CHEMICAL ENGINEERING TRANSACTIONS
VOL. 38, 2014
A publication of AIDIC
The Italian Association of Chemical Engineering
www.aidic.it/cet
Guest Editors: Enrico Bardone, Marco Bravi, Taj Keshavarz

ISBN 978-88-95608-29-7; **ISSN** 2283-9216
DOI: 10.3303/CET1438047

Optimization of Cellulase Production by *Bacillus Sp*. Isolated from Sugarcane Cultivated Soil

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Cellulases are glycoside hydrolases involved in the depolymerization of cellulosic materials and have wide industrial applications, such as in the food, textile and feed industry. In addition, the animal feed industry uses cellulases to improve the digestibility of plant products. A technological application currently under study is the production of second-generation bioethanol, as a result of the search for alternative energy sources. The objective of this work was to use bacteria isolated from tropical soil used by the sugar and alcohol industry in the State of Paraíba (Brazil). These bacteria are *Bacillus* sp C1AC55.07 selected as producers of cellulolytic enzymes for the production of cellulases. The factorial planning methodology and the response surface analysis were used to evaluate the effect of cultivation temperature, yeast extract concentration and inoculum concentration on carboxymethylcellulase production. The experimental design used has three factors (2^3), resulting in 11 experiments, since three of them were central point replications. The cultures were grown in 500mL Erlenmeyer flask containing 250mL of culture medium. Samples were collected at regular time intervals for analysis of the carboxymethylcellulase activity. The results showed that the highest carboxymethylcellulase activity (366 U / L) was obtained in 2 g / L yeast extract, 30% inoculum (v/v), and temperature of 32°C for 54 hours. *Bacillus* sp C1AC55.07 showed good cellulase activity and may be used in industrial applications.

1. Introduction

Biorefinery is a branch of industry which aims to convert low-value resources (lignocellulosic materials such as sugar cane bagasse, cassava and wood residues, etc) into basic products such as starch, sugar and cellulose. These basic products are later used in the manufacture of products with high added value such as bioethanol, biobutanol and lactic and citric acids (Chinnawornrungsee et al, 2013; Garcia, 2013).
Thus, biorefinery plays an important role in the local economy, as it allows the use of natural resources in the region where it operates and does not increase the demand for food products, since the raw material used for this type of industry is not consumed as food by humans.
The ethanol production for fuel use from lignocellulosic biomass has proven to be one of the most important applications of the biorefinery concept, since it represents a sustainable energy production. Moreover, the burning of ethanol represents a reduction in the emission of greenhouse gases when compared with the burning of traditional fuels (Lin and Tanaka, 2006).
Microorganisms isolated from soil such as fungi and bacteria produce several enzymes that degrade lignocellulosic biomass (Bruce et al, 2010).
The biological conversion of lignocellulosic materials into industrial products requires the use of cellulolytic and hemicellulolytic enzymes to release fermentable sugars (Jourdier et al, 2012).

Please cite this article as: Dias P., Ramos K., Padilha I., Araujo D., Santos S.F.M., Silva F.L.H., 2014, Optimization of cellulase production by bacillus sp. isolated from sugarcane cultivated soil, Chemical Engineering Transactions, 38, 277-282 DOI: 10.3303/CET1438047

Considering the need to reduce the costs for obtaining cellulases of industrial interest, this study evaluated the optimization of cellulase production by *Bacillus* sp., C1AC55.07 strain, isolated from sugarcane cultivated soil using 2^3 factorial design methodology.

2. Methodology

2.1 Microorganism

The bacterial C1AC55.07 strain used was isolated from sugarcane cultivated soil at the Japungu Agroindustrial S.A. located in the Santa Rita city (Brazil) and classified as *Bacillus* sp. by 16S rDNA sequence analysis. The bacterial strain was evaluated for enzyme production of carboxymethylcellulase (CMCase) using carboxymethylcellulose (CMC)-containing agar medium and incubation at 55°C for 3 days. Cellulolytic activity was detected by the formation of clear zones around colonies through the Congo red staining.

2.2 Cellulase production

C1AC55.07 strain was cultured in 500 ml Erlenmeyer flasks containing 250 ml of culture medium (g / L): CMC, 10; yeast extract (according to design); KH_2PO_4, 1; K_2HPO_4, 1.145; $MgSO_4.7H_2O$, 0.4; $CaCl_2.2H_2O$, 0.05; $FeSO_4.7H_2O$, 0.00125. The cultures were shaken at 180 rpm at temperature according to design. The inoculum was done on LB (Luria - Bertani) medium and was grown for 18 hours at 37°C and 180 rpm, being standardized at absorbance of 1 ± 0.1. The inoculum concentrations were evaluated according to design. Thus, samples were collected and centrifuged at 5,000 rpm at 30 ° C for 5 minutes in times 6 h, 12 h, 24 h, 30 h, 36 h, 48 h, 54 h and 72 h to measure the enzymatic activity. Table 1 shows the experimental range values of variables used in the experimental design.

Table 1: Real and coded values of *variables used in the 2^3 factorial design*

Variable	-1	0	1
Temperature (ºC)	32	37	42
Yeast extract concentration (g/L)	2	3	4
Inoculum concentration (%volume/volume)	10	20	30

2.3 Quantification of the activity of cellulases

Enzymatic activity quantification was based on methodology proposed by Ghose (1987). Thus, 250 µL of enzyme extract were mixed with 250 µL of a 4% CMC solution in phosphate buffer (pH 7) at 50°C for 10 minutes. Subsequently, 500 µL of dinitrosaliscylic acid (DNS) were added, with contents were homogenized and heated at 100°C for 5 minutes. The samples were cooled on ice to room temperature and the absorbance was read at 540nm in a spectrophotometer. One unit of CMCase was defined as the amount of enzyme that catalyzes the release of 1µmol of reducing sugar glucose equivalent per minute under the specified assay conditions. All activity measurements were repeated three times.
The concentration of enzymes present in the enzyme extract was calculated using Eq.1.

$$CMCase\ \left(U/L\right) = \frac{(A-B)\times f \times d \times 0.5}{0.18 \times 10 \times 0.25} \times 1{,}000 \quad (1)$$

where:
A = Absorbance of the sample;
B = Absorbance of sample control;
f = Conversion factor of the calibration curve (mg / mL);
D = Sample dilution
0.5 = Total volume of the reaction medium (µL);
0.18 = Conversion factor from mg to µmol of glucose;
10 = Reaction time (min);
0.25 = Enzyme volume in the reaction medium (mL);
1.000 = Conversion factor from 1/ml to 1 / L.

3. Results and discussion

The experimental design used has three factors, i.e., it is a 2^3 factorial design totaling 11 trials, three of them central point replicates, displaying enzymatic activity values for cultivation times of 6 h, 12 h, 24 h, 30 h, 36 h, 48 h, 54 h, 60 h and 72 h.

Thus, the cultures were made by varying the cultivation temperature, yeast extract concentration and inoculum concentration, with enzymatic activity being measured in each experiment, as shown in Table 2, which shows the enzymatic activity values for 54 h of cultivation due to its statistical significance associated to presence of the maximum activity value (Run 5).

Table 2: Identification of 2^3 factorial design experiments with the respective enzymatic activity values at 54 h of cultivation.

Run	Temperature (ºC)	Yeast extract concentration (g/L)	Inocumum concentration (%vol.)	Activity (U/L) (54 h of cultivation)
1	32	2	10	341.9
2	42	2	10	246.3
3	32	4	10	239.1
4	42	4	10	208.2
5	32	2	30	366.5
6	42	2	30	316.2
7	32	4	30	217.5
8	42	4	30	200.0
9	37	3	20	232.9
10	37	3	20	228.8
11	37	3	20	245.2

Analyzing data shown in Table 2, it was observed that temperature had the greatest influence on the CMCase production by C1AC55.07 strain, in which the highest activity values were obtained when the strain was grown at 32°C.

The yeast extract concentration is also important for the process. Yeast extract concentration of 2 g / L leads to higher activity values, and when grown in higher values, the bacterium showed lower enzymatic activity, which could be related to some kind of enzyme production inhibition.

In the concentration range of 10, 20 and 30 (% vol), the inoculum concentration showed no significant activity variations, requiring a broadening of this range for better evaluation of this variable in the process.

Table 3: Analysis of the significance of models, where EA represents the enzymatic activity value in U / L, T is the cultivation temperature in °C, [E] is the yeast extract concentration in g / L and [I] is the inoculum concentration in % volume / volume.

Time (h)	Model	Fcalc/Ftab	R^2
6	**EA** = 125.9114-26.0827**T**-4.4970**[E]**-9.8934**[I]**+1.4133T**[E]**+11.9492**T[I]**+7.8377**[E][I]**	1.01	0.903
12	**EA** = 169.55-54.0927**T**-42.2719**[E]**+11.1783**[I]**+38.1604**T[E]**-24.0269**T[I]**+9.3795**[E][I]**	4.09	0.974
24	**EA** = 151.0480-66.1703**T**+16.0608**[E]**+14.5189**[I]**+4.4970**T[E]**-15.5468**T[I]**+7.5807**[E][I]**	0.93	0.900
30	**EA** = 198.9850-26.3397**T**-14.2620**[E]**-21.2002**[I]**-5.0110**T[E]**+69.7680**T[I]**-8.0946**[E][I]**	0.15	0.577
36	**EA** = 197.4899+8.7371**T**+6.4243**[E]**+57.0478**[I]**+33.9203**T[E]**+13.1056**T[I]**-7.7092**[E][I]**	0.05	0.322
48	**EA** = 256.1730+1.6703**T**+43.0428**[E]**-1.6703**[I]**-0.3855**T[E]**+25.8257**T[I]**-16.0608**[E][I]**	0.19	0.640
54	**EA** = 258.4157-24.2839**T**-50.7520**[E]**+8.0946**[I]**+12.2062**T[E]**+7.3237**T[I]**-15.5468**[E][I]**	1.32	0.924
72	**EA** = 194.5931+2.0558**T**+14.1335**[E]**+2.0558**[I]**+0.5139**T[E]**+12.5916**T[I]**	0.15	0.580

Statistical analysis consisted of analysis of each culture time through the Fisher test (F test), if the model generated for each time showed statistical significance at confidence level of 95 %. Therefore, ANOVA

was used to define the calculated F value and relate it to the tabulated F value for Regression Degree of Freedom equal to 6 and Residue Degree of Freedom equal to 4. The analysis of the significance of models shown that the models of times 6 h, 12 h and 54 h are statistically significant, as they have Fcalc/Ftab > 1 (Table 3). Thus, for these models, response surfaces shown in Figure 1, Figure 2 and Figure 3, respectively, were made.

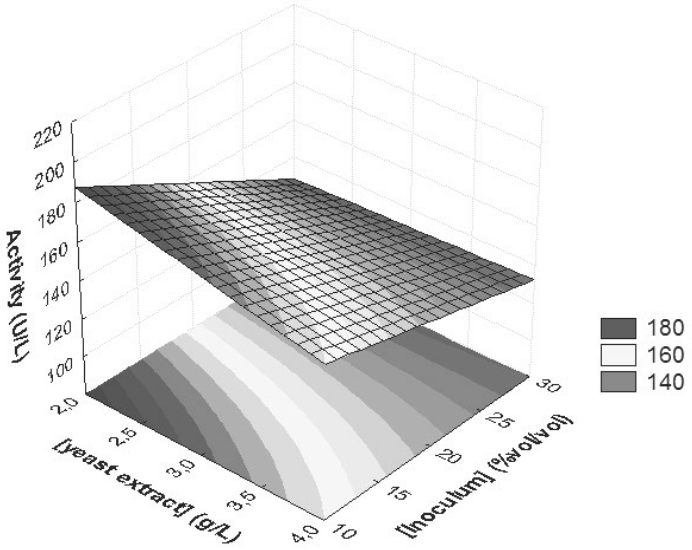

Figure 1: Response surface showing the effect of variation and interaction of the yeast extract concentration and inoculum concentration on the enzymatic activity at 6 h of cultivation using Bacillus sp. C1AC55.07 strain.

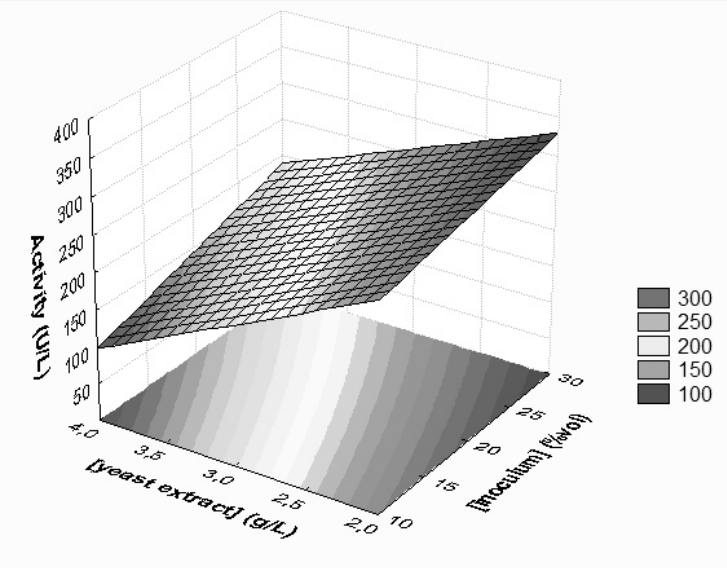

Figure 2: Response surface showing the effect of variation and interaction of the yeast extract concentration and inoculum concentration on the enzymatic activity at 12 h of cultivation using Bacillus sp. C1AC55.07 strain.

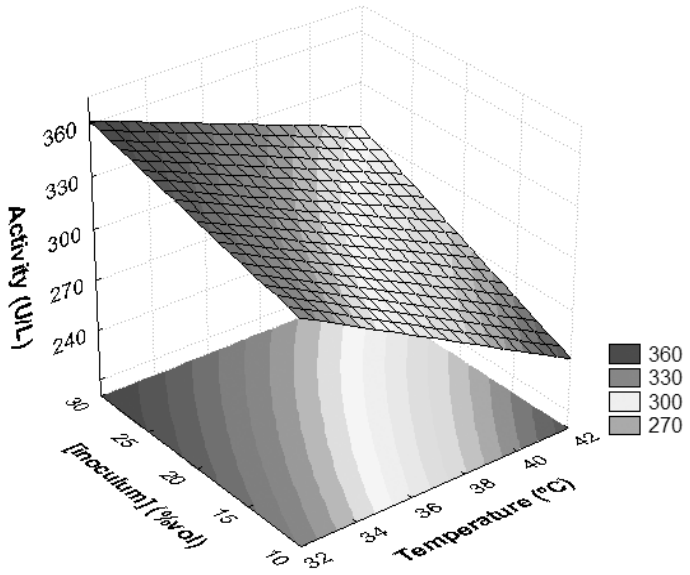

Figure 3: Response surface showing the effect of variation and interaction of the inoculum concentration and cultivation temperature on the enzymatic activity value in medium containing 2 g / L of yeast extract at 54 h of cultivation of Bacillus sp. C1AC55.07 strain.

As shown in Table 3, using the parameters 54 h of cultivation time and yeast extract concentration (2 g/L) showed the greatest effect. Analyzing the model shown in Table 3, it was concluded that increasing temperature and yeast extract concentration reduced the enzymatic activity value, while increasing the inoculum concentration increased the enzymatic activity value. This is confirmed by observing Table 2 and Figure 3, which show that the peak enzymatic activity of 366.5 U/L is obtained in experiment number 5.
Deka et al (2011) evaluated the increased production of cellulases from a strain of Bacillus subtilis by optimizing the production medium. Therefore, the significance of each component's concentration was evaluated and also determined the optimal concentration of statistically significant components. The peak of activity was 430 U / L to 18 g / L of CMC, 8 g / L of peptone and 4.79 g / L of yeast extract. Although the enzymatic activity was about 17 % higher than the one presented in this work, the production medium used has a high cost due to high concentrations of CMC (80% higher), peptone and yeast extract (140% higher), which is not interesting for industry.

4. Conclusion

The optimization of the production of enzymes are important for increasing productivity and reducing costs strategies. Further studies with expanded scale are needed, as well as the evaluation of other parameter. *Bacillus* sp. C1AC55.07 strain is promising for industrial application. Thus, CMCase produced could be a good candidate to use for industrial purpose.

5. Acknowledgments

The authors would like to thank the financial support from the National Council for Scientific and Technological Development (CNPq).

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