https://dx.doi.org/10.22161/ijaers.6741 ISSN: 2349-6495(P) | 2456-1908(O)

Optimization of Food-Waste Based Culture Medium for Cellulase Production by Thermophilic *Bacillus* sp SMIA-2 and effect of Divalent Metal Ions on Activity and Stability of the Enzyme at Higher Temperatures

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Abstract*— *In general, the costs for cellulases production are associated with the value of the carbon source used in the process. Waste from the food processing industry contains several reusable substances that can replace the expensive components used in culture media for the production of cellulases. In this work, it was studied the combined interactive effect of different concentrations of sugarcane bagasse treated with alkali, passion fruit rind flour and corn steep liquor for maximal avicel-hydrolyzing enzymes – avicelases by thermophilic Bacillus sp SMIA-2, using statistical methodology. The influence of metal ions on the activity and stability of the enzyme was also investigated in order to increase the industrial applicability of enzymes. A concentration of 0.3% (w /v) of these three components in the production medium can be used successfully to obtain high levels of avicellase activity. The avicelase displayed enhanced activity in the presence of 10 Mm CoCl2 after incubation at 90ºC for 1 h, indicating that this enzyme depended on the metal ions to promote its activity and stability at higher temperatures.

Keywords*— *Avicelase, Bacillus, Food wastes, Metal ions, Thermostability.

I. INTRODUCTION

Lignocellulose constitutes 60% of the plant cell wall [1]. Cellulose is a major polysaccharide found in lignocellulose and is made up of repeating glucose units. Besides cellulose, plant-based food wastes are rich in pectin, inulin, xylan, mannan, glucan, starch, etc., depending upon the nature of the waste product [2]. The sugarcane bagasse, a fibrous residue of cane stalks left over after the crushing and extraction of the sugar from sugarcane is rich in cellulose and hemicellulose [3]. It can be regarded either as a waste, affecting the environmentor, as a resource when suitable valorization technologies are implemented. Bagasse consists of cellulose 43.8%, hemicellulose 28.6%, lignin 23.5%, ash 1.3% and other components 2.8% and is abundantly and cheaply available as a byproduct from the sugar industry in Brazil [3]. Another food waste produced abundantly in Brazil is yellow passion fruit peel, which is rich in cellulose and hemicellulose contents, besides minerals, and especially pectin [4]. Like many other agro-industrial by-products, passion fruit waste has low commercial value and its deposition on a large scale may result in a negative environmental impact. Currently, its main use in Brazil is as a supplement to animal feed, which presents several transport and storage problems due its high moisture content [5]. Therefore, being rich in polysaccharides, sugarcane bagasse and yellow passion fruit peel have been studied as a potential media component for the production of various industrially important enzymes, in particular, cellulose-degrading enzymes [6,7].Cellulases are the third most abundant industrial enzyme [8]. Thermophilic cellulases are ideal biocatalysts for modern biotechnology because of their thermostability and better yields under extreme operational conditions [9,10]. The wide range of applications of thermophilic cellulases include the food, textile, chemical, pulp and paper industries, laundry detergents, and second generation ethanol production

https://dx.doi.org/10.22161/ijaers.6741 *ISSN: 2349-6495(P) | 2456-1908(O)*

[11-16]. The thermophilic *Bacillus* sp. SMIA-2 expressed a promising level of avicel-hydrolyzing enzymes — avicelases) in cultures shaken at 50°C containing sugarcane bagasse, as cellulosic substrate, in combination with passion fruit rind flour, as co-substrate, and corn steep liquor as nitrogen source [7]. In this work, it was studied the combined interactive effect of the concentration of these three components for maximal avicelases by thermophilic *Bacillus* sp SMIA-2, using statistical methodology. The influence of metal ions on activity and thermostability of the avicelases was also studied in order to increase the industrial applicability of enzymes.

II. MATERIALS AND METHODS

2.1. Organism

The present study used a thermophilic *Bacillus* sp strain SMIA-2, previously isolated from a soil sample collected in the city of Campos dos Goytacazes, Rio de Janeiro, Brazil. Phylogenetic analysis revealed that the bacteria were closely related to *Bacillus caldoxylolyticus* and *Bacillus* sp strain AK1, and these three organisms exhibited levels of ribossomal DNA sequence homology of 94% [17].

2.2. Enzyme Production

The culture medium used in this work for cellulase production contained (g/L): KCl - 0.3, $MgSO_4$ - 0.5, K_2HPO_4 - 0.87, $CaCl_2$ - 0.29, ZnO - $2.03x10^{-3}$, $FeCl_3.6H_2O$ - $2.7x10^{-2}$, $MnCl_2.4H_2O$ - $1.0x10^{-2}$, $CuCl_2.2H_2O$ - $8.5x10^{-4}$, $CoCl_2.6H_2O$ - $2.4x10^{-3}$, $NiCl_3.6H_2O$ - $2.5x10^{-4}$, H_3BO_3 - $3.0x10^{-4}$. Sugarcane bagasse (SCB) treated with alkali (81.05% cellulose, 18.75% hemicellulose, 5.45% lignine) was used as a source of cellulose [3] and commercial corn steep liquor (Sigma Aldrich), as a nitrogen source. Passion fruit rind flour (obtained from a local market) was used as co-substrate. The SCB, corn steep liquor (CSL) and passion fruit rind flour (PFRF) concentrations were adjusted for each value according to central composite design (CCD), as presented in Table A.1. The pH was adjusted to 7.2 with 1.0 M NaOH and the medium was sterilized by steam-autoclaving at 121 °C, 1 atm for 15 minutes. The medium (50 mL in 250 mL Erlenmeyer flasks) was inoculated with 1 mL of an standard overnight culture (initial number of cells 10^4) and incubated at 50 °C in an orbital shaker (Thermo Forma, Ohio, USA) operated at 150 rpm. After 168 h triplicate flasks were withdrawn and the contents were then centrifuged (HERMLEZ 382K, Wehingen, Germany) at 15,500 g for 15 min, at 4 °C, and the cell free supernatant was used as crude enzyme preparation.

2.3. Enzyme Assay

The cellulolytic enzyme activities were determined using the dinitrosalicyclic acid method [18], which measures reducing sugars. The reaction mixture containing 0.5 mL of 1% (w/v) avicel, PH-101 prepared in 10 mM sodium phosphate buffer, pH 7.5, and 0.5 mL of appropriate concentration of enzyme solution, was incubated at 70 °C. After 10 min of reaction, 1 mL of dinitrosalicyclic acid reagent was added and boiled in water bath for 5 min. The resulting samples were then cooled to room temperature, and the absorbance was measured at 540 nm. When the activity was tested using avicel as substrate, the assay tubes were agitated during the course of the assay to keep the substrate suspended. One unit (U) of activity toward the substrates mentioned above was defined as 1 µmole of glucose equivalent released per minute under the above assay conditions, by using a glucose standard curve. Appropriate controls were conducted in parallel with all assays. Enzyme blank containing 0.5 mL of 10 mM sodium phosphate buffer and 0.5 mL of 1% (w/v) substrate solution were run. To exclude the background of reducing sugars found in the enzyme supernatant from the results, a substrate blank containing 0.5 mL of 10 mM sodium phosphate buffer and 0.5 mL enzyme solution was also run. The absorbance of the enzyme blank sets and the substrate blank were subtracted from the absorbance of the activity assay. All of the samples were run in triplicate, while the blanks were run in duplicate.

2.4. Experimental Design and Statistical Analysis

The surface-response methodology (SRM) was used to obtain a model for cellulase activity. To evaluate the effects of SCB, PFRF and CSL concentration on the production of cellulase, a central composite design (CCD) 2^3 was constructed. The factorial planning had five central points and yielded a total of 19 treatments. The factors and levels studied are described in Table A.1.The results were analyzed using the Statistica software system, version 5.0. In this context, the F test was used as a validation criterion of statistical significance of the models obtained at a confidence level of 95%.The optimization of condition was performed using CCD and surface-response was produced with fixed central points of 0.575% SCB, 0.575% PFRF and 0.575 CSL. The experimental model can be expressed as follows (Eq. A.1):

$$Y = bo + \sum_{i=1}^{4} bi\, xi + \sum_{i=1}^{4} bii\, xi^2 + \sum_{i \neq j=1} \sum bij\, xi\, xj$$

Eq. (A.1)

https://dx.doi.org/10.22161/ijaers.6741 *ISSN: 2349-6495(P) | 2456-1908(O)*

Where *bo*, *bi*, *bii* and *bij* are the intercept terms, linear, quadratic coefficient and interactive coefficient, respectively, and *xi* and *xj* are coded independent variables.

2.5. Effect of metal ions on avicelase activity

The effect of different metal ions on avicelase activity was determined by the addition of the corresponding ion at a final concentration of 1mM and 10 mM to the reaction mixture, and assayed under standard conditions. The enzyme assay was carried out in the presence of $CoCl_2$, $CaCl_2$, $ZnSO_4$, $CuSO_4$, $MgSO_4$, $BaCl_2$ and $MnSO_4$.

Thermostability of avicelase was determined by incubation of crude enzyme at temperature of 60ºC, 70 ºC and 90ºC in a constant-temperature water bath. The residual activity (%) was measured at various intervals of time under standard assay conditions. The enzyme preparation was also incubated in the presence of $CoCl_2$ (10 mM) and $CaCl_2$ (10mM) at 70ºC and 90ºC. The residual activity (%) was measured at various intervals of time under standard assay conditions.

III. RESULTS AND DISCUSSION

3.1. Optimization of food-waste concentrations in the culture medium

The thermophilic *Bacillus* sp SMIA-2 produced avicelase (avicel-hydrolyzing enzymes) in 120 h submerged cultures containing SCB treated with alkali as source of cellulose and PFRF as co substrate (Table A.1). CSL was used in place of meat and yeast extracts, which are nitrogen sources of high cost. The use of waste from the food industry as raw materials for culture media, promote economic advantages, because they reduce environmental pollution and stimulate new research for science sustainability [19]. Passion fruit processing generates a substantial amount of residues, including peel. Its use as an additional source of carbon can enhance the growth and production of cellulases by *Bacillus* sp SMIA-2, eliminating the need for additional nutrients as it is rich in fiber, minerals and mainly pectin [7]. Whereas it is essential to study and quantify the effects of these three components on cellulase production and strike a balance between them to enhance the enzyme activity, a central composite design (CCD) 2^3 was constructed (Table A.1). A variation in avicelase activity was observed from 0.820 (Treatments 16) to 1.96 – 1.97 $U.mL^{-1}$ (Treatment 1, 2 and 7).

Table A.1 - Matrix of CCD 2^3 (real and coded values) used and its response (avicelase activity).

Essays	PFRF (%, w/V)	SCB (%, w/V)	CSL (%, w/V)	Avicelase (U/mL)
1	0.322 (-1)	0.322 (-1)	0.322 (-1)	1.961
2	0.828 (+1)	0.322 (-1)	0.322 (-1)	1.964
3	0.322 (-1)	0.828 (+1)	0.322 (-1)	1.044
4	0.828 (+1)	0.828 (+1)	0.322 (-1)	1.083
5	0.322 (-1)	0.322 (-1)	0.828 (+1)	1.214
6	0.828 (+1)	0.322 (-1)	0.828 (+1)	0.857
7	0.322 (-1)	0.828 (+1)	0.828 (+1)	1.974
8	0.828 (+1)	0.828 (+1)	0.828 (+1)	1.379
9	0.150 (-1.68)	0.575 (0)	0.575 (0)	1.575
10	1.00 (+1.68)	0.575 (0)	0.575 (0)	1.752
11	0.575 (0)	0.150 (-1,68)	0.575 (0)	1.360
12	0.575 (0)	1.000 (+1,68)	0.575 (0)	1.741
13	0.575 (0)	0.575 (0)	0.150 (-1.68)	0.871
14	0.575 (0)	0.575 (0)	1.000 (+1.68)	1.662
15	0.575 (0)	0.575 (0)	0.575 (0)	0.849
16	0.575 (0)	0.575 (0)	0.575 (0)	0.820
17	0.575 (0)	0.575 (0)	0.575 (0)	0.916
18	0.575 (0)	0.575 (0)	0.575 (0)	0.920
19	0.575 (0)	0.575 (0)	0.575 (0)	0.852

The statistical significance of the model equation was assessed by an F-test (ANOVA) and the data are shown in (Table A.2). An equation for avicelase activity (Eq. A.2) was developed based on a regression analysis of the following experimental data:

$$Y = 0.874863 - 0.044671\ x_1 + 0.259512\ x_1^2 + 0.09221\ x_2 + 0.219573\ x_2^2 + 0.051560\ x_3 + 0.119182\ x_3^2 - 0.025154\ x_1 x_2 - 0.124228\ x_1 x_3 + 0.384825\ x_2 x_3 \quad \text{Eq. (A. 2)}$$

Where x_1 is the PFRF, x_2 is the SCB and x_3 is the CSL concentration.

The outcome of ANOVA analysis revealed that the adjusted model was significant, according to the analysis of the F test. The regression model for avicelase production was highly significant ($p < 0.05$), with a satisfactory value of determination coefficient (R^2 = 83.11). The response surface was produced according to (Rodrigues and Lemma, 2009) [20].

Table A.2 - ANOVA for the variables of response surface quadratic model for avicelase production

Variable	Mean square	(Degrees of freedom)	Sum of squares	F Value	F Statistic 0.05
Regression	2.149	9	0.239	3.771	3.18
Residues	0.570	9	0.063		
Lack of adjustement	0.562	5	0.112	56.759	6.26
Pure error	0.008	4	0.002		
Total error	3.290	18			
				R^2 =	83.11%

Response surface and contour plot figures obtained by the analysis of the experimental data of CCD showed a relationship between two variables at time. The non-

https://dx.doi.org/10.22161/ijaers.6741 *ISSN: 2349-6495(P) | 2456-1908(O)*

explicit variables were fixed at the central point (level 0) for the surface construction. The interaction effect between CSL and PFRF concentration on avicelase activity, when the SCB concentration was kept constant (0.575%, w/v) is displayed in Fig. A.1 (A). The activity of the enzyme increased both, when the CSL concentration was increased simultaneously with the decrease in the PFRF concentration and when the PFRF concentration was increased simultaneously with the decrease in the CSL concentration. The interaction effect between SCB and CSL concentrations on avicelase production, at constant PFRF concentration (0.575%, w/v), is presented in Fig. A.1 (B). Higher levels of avicelase activity were found as when higher as lower concentrations of SCB and CSL were utilized simultaneously. Finally, the interaction effect between SCB and PFRF concentrations on avicelase production was presented in Fig. A.1 (C). The 3D graph shows that when keeping the CSL concentration constant at 0.575% (v/v), irrespective of the increase or decrease of SCB and PFRF concentrations at the levels studied, the avicelase activity increased. According to these results, seems that lower concentrations of these three components in the production medium can be used to obtain high levels of avicellase activity.

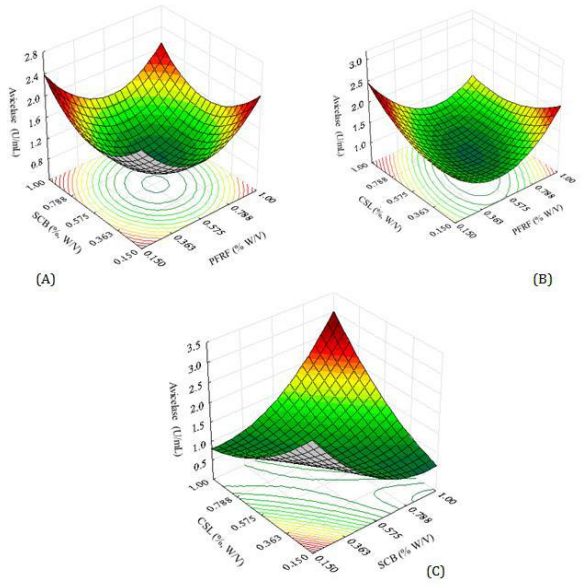

Fig. A.1 - Three-dimensional response surface plot for: (A) - Effect of SCB and PFRF concentration on avicelase production by Bacillus sp SMIA-2 at a constant CSL (0.575%, w/v); (B) - Effect of CSL and PFRF concentration on avicelase production by Bacillus sp SMIA-2 at a constant SCB (0.575%, w/v); (C) - Effect of SCB and CSL concentration on avicelase production by Bacillus sp SMIA-2 at a constant PFRF (0.575%, w/v).

The avicelase activity is low when the graphic has dark red color indicates high activity, while green and yellow color indicates low avicelase activity.

In fact, the maximum avicelase activity was obtained when *Bacillus* sp. SMI-2 was growth for 120 h in the culture medium containing 0.3% (w/v) of each of the three components (Fig. A.2). At higher concentrations, the avicelase activity was comparatively lower or was not affected. Besides, the concentration of 0.3% (w/v) of these three components gave better reproducibility in production experiments.

[7] Costa et al. (2017) reported that higher avicelase activities (2.73 U.mg protein^{-1}) were obtained when *Bacillus* sp SMIA-2 was grown in liquid medium containing 0.625% (w/v) treated SCB and 0.625% (w/v) CSL. The supplementing of this culture medium with 0.75% (w/v) passion fruit rind flour (PFRF) supported maximal enzyme activity. However, the combined interactive effect of the concentration of these three components was not investigated by the authors.

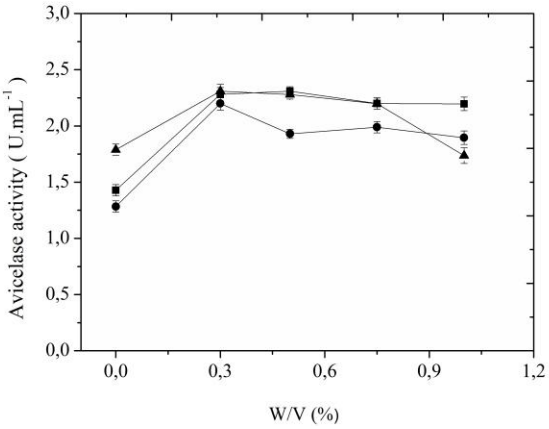

Fig. A.2 - Activity of avicelase in submerged cultures of Bacillus sp SMIA-2 (120 h) containing: (▲) 0.3% (w/v) SCB and 0.3% (w/v) PRFR and different concentrations of CSL; (●) 0.3% (w/v) PRFR and 0.3% (w/v) CSL and different concentrations of SCB (■); 0.3% (w/v) SCB and 0.3% (w/v) CSL and different concentrations of PRFR.

Comparison between the activities obtained and the published literature was hindered by the different definitions of enzymatic activity and different levels of enzyme purity used. Thus, a concentration of about 0.3% (w/v) of these three components in the production medium can be used successfully to obtain high levels of avicellase activity.

3.2. Effect of metal ions on activity and stability of avicelase

https://dx.doi.org/10.22161/ijaers.6741 *ISSN: 2349-6495(P) | 2456-1908(O)*

The activity of the avicelase was increased in the presence of 1 and 10 mM $CoCl_2$ and inhibited by Ba^{2+} and Mn^{2+} (Table A.3). Cellulases from different microbial sources show diverse behavior towards metal ion requirement for their activity. Some enzymes require metal ions, some are inhibited by metal ions, while some are independent of their presence. [21] Duman et al. (2016) reported that the presence of Co^{2+} metal ions in the reaction mixture enhanced the cellulase activity of *Bacillus methylotrophicus* Y37 to 319% of the original level, while Mg^{2+} increased the activity at moderate levels (148% and 141%, respectively). [22] Gaur and Tiwari (2015) reported that cellulase activity of *Bacillus vallismortis* RG-07 was strongly stimulated by $CaCl_2$ and NaCl. The cellulolytic and hemicellulolytic enzymes activities from *Enterobacter* sp. SUK-Bio increased after the addition of $MnCl_2$ and $CoCl_2$ in the reaction mixture [23].

Table A.3 - Effect of various divalent metal ions on avicelase activity

	Residual avicelase activity (%)	
Divalent metal ions	1mM	10mM
Control	100 ± 0.019	100 ± 0.012
$CoCl_2$	109.8 ± 0.016	112.75 ± 0.011
$CaCl_2$	96.57 ± 0.017	98.72 ± 0.015
$ZnSO_4$	95.09 ± 0.011	108.13 ± 0.012
$CuSO_4$	92.65 ± 0.019	98.00 ± 0.019
$Mg\ SO_4$	91.67 ± 0.017	99.94 ± 0.019
$BaCl_2$	84.8 ± 0.013	65.20 ± 0.012
$MnSO_4$	72.06 ± 0.011	82.35 ± 0.014

The thermostability of avicelase from *Bacillus* sp at 60ºC, 70ºC and 90ºC is showed in Fig. A.3. The results are expressed as percentage of residual activity, taking into account the activity determined with the non-treated enzyme samples. The thermostability profile indicated that the enzyme remained 100% stable at 60ºC for 1 h, but lost about 47% and 68% of the original activity after 1 h heat treatment at 70ºC and 90ºC, respectively (Fig. A.3). It's known that at very high temperatures, inactivation of enzymes may occurs mainly due to thermal denaturation (loss of tertiary structure) and degradation (loss of primary structure) [24], and that metal ions are known to be important to the catalytic activity and stability of many enzymes [24,25]. In fact, after incubation of avicelase at 70º and 90ºC for 1 h, in the presence of $CaCl_2$ the avicelase lost about 28.9% and 54.3% of its original activity, but in the presence of $CoCl_2$ the enzyme displayed enhanced activity after incubation for 1 h at both temperatures, indicating that this enzyme depended on the metal ions to promote its activity and stability at higher temperatures. Thus, apparently the $CoCl_2$ protected the enzyme against thermal denaturation and play an important role in modulating both the stability and the activity of avicelase produced by *Bacillus* sp SMIA-2. According to [26] Vasconcellos et al. (2016), high activity and stability are essential for (hemi) cellulolytic enzymes used in biomass conversion and one potential strategy is the addition of inexpensive metal ions to improve its activity, thermostability, and saccharification efficiency.

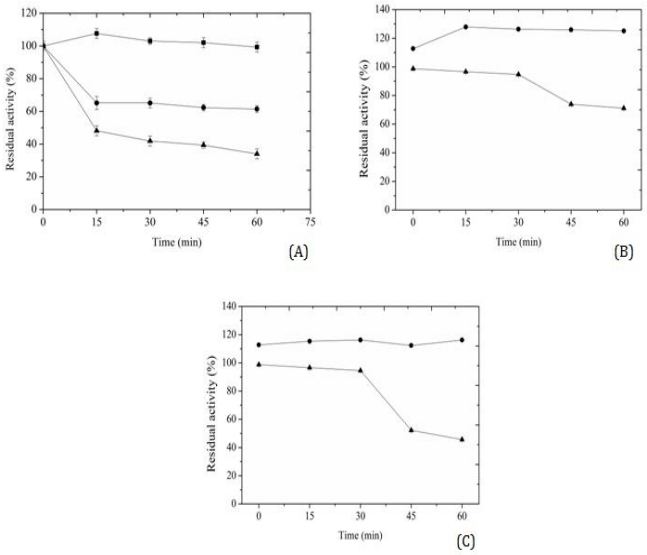

Fig. A.3 - (A) - Thermostability of avicelase at 60ºC (■), 70ºC (●) and 90ºC (▲); (B) - In the presence of 10 mM $CoCl_2$ (●) and $CaCl_2$ (▲) at 70ºC (C) – In the presence of 10 mM $CoCl_2$ (●) and $CaCl_2$ (▲) at 90ºC (C). (100% of enzyme activity = 1.98 UmL^1).

IV. CONCLUSION

Our results showed that the combination of 0.3% sugarcane bagasse, passion fruit rind flour and corn steep liquor in a basic mineral media provides the achievement of high levels of cellulase activity by *Bacillus* sp SMIA-2. This opens perspectives for use of these agricultural byproducts as novel industrial culture mediums for the production of cellulase, combinating environmental concern with sustainable processes to reduction of production cost. The avicelase displayed enhanced activity in the presence of 10 mM $CoCl_2$ after incubation at 70º and 90ºC for 1 h, indicating that this metal ion apparently protected the enzyme against thermal denaturation and play an important role in modulating both the stability and the activity of the enzyme. This result is very important because increase the industrial applicability of avicelase from *Bacillus* sp SMIA-2 in processes that operate at high temperature.

ACKNOWLEDGEMENTS

The authors thank the Brazilian Agencies CNPq and CAPES for financial support.

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