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# Simultaneous production of amylases and proteases by *Bacillus subtilis* in brewery wastes

*Alina Sánchez Blanco*[a], *Osmar Palacios Durive*[a], *Sulema Batista Pérez*[a], *Zoraida Díaz Montes*[a], *Nelson Pérez Guerra*[b,*]

[a] *Departamento de Fundamentos Químicos y Biológicos, Facultad de Ingeniería Química, sede Mella, Universidad de Oriente, Santiago de Cuba, Cuba*

[b] *Departamento de Química Analítica y Alimentaria, Facultad de Ciencias, Campus de Ourense, Universidad de Vigo, Spain*

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ABSTRACT

The simultaneous production of amylase (*AA*) and protease (*PA*) activity by *Bacillus subtilis* UO-01 in brewery wastes was studied by combining the response surface methodology with the kinetic study of the process. The optimum conditions ($T$ = 36.0 °C and pH = 6.8) for high biomass production (0.92 g/L) were similar to the conditions ($T$ = 36.8 °C and pH = 6.6) for high *AA* synthesis (9.26 EU/mL). However, the maximum *PA* level (9.77 EU/mL) was obtained at pH 7.1 and 37.8 °C. Under these conditions, a considerably high reduction (between 69.9 and 77.8%) of the initial chemical oxygen demand of the waste was achieved. In verification experiments under the optimized conditions for production of each enzyme, the *AA* and *PA* obtained after 15 h of incubation were, respectively, 9.35 and 9.87 EU/mL. By using the Luedeking and Piret model, both enzymes were classified as growth-associated metabolites. Protease production delay seemed to be related to the consumption of non-protein and protein nitrogen. These results indicate that the brewery waste could be successfully used for a high scale production of amylases and proteases at a low cost.

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## Introduction

The amylases and proteases produced by the *Bacillus* species are of world-wide interest for their important industrial applications.[1] Therefore, production of these enzymes should be carried out at a low production cost by using economically available culture media (such as food wastes or agro-industrial residues) and optimized fermentation conditions.[2,3]

Different studies have shown that production of amylases and proteases is affected by a variety of physicochemical factors, including the type and composition of the substrate, incubation time and temperature, pH, agitation and the concentration and type of the carbon and nitrogen sources.[1,3] However, in some cases, the effects of these variables on enzyme production have been studied by using the “one variable at a time” method[1,3,4] rather than using response surface methodology (RSM). The first method is time

* *Corresponding author.*
E-mail: nelsonpg@uvigo.es (N. Pérez Guerra).
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consuming and could lead to an incomplete interpretation of the behavior of the system, resulting in a lack of predictive ability, mainly when there are interactions between the independent factors.[5] In contrast, the use of RSM allows the reduction in the number of experiments and obtains empirical mathematical models describing the effect (both linear and quadratic) of each independent factor and their interactions on the response variables.[5]

Considering the substantial availability of brewery wastes (BWs) with a high chemical oxygen demand (*COD*) at very low prices from a brewery plant in Santiago de Cuba (Cuba), the use of this effluent as a fermentation substrate could offer an attractive alternative for the low cost production of amylases and proteases by *Bacillus subtilis* UO-01. However, to the best of our knowledge, there is no information available on the use of brewery waste for production of amylases and proteases by *Bacillus* strains.

In the present study, we investigated the suitability of this waste to support both the growth and enzyme production by *B. subtilis* UO-01 at different initial values of pH and incubation temperatures. For this purpose, the time courses of the synthesis of amylases, proteases, biomass and total sugars consumption were followed for 30 h. Subsequently, the concentrations of enzymes and biomass obtained were modeled by using the corresponding logistic model to smooth the experimental data obtained and reduce the experimental error. The data predicted by the logistic model in each case at the appropriate fermentation time were then used for the RSM analysis to obtain the optimum pH and temperature values for high production of amylases, proteases and biomass. In addition, the enzyme production system was studied under the optimum conditions to verify the effectiveness and the accuracy of the empirical enzyme model obtained. After typifying both the amylase and protease production with the Luedeking and Piret model,[6] the relationship between protease production and consumption of total nitrogen and proteins was studied at different culture pH values and at the optimum temperature for growth of *B. subtilis* UO-01.

## Materials and methods

### *Bacterial cultures*

*B. subtilis* UO-01, the amylase- and protease-producing strain, was acquired from the Biotechnology Center of the University of Oriente (Santiago de Cuba, Cuba). Stock cultures were maintained at 4 °C on nutrient agar slants. The working cultures were prepared monthly from frozen stock cultures and maintained at 4 °C on nutrient agar (Cultimed Panreac Química S.A.).

### *Culture medium preparation, inoculum and fermentation conditions*

Brewery wastes (BWs), which were used to prepare the culture media, were obtained from a local brewery in Santiago de Cuba, Cuba. The wastes were centrifuged at 12,000 × *g*/15 min to remove the solids in suspension. The supernatant obtained from the BWs contained (g/L): *COD*, 3.40; total sugars, 1.98; reducing sugars, 1.46; total nitrogen, 0.095; total phosphorus, 0.034.

**Table 1 – Experimental domain and codification of the independent variables (*T*: temperature and pH) used in the experimental design.**

| Coded values | Actual values | |
|---|---|---|
| | *T* (°C) | pH |
| −1.267 | 28.0 | 4.0 |
| −1 | 30.0 | 4.5 |
| 0 | 37.8 | 6.2 |
| 1 | 45.5 | 8.0 |
| 1.267 | 47.6 | 8.5 |

Inoculum was prepared by transferring (with a sterile inoculation loop) some colonies of a 24-h old slant culture into 250 mL Erlenmeyer flasks containing 50 mL of sterile medium composed of (g/L): glucose, 20; bacteriological peptone, 2.5; $KH_2PO_4 \cdot 3H_2O$, 1.5; $Na_2SO_4$, 1.5; $MgSO_4 \cdot 7H_2O$, 0.15, $FeSO_4 \cdot 7H_2O$, 0.03; $MnCl_2 \cdot 4H_2O$, 0.1; $CaCl_2 \cdot 2H_2O$, 0.45. After inoculation, the medium was adjusted to pH 6.8 and sterilized at 121 °C/15 min. The inoculated culture was then incubated at 36 °C/12 h (200 rpm).

The production BWs medium was supplemented with the same ingredients as the medium used to prepare the inoculum, but in this case, soluble potato starch (at a concentration of 10 g/L) was used instead of glucose. The media were buffered at different initial pH values with the appropriate buffer (0.1 M potassium hydrogen phthalate–HCl buffer for pH values of 4.0, 4.5 and 6.2 or 0.1 M sodium phosphate buffer for pH values of 8.0 and 8.5) according to the experimental design defined in Table 1 and then sterilized (121 °C/15 min).

Batch cultures were performed in triplicate in 250 mL Erlenmeyer flasks containing 50 mL of the corresponding buffered medium. Each flask was inoculated with a 2% (v/v) inoculum level (with an absorbance of 0.5 at 600 nm) of a 12-h inoculum culture and incubated in an orbital shaker (200 rpm) for 30 h at the corresponding temperature according to the experimental matrix defined in Table 1.

To study the relationship between nitrogen and protein consumption and protease production by strain UO-01 at different initial pH values, the BWs media were buffered with the same buffers used in the former experiment to obtain initial pH values of 4, 5, 6, 7, and 8. The media were incubated (200 rpm) at the optimum temperature for growth of the enzyme-producing bacterium for 21 h.

### *Analytical methods*

In each fermentation, three flasks were collected each 3 h, and triplicate samples (runs) were taken from each flask to perform analytical determinations. The *COD* values were measured at the end of the culture period (30 h).

Growth was monitored by absorbance at 600 nm and converted to cell dry weight (*CDW*) from a standard curve. Cells were harvested by centrifugation (12,000 × *g* for 15 min at 4 °C) of culture samples and washed twice with saline (0.8% NaCl). The culture supernatants were used to measure total sugars (TS), total nitrogen (TN), proteins, enzyme production and *COD*. The methods for determining total sugars

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