	1.30	1.6	5.0	2.0	9.3	7.7	4.6

TABLE II
Values of independent variables at different levels of 2^3 factorial design.

Independent variables	Symbol	Levels				
		-1.68	-1.0	0	1.0	1.68
Initial pH	x_1	5.2	5.5	6.0	6.5	6.8
Glucose (g/l)	x_2	0.8	2.5	5.0	7.5	9.2
$CaCl_2$ (ng/l)	x_3	6.5	8.3	11.0	13.7	15.5

where x_i is the coded value of the ith independent variable, X_i the real value of the ith independent variable, X_0 the real value of the ith independent variable at the center point and DX_i is the step change value. For a three-factor system, the model equation was:

$$Y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3$$

where Y is the predicted response; b_0 is the intercept; b_1, b_2, b_3 are the linear coefficients; b_{11}, b_{22}, b_{33} are the squared coefficients and b_{12}, b_{13} and b_{23} are the interaction coefficients.

Results were analyzed by Design Expert,® version 8.0.7.1 trial version software. The model permits the evaluation of the effects of linear, quadratic and interactive terms of the independent variables on the dependent variable. The statistical significance of the regression coefficients was determined by Student`s t-test and the second order model equation was determined by Fisher`s test. Three-dimensional surface plots were drawn to illustrate the main and interactive effects of the independent variables on keratinolytic protease production. The optimum values of the selected variables were obtained by solving the regression equation and also by analyzing the response surface contour plots.[16] The existence of a correlation between the predicted and the experimental values justified the model validity.[16] Four glucose and $CaCl_2$ concentrations and initial pH points were chosen, and for these points, the predicted enzyme activities were calculated using the generated equation. For model validation, these activity values were then compared with enzyme activities obtained in actual experiments.

Biochemical properties of crude enzyme

Detection of keratinolytic activity on polyacrylamide gels (casein and keratin-zymograms)

Casein and keratin-zymograms were performed to test protease and keratinase activity, respectively. Zymograms were performed in conjunction with SDS-PAGE according to the method of García-Carreño et al.[17] with slight modification. SDS-PAGE was performed as described by Laemmli,[18] using 5% (w/v) stacking gel and 12 % (w/v) separating gel. In the case of SDS-PAGE, samples heated and not heated were used and for zymograms the sample was not heated prior to electrophoresis. For zymograms, after electrophoresis, the gel was submerged in 100 mM Tris-HCl buffer (pH 9.0) (buffer A) containing 2.5% Triton X-100 for 60 min, with constant agitation in an orbital shaker (New Brunswick Scientific Co, New Jersey, USA) at 50 rpm and 28ºC in order to remove SDS. Triton X-100 was then removed by washing the gel three times with buffer A. A portion of the gel was then incubated with 1% (w/v) casein or soluble keratin in buffer A at 28ºC for 30 min and 90 min, respectively. Finally, gels were stained with Coomassie Brilliant Blue R-250 for zymography analysis. The development of clear zones on the blue background of the gels indicated the presence of protease or keratinase activity. The molecular mass markers used were: phosphorylase b (97 kDa); albumin (66 kDa); ovoalbumin (45 kDa); carbonic anhydrase (30 kDa); trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa).

Substrate specificity

The enzyme activity was tested on different keratinous substrates. Activity on feather meal, hair waste, feather and wool was essentially as follows: the enzyme solution (100 μl; 20 U ml^{-1}) was added to 900 μl of substrate suspension (5 mg ml^{-1} and 30 mg ml^{-1} in the case of feather meal) in reaction buffer 100 mM Tris-HCl buffer (pH 9.0). The admixture was incubated at 37ºC for 30 min in the case of feather meal and for 90 min for the other substrates, stopped by dipping the preparation into ice-cold water for 30 min, centrifuged at 10,000 g for 10 min at 4ºC, and absorbance at 280 nm was measured. One unit of enzyme activity was defined as the amount of protein that produce an increase of absorbance at 280 nm of 0.01 under the assay conditions used.[19]

RESULTS

Optimization using one-variable-at-a-time approach

Firstly, the determination of optimal growth conditions yielding the highest proteases activities was achieved by analyzing the influence of several factors using one-variable-at-a-time approach. Keratinolytic protease production was investigated at two temperatures (28 and 37ºC). Maximum protease production of 2.46 U ml^{-1} was achieved around 111-117 h of incubation at 28ºC, meanwhile at 37ºC, poor growth of *P. lilacimun* LPS#876 and hence a poor keratinase activity was observed (Table III). The effect of initial pH on enzyme activity was investigated in a pH range of 5.0-8.0. Medium was adjusted to the required pH before sterilization. As can be seen in Table III, *P. lilacimun* was able to grow and produce proteases in a broad pH range. However, optimum production was obtained at pH 6.0.

TABLE III
Effect of temperature (ºC) and initial pH of the medium on proteases production. Values are the average of three independent experiments ±standard deviations

	Protease activity (U_c/ml)
T (ºC)	
28	2.46 ± 0.04
37	0.31 ± 0.05
initial pH *(T:28ºC)*	
5	1.35 ± 0.03
6	4.23 ± 0.30
7	2.46 ± 0.04
8	2.16 ± 0.04

In order to study the effect of reducing the concentration of metal ions present in the basal mineral medium, several submerged cultures were carried out using diluted concentrations of four metal ions present on basal mineral medium (Ca^{2+}, Fe^{3+}, Zn^{2+} and Mg^{2+}). It is clear from the results presented on Figure 1A that the enzyme production was considerably enhanced when the concentration of these four elements were reduced up to 1:10. In addition to the above study, the effect of the supplementation with divalent cations (Co^{2+} and Mn^{2+}) was also included. Cations were individually added to the ***hair basal medium*** at a concentration of 2 mg l^{-1}, and a mixture of them was also tested (2 mg l^{-1} each). The initial pH of the medium was set at 7.0. As shown in Figure 1B, protease activity decreased when the hair basal medium was supplemented with $CoCl_2.6H_2O$, $MnCl_2.4H_2O$ and a mixture of these two metals, compared to the control.

The last experiment conducted using one-variable-at-a-time approach was the study of the effect of nitrogen sources on *P. lilacinum* enzymes production. In these experiments, 17 mM of nitrogen of each co-nitrogen source was added to the ***hair basal medium***. Figure1C showed that addition of yeast extract and $(NH_4)_2SO_4$ increased keratinase production 1.56-fold and 1.13-fold, respectively. However, the addition of others co-nitrogen sources, such as Urea or KNO_3, repressed keratinase production.

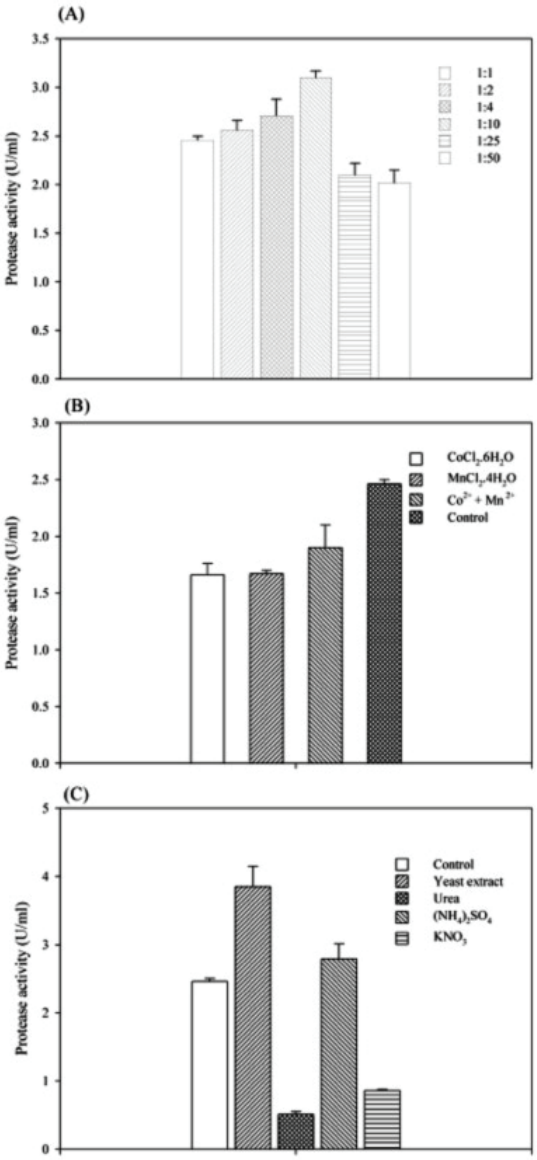

Figure 1. **(a)** Influence of various dilutions of the metal ions present in the mineral medium on enzyme production. **(b)** Effects of the supplementation with $CoCl_2.6H_2O$, $MnCl_2.4H_2O$ and a mixture of these two metals on basal hair medium. **(c)** Influence of co-nitrogen sources on keratinolytic enzyme production by *P. lilacinum* in basal hair medium. Values are the average of three independent experiments ±standard deviations.

The maximum yield of the enzyme using one-variable-at-a-time approach was 15.96 U ml^{-1}; this value was about 6.5-fold higher than the yield in the hair basal medium. Although one-variable-at-a- time methodology is a tedious methodology, it gave us the range of concentrations, which we should focus. For example, it was useful in order to see that the original concentration of metal ions present in the basal mineral medium was higher than the fungi needs to grow and produce crude enzyme.

Selection of significant variables by Plackett-Burman design

After optimization using one-variable-at-a-time approach, a total of seven variables were analyzed with regard to their effects on keratinase production using PB design. The design matrix to the screening of significant variables for keratinases production and the corresponding responses are shown in Table I. Although enzyme activities were tested after 94, 110 and 118 h of incubation, the adequacy of the model was calculated at 94 h where treatment 9 showed the highest level of enzyme activity (Table IV). The variables tested were screened for their statistically significance via Student's *t*-test (ANOVA). Analysis of the results of PB design showed that pH (p = 0.009) and glucose (p = 0.100) had significant effects on keratinase production. The total R^2 was 0.9432, indicating that 94.32% of the experimental results could be explained by this model.

TABLE IV
Identification of significant variables for keratinolytic enzyme production by *P. lilacinum* using Plackett-Burman design *(where SS: sum of squares; df: freedom degrees; MS: mean square and F-value: Fisher number)*

	SS	df	MS	F-value	*P*-value
pH	97.35	1	97.35	36.53	0.009 [a]
$CaCl_2$	12.68	1	12.68	4.76	0.117 [c]
$MgCl_2$	5.11	1	5.11	1.92	0.260 [c]
$ZnCl_2$	0.00	1	0.00	0.00	0.997 [c]
$FeCl_3.6H_2O$	1.87	1	1.87	0.70	0.464 [c]
Glucose	14.50	1	14.50	5.44	0.100 [b]
Yeast extract	1.24	1	1.24	0.47	0.543 [c]
Error	7.99	3	2.66		
Total SS	140.74	10			

[a] significant negative effect
[b] significant positive effect
[c] non-significant at P<0.1

Optimization by response surface methodology (RSM)

Central composite design (CCD) was used in order to determine the optimum concentration and value of three factors selected from the PB design (pH, glucose and $CaCl_2$). Although $CaCl_2$ showed no significant effect, it was selected because the experimental results suggested a relation between this salt and the protease activity (data not shown). A total of 20 experiments with different combinations of the selected variables were performed. The design matrix with the corresponding results of CCD experiments, as well as the predicted results is presented in Table V. The regression equation coefficients were calculated and the data was fitted to a second-order polynomial equation.

The response of protease production (Y) by *P. lilacinum* LPS # 876 can be expressed in terms of the following regression equation:

$$Y= -368.713+132.487\ x_1+15.7193\ x_2-8626.28\ x_3-10.937\ x_1^2- 1.7119\ x_1x_2 + 562.873\ x_1x_3- 0.3953\ x_2^2- 17.0191\ x_2x_3+ 211035.0\ x_3^2$$

Where x_1 is initial pH; x_2 is glucose (g/l) and x_3 is $CaCl_2$ (mg/l).

Statistical analysis of the optimization study indicated that, among the three variables selected by PB design experiment, glucose (x_2) and $CaCl_2$ (x_3) were found to have significant effect on enzyme production. Furthermore, the interaction between initial pH and glucose (x_1x_2), as well as squared terms x_1^2, x_2^2 and x_3^2 were also significant (limit of significance for 95% of significance) (Fig. 2).

The regression equation obtained from the ANOVA analysis showed that the value of R^2 (multiple correlation coefficient) is 0.892. This value indicated that only 10.8% of the total variation could not be explained by the model. The adjusted value determination coefficient (Adj-R^2 = 0.783) further confirmed the significance of the model.

Response surface and contour plot figures obtained by the analysis of the experimental data of CCD showed the relationship between two of the three variables at time while maintaining third variable at fixed level. These figures are helpful in understanding both the linear and interaction effects of two variables. The 3-D response surface plot described by the regression model was drawn to illustrate the combined effects of the independent variable upon the response variable. Figure 2a shows the interaction of initial pH and glucose concentration with $CaCl_2$ concentration fixed at its middle level. It can be seen that a linear increase on protease production was observed when glucose concentration and initial pH increased up to 9.0 g l^{-1} and 6.10, respectively. The elliptical nature of the contour plot shows the existence of a significant interaction relationship between initial pH and

TABLE V
Experimental design and results of the 2^3 factorial design.

Run number				Protease activity (U/ml)	
	pH	Glucose (g/l)	$CaCl_2$ (ng/l)	Observed	Predicted
1	6.5	2.5	8.3	10.1	12.3
2	6.0	5.0	11.0	17.9	16.7
3	6.5	7.5	13.7	13.5	12.4
4	5.2	5.0	11.0	8.3	9.9
5	6.5	7.5	8.3	15.1	14.8
6	5.5	2.5	8.3	10.4	10.7
7	6.0	5.0	11.0	16.9	16.7
8	6.5	2.5	13.7	11.2	10.3
9	6.0	0.80	11.0	5.3	4.2
10	5.5	2.5	13.7	6.3	5.7
11	6.0	5.0	11.0	15.1	16.7
12	5.5	7.5	13.7	19.4	16.3
13	5.5	7.5	8.3	27.4	21.7
14	6.8	5.0	11.0	8.4	8.0
15	6.0	5.0	11.0	17.2	16.7
16	6.0	5.0	6.5	25.9	24.2
17	6.0	5.0	11.0	14.7	16.7
18	6.0	5.0	15.5	14.9	17.9
19	6.0	9.2	11.0	12.9	15.2
20	6.0	5.0	11.0	18.5	16.7

glucose concentration. The data observed when concentration of $CaCl_2$ and the initial pH were varying, keeping glucose concentration at 5 g l^{-1}, was plotted (Figure 2b). It shows that initial increase in pH with simultaneous decrease in $CaCl_2$ concentration resulted in an increase of protease production. However, the increase beyond initial pH of 6.0 affected the enzyme production. Similar behavior was observed when the effect of glucose concentration and $CaCl_2$ concentration was studied keeping initial pH constant at 6.0.

The plot revealed that the protease production was high at lower concentration of $CaCl_2$ and increasing glucose concentration up to 8.0 g/l resulted in an increase of keratinase production, after that limit it can be seen that as long as glucose concentration increase the production of the enzyme decreases (Figure 2c). The model predicted that maximum keratinolytic enzyme production of 26.47 U/ml was achieved in an ***optimum hair medium*** containing (per liter) 10 g hair waste, 496 mg NaH_2PO_4, 2.486 g K_2HPO_4, 16 mg $FeCl_3.6H_2O$, 13 mg $ZnCl_2$, 10 mg $MgCl_2$, and 0.0065 mg $CaCl_2$, 2 g of yeast extract and 7.10 g of glucose (pH 5.6). To validate the RSM, four points were chosen and the experimental and predicted enzymatic activities were determined to these points (Table VI). In biological processes, a difference of about 20% between experimental and predicted results is acceptable.[20] As all values are within this range, the model described adequately the influence of glucose concentration, initial pH and $CaCl_2$ concentration on enzyme production.

Detection of keratinolytic activity on polyacrylamide gels (casein and keratin-zymograms)

Zimograms of the crude enzyme preparation revealed the presence of at least two clear zones when casein was used as substrate and just one clear zone when soluble keratin was used as substrate, suggesting the presence of just one keratinase (the development of clear zones on the blue background of the gels indicated the presence of protease or keratinase activity) (Figure 4a).

Substrate specificity

Keratinolytic activity was tested on several substrates. Incubation was carried out using the substrates at the concentration of 5 mg ml^{-1} for hair waste, feather and wool and in the case of feather meal the concentration was 30mg ml^{-1}. Among the natural keratinous proteins tested, the enzyme was active against all the proteins tested with high keratinolytic activity against feather meal follow by hair waste (Figure 4b).

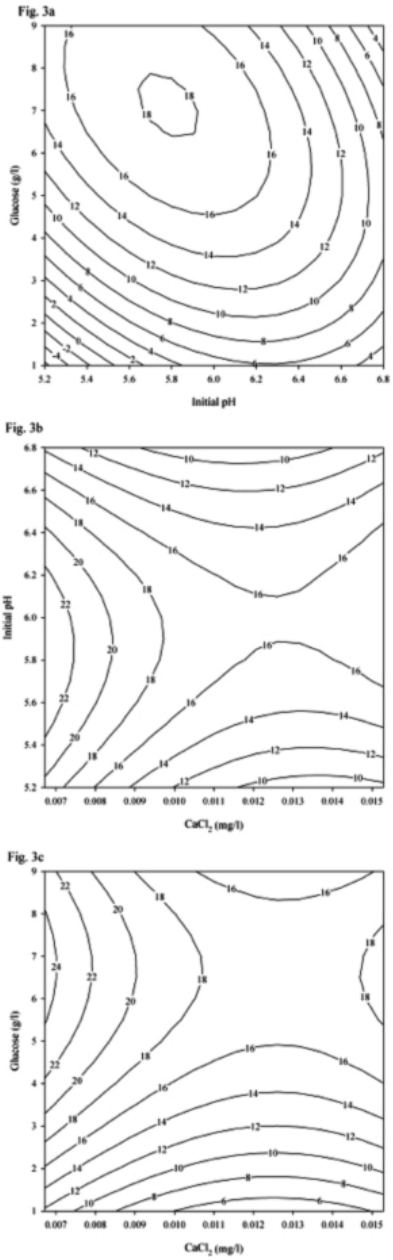

Figure 2: (a) Protease production (U/ml) observed as a response to the interaction of glucose concentration (g/l) and initial pH as variables and $CaCl_2$ concentration at central point. (b) Protease production (U/ml) observed as a response to the interaction of $CaCl_2$ concentration (g/l) and initial pH as variables and glucose concentration at central point. (c) Protease production (U/ml) observed as a response to the interaction of glucose concentration (g/l) and $CaCl_2$ concentration (mg/l) as variables and initial pH at central point.

DISCUSSION

Improving the microbial protease production is the aim of several investigations, where the production capacity of the organism depends on the successful selection of growth conditions and substrate.[21] Several researches attempted to induce protease production by using glucose and starch, coupled with expensive nitrogen sources such as yeast extract, peptone or casamino acids. However, few studies have been made to induce protease production using inexpensive carbon and nitrogen sources.[22,23] Since hair waste is readily available, abundant and polluting the environment, its utilization as substrate by *P. lilacinum* LPS # 876 could result in a cost-effective process.

TABLE VI
Experiments for model validation

Run	initial pH	Glucose (g/l)	$CaCl_2$ (ng/l)	Experimental	Predicted	Difference (%)
1	6.0	5.0	8.3	19.9	20.1	0.8
2	6.5	3.0	5.0	17.8	20.7	14.2
3	6.0	10.0	5.0	20.4	25.1	18.6
4	5.2	2.0	5.0	15.0	13.1	12.6

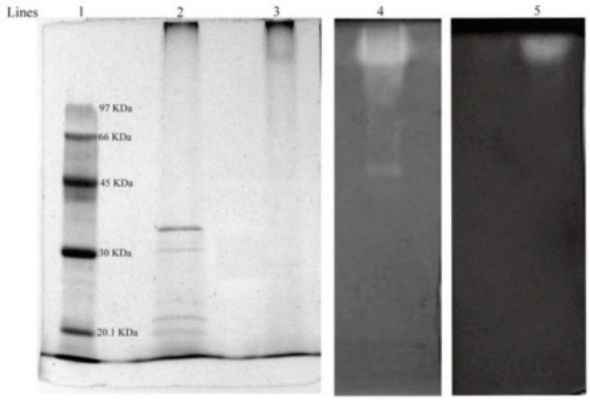

Figure 3: SDS-PAGE and zymograms analysis of the crude extract. Line 1: molecular marker. Line 2: heated crude extract supernatant. Line 3: not heated crude extract supernatant. Line 4: casein zymograms and Line 5: keratin zymograms.

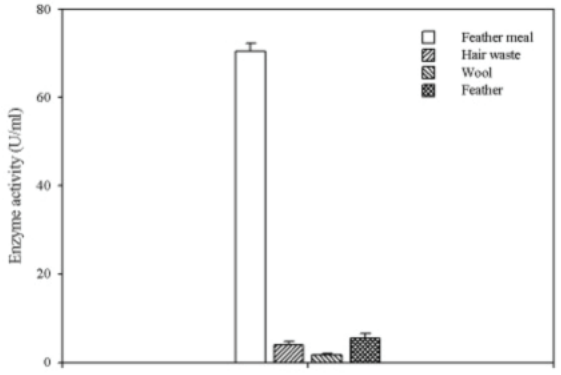

Figure 4b: Substrate specificity of *P. lilacinum´s* crude extract

In general, both organic and inorganic nitrogen sources were used effectively for protease production. The maximum enzyme activity produced by *P. lilacinum* was achieved with yeast extract as nitrogen co-source (1.56-fold), followed by $(NH_4)_2SO_4$ (1.13-fold) compared to the control without nitrogen co-source (2.46 U ml^{-1}). Through these results, it can be concluded that the addition of yeast extract increased significantly the production of extracellular proteases. This is in line with previous findings reported that yeast extract enhanced enzyme production.[4,24] Few studies have demonstrated that extracellular enzyme production is. substantially influenced not only by carbon and nitrogen sources, but also by trace elements.[12,13].In our case, the effect in diluting the metal ions present in the ***basal hair medium*** up to 1:10, enhanced the enzyme production considerably. Although Mizusawa et al.[12] and Ferreyra et al.[13] reported that the addition of Mn^{+2} and Co^{+2} improved enzyme production, the same behavior was not observed in our case where the presence of these metal ions decreased enzyme production; similar results were reported by Fakhfakh-Zouari et al.[23] when the effect of $MnSO_4$ on enzyme production was tested.

Optimization by a conventional "one-at-a-time-approach" leads to a substantial increase in enzyme yields (6.5-fold higher than the yield in the basal hair medium), however, this approach is not only awkward and time-consuming, but also has the limitations of ignoring the importance of interaction of the parameters [16]. The use of statistical models to optimize culture medium components and conditions has increased in present-day biotechnology, due to its easy applicability, reliability and validity. In the current study, the significant variables necessary for the enhancement of keratinolytic enzyme production were selected using the Plackett-Burman design. The RSM used for optimization of enzyme production by *P. lilacinum* LPS # 876 indicated a significant interaction between initial pH and glucose concentration. Analyzing RSM it can be seen that at lower concentration of $CaCl_2$ the best values of glucose concentration or initial pH for the best enzyme production were in the middle range. The CCD used in this study enabled us to improve keratinolytic enzyme production by about 10.8-fold. This improvement seems to be higher was reported for *Microbacterium sp.* kr10 [22] where a 3.6 fold increase in protease production was obtained. For *Bacillus mojavensis*, a 4.2-fold increase was observed [25]. A high degree of similarity was observed between the predicted and the experimental values reflecting the accuracy and applicability of RSM to optimize the process for enzymes production. RSM was successfully applied in the production of keratinase by *Kocuria rosea* [6]; *Microbacterium sp.*;[22] *Bacillus pumilis* A1[23] and *Aspergillus niger.*[9]

Finally, a maximum keratinolytic enzyme production (26.47 U ml^{-1}) was predicted at the following conditions: temperature 28ºC, speed agitation 200 rpm, pH 5.60; 10 g/l of hair waste, 7.10 g l^{-1} of glucose, 2.0 g l^{-1} yeast extract, 0.0065 mg l^{-1} $CaCl_2$ and other salts which were taken at central level as shown in PB design. By optimizing the medium composition, not only the production of the enzymes was enhanced from 2.46 U ml^{-1} to 26.47 U ml^{-1} but also the cost/benefit of the process was reduced since a higher productivity was achieved. In this study, *P. lilacinum* LPS#876 was found to produce at least two proteases but just one of them has keratinolytic activity as revealed in its casein and keratin zymograms. These results are in line with those reported by Huang et al.[26] where just one protease was produced by *B. pumilis* with dehairing capabilities. Nevertheless, several works reported the production of more than one extracellular protease. Agrebi et al.[27] reported the production of at least seven proteases by *B. pumilis* A 26; Xie et al.[28] reported the presence of five proteases in the culture supernatant of *Streptomyces sp.* strain 16 growing on human foot skin medium. Mazotto et al.[29] reported the production of seven proteases in the culture supernatant of human hair medium supplemented with yeast extract by *B. subtilis* AMR from which three resulted to be keratinases according to the keratin zymograms. Interestingly, when the supernatant was subjected to SDS-PAGE and to the corresponding zymograms it could be seen that enzyme activities were not affected by the presence of β-mercaptoethanol neither by SDS (in SDS-PAGE solutions) and the apparent molecular weight seems to be high. But when the enzyme was heated its apparent molecular weight resulted to be near 37 KDa. Moreover when the supernatant was ran through a Superdex 75 chromatography the active fraction collected was one that corresponds to a molecular mass near to 35 KDa (data not shown), suggesting that the presence of SDS could induce conformational changes that did not affect enzyme activity. At high temperatures, denaturation "opens" the polypeptide chain, rearranging the pattern of intra and intermolecular interactions within the protein and with the solvent.[30] Similar behavior was reported by Muga et al.[31] about the interaction of SDS with β-Galactosidase.

Different keratinous substrates were incubated with the crude extract, showing that feather meal was the most hydrolyzed followed by feather and hair waste. With sheep wool lower amounts of degradation products could be detected after 90 min incubation. The high activity against feather meal in comparison to the other keratinous materials tested may be attributed to the thermal and mechanical treatment suffered by this substrate. The crude extract showed that has the ability to hydrolyze several keratinous proteins, like observed for proteases of *Chryseobacterium* sp.[32] and *Doratomyces microsporum*.[33]

Conclusion

The statistical methods used in the present study enabled us to optimize the keratinase production medium, giving a 10.8-fold increase compared with the un-optimized medium. Using an optimized formulation will provide a basis for the development of new technology for the large-scale production of keratinolytic enzymes. Optimization is an essential step for the industrial production of large amount of these important bioproducts.

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