

**Bioprospection of cellulose-decomposers soil bacteria**  
**Bioprospecção de bactérias do solo degradadoras de celulose**

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**ABSTRACT**

Soil microorganisms have a high bacterial biodiversity and an important group is the cellulose-decomposers bacteria, which through their endoglucanases, exoglucanases and  $\beta$ -glycosidases enzymatic complexes are responsible for the planet organic biomass degradation. Therefore, to understand and apply their potentials in the biotechnology area, is necessary to know their biologic,

biochemical and environmental characteristics and proprieties of the microorganisms involved in these degradation processes. Ten bacteria isolates were obtained. To verify the degradation, the filter paper and solid medium Luria-Bertani was used. In the extracellular enzymatic production analysis, the substrate used was the Whatman N°1 filter paper for the  $\beta$ -1,4 exoglucanase and carboxymethyl cellulose for the  $\beta$ -1,4 endoglucanase. Also the microbial biomass analysis was made. The results showed that, the most effective cellulose degradation isolates having as substrates, the BC5 and the BC2 bacteria respectively. In the endoglucanase  $\beta$ -1,4 enzymatic activity was the BC5 and for the  $\beta$ -1,4 exoglucanase was the BC8, meanwhile for the microbial biomass production, the best result was for the BC9 isolate bacteria. The results showed that between the different bacteria species from the different locals, significant differences were presented, when each of the analysis were compared and therefore this can be associated to the different types of ecosystems and to the biochemical and environmental characteristics in each type of soil, having different mechanisms directly related with the cellulose degradation. Therefore, these microorganisms have a potential use in diverse biotechnological and bioremediation cellulose degradation processes.

**Key-words:** cellulolytic enzymes, cellulolytic microorganisms, endoglucanase, exoglucanase, soil, microbiology environmental.

## RESUMO

Os microrganismos do solo apresentam alta biodiversidade bacteriana e um grupo importante são as bactérias decompositoras de celulose, que por meio de seus complexos enzimáticos endoglucanases, exoglucanases e  $\beta$ -glicosidases são responsáveis pela degradação orgânica da biomassa do planeta. Portanto, para entender e aplicar seus potenciais na área de biotecnologia, é necessário conhecer suas características biológicas, bioquímicas e ambientais e as propriedades dos microrganismos envolvidos nesses processos de degradação. Seis bactérias foram isoladas. Para verificar a degradação, foi utilizado o papel de filtro e o meio sólido Luria-Bertani. Na análise da produção enzimática extracelular, o substrato utilizado foi o papel de filtro Whatman N ° 1 para a  $\beta$ -1,4 exoglucanase e carboximetilcelulose para a  $\beta$ -1,4 endoglucanase. Também foi feita a análise da biomassa microbiana. Os resultados mostraram que a degradação mais eficaz da celulose isolada tendo como substrato as bactérias BC5 e BC2, respectivamente. Na atividade enzimática  $\beta$ -1,4 da endoglucanase foi o BC5 e para a exoglucanase  $\beta$ -1,4 foi o BC8, enquanto a produção de biomassa microbiana foi o melhor resultado para a bactéria isolada BC9. Os resultados mostraram que, entre as diferentes espécies de bactérias dos diferentes locais, foram apresentadas diferenças significativas quando cada uma das análises foi comparada e, portanto, isso pode estar associado aos diferentes tipos de ecossistemas e às características bioquímicas e ambientais em cada tipo de solo. , possuindo diferentes mecanismos diretamente relacionados à degradação da celulose. Portanto, esses microrganismos têm um uso potencial em diversos processos de degradação de celulose biotecnológica e de biorremediação.

**Palavras-chave:** enzimas celulolíticas, microrganismos celulolíticos, endoglucanase, exoglucanase, solo, microbiologia ambiental.

## 1 INTRODUCTION

The cellulose is a linear polysaccharide of glucose residuals with  $\beta$ -1, 4- glycosidic bonds. Among natural materials is the most abundant biopolymer and it can be hydrolyzed by the enzyme denominated cellulase, found as a multi-enzymatic complex where the final product result is the

glucose. In nature, these process represent the highest source of carbon in the soil (LYNCH, 1981), having a wide variety of microorganisms, such as fungi and bacteria able to produce cellulase.

Although, bacteria has a high potential in allowing a better separation of the lignin from the cellulose, having the genres; *Pseudomonas*, *Cellulomonas*, *Streptomyces* and *Actinomycetales*, as the better known (LYND, 2002) and (PERÉZ, 2002), employing; cellulases, laccases and extracellular peroxidases. In laboratory conditions, cotton and filter paper and other types of substrates inducers, are the ones commonly used to stimulate the exo-glycosidases enzyme production and also for the total cellulolytic complex activity measurement (ROBSON, 1989). For each bacterial species, the cellulose degradation can be related with the genetic feature and the specie's biochemical characteristics, related with the soil physicochemical properties, ecological and environmental factors, from the place of origin.

Its abundant availability becomes a raw attractive material for the production of a lot of important industrial products, through cheapest process and favorable ecological (GUPTA, 2012). In first place there is a residual degradation and pollution reduction of the environment. In second place, due to its large scale applicability, it can be used in industrial processes, such as the production of biofuels, the bioethanol (EKEPERIGIN, 2007)(VAITHANOMSAT, 2009), the agriculture management of vegetal residuals (LU, 2004); and the fermentation and bioremediation processes. Therefore the aim of this research was the soil's bacteria cellulose-decomposers characterization and comparison from the cities of Canguçu and Arroio do Padre from the state of Rio Grande do Sul and the city of Belém do Pará from the state of Pará, in Brazil.

## **2 MATERIALS AND METHODS**

### **2.1 OBTAINMENT OF THE BACTERIAL ISOLATES**

#### **2.1.1 Sample origin**

The soil samples were obtained from different cities belonging to the states of Pará and Rio Grande do Sul, Brazil, comparing the cellulose degradation differences for each isolate of each local place. The soil samples from Belém do Pará (Pará) were collected from the Amazon rainforest, from Arroio do Padre (Rio Grande do Sul) were collected from the familiar agro ecosystems, under the Lavoura-Pecuária integration and from Canguçu (Rio Grande do Sul) were collected from a rural open field. The soil was collected from soil layers (0,00-0,20 cm of deep) cutting shovel, conditioned in plastic bags and taken to the Environmental Microbiology Laboratory in the Biology Institute/UFPel for further analysis (Embrapa, 2011). For each positive isolate, the initials (BC) were named, for the cellulolytic bacteria.

### 2.1.2 Experimental design

The experimental design used was completely randomized design, with three repetitions for all the dependent variables. For the filter paper degradation variable, the experiment was arranged in a unifactorial scheme, the treatment factor tested were; the cellulolytic bacteria isolates (BC1 to BC10, beyond the controls, 1 and 2). For the colony diameter, the inhibition halo and the enzymatic index, the unifactorial scheme was adapted, the tested treatment factor was the cellulolytic bacteria isolates (BC1 to BC10).

For the microbial biomass variable, the bifactorial scheme was adapted. The treatment factor A tested the cellulolytic bacterial isolates (BC1 to BC10, beyond the controls) and, the factor B tested the evaluation times (0, 6, 12, 18, 24 and 30 h). In the glucose-cellobiose's variable activity, the experiment was arranged in a bifactorial scheme. The factor of treatment A tested the evaluation times (0, 6, 12, 18, 24 and 30 h), and the factor B tested the doses (0,1, 0,4 and 07 ml), that comparison was realized for separated, for both the  $\beta$ -1,4-exoglucanase and the  $\beta$ -1,4-endoglucanase, for each cellulolytic bacterial isolate.

### 2.1.2 Isolated method and morphologic characterization

For each bacterial isolate, 1 g of each soil sample was weighted and collocated in jars containing saline solution (0,85%). The solutions were agitated in a (Orbital Shaker Warmnest) shaker for 1 h at 120 g and after that, the serial dilution was performed. Posteriorly, aliquots of 100  $\mu$ l were inoculated in the plates containing Luria-Bertani (0,2 % of Carboximetilcellulose-CMC) solid media and they were incubated at 30 °C for 24 h. Were considered as positive, the colonies that formed a clear zone around (degradation halo). The bacterial cells were stored at -6 °C, using a glycerol solution at (10 %) as cryoprotectant. Each of the positive colonies was morphological characterized in appearance and arranged using the Gram staining method.

## 3 CELLULOSE'S DEGRADATION ANALYSIS

### 3.1 CELLULOSE'S DEGRADATION DETERMINATION WITH FILTER PAPER

For each isolate, a bacterial inoculum with a concentration of  $10^8$  UFC ml<sup>-1</sup> was prepared, indirectly quantified by optic density at OD<sub>600</sub>, incubated under agitation of 100 (g) at 37 °C in mineral medium (2,5 g of NaNO<sub>3</sub>; 2 g of KH<sub>2</sub>PO<sub>4</sub>; 0,2 g of MgSO<sub>4</sub>; 0,2 g of NaCl; 0,1 g of CaCl<sub>2</sub>·6H<sub>2</sub>O, pH 6,8-7,2) containing (Whatman Filter Paper N°1) filter paper as the only cellulose source and using as negative control a saline solution inoculum. The filter paper's patronization was made cutting tapes in size of 1x6 cm and 12,5 mg in weigh, each jar contained the inoculum and the

negative control. After ten days of incubation, each sample was taken for dry and for weighing of the filter paper. The results were shown in mg (GUPTA, 2012).

### 3.2 PROOF OF THE CELLULOSE'S DEGRADATION IN SOLID MEDIUM

For the potential cellulose's degradation confirmation, the bacteria were incubated in plates containing Luria-Bertani medium, supplemented with 0,2 % of CMC. After three days of incubation at 30 °C, the plates were immersed in *Lugol's solution* (1 mg ml<sup>-1</sup>) for 3 min, according with the Ramesh's method et al 2008. The solution was then washed with NaCl 1 M (REINHOLD-HUREK et al., 1993). The positive isolates cellulose's degradation potential was evaluated qualitatively, estimating the enzymatic index (I.E) calculation: (Colony Diameter + halo Diameter)/Colony Diameter.

### 3.3 EXTRACELLULAR ENZYMATIC PRODUCTION

For the extracellular enzymatic quantification 1 mL of bacterial concentration (10<sup>8</sup> UFC ml<sup>-1</sup>) of each one of the eleven bacterial isolates obtained was used and collocated in 3 ml of liquid Luria-Bertani medium supplemented with CMC 10 %. The tubes were incubated at 28 °C in constant agitation at 130 (g) in a (Orbital Shaker Waemnest) shaker for the extracellular enzymatic quantification analysis, aliquots of 2 ml for each tube were removed in intervals of 6 h during 24 h and collocated in microtubes for further centrifugation (refrigerated digital micro processed centrifuge CT-15000R) at 6.000 (g). After the centrifugation each microtube of each isolate was separated in different microtubes containing the supernatant and the *pellet* respectively (MIKÁN; CASTELLANOS, 2004).

#### *β-1,4 exoglucanase's activity*

For each bacterial isolate, 800 µl of the supernatant, 12,5 mg of the Whatman N°1 filter paper, 200 µl of the sodium acetate tampon solution 0,6 M with pH 6,0 were collocated in microtubes and incubated at 50 °C for 50 min (RAMÍREZ; COHA, 2003).

#### *β-1,4 endoglucanase's activity*

For each bacterial isolate, 200 µl of the supernatant, 875 µl of CMC (1 %) and 25 µl of the sodium acetate tampon solution (1,0 M with pH 6,0) were collocated in microtubes and incubated at 50 °C for 50 min (RAMÍREZ; COHA, 2003).

### 3.4 MICROBIAL BIOMASS DETERMINATION AND SUGAR REDUCERS

Microbial biomass was determined by the *pellet* drying and weighting of each bacterial isolate. The sugar reducers liberation was determined by the Somogyi-Nelson's method (SOMOGYI,

1952; NELSON, 1994) using a spectofotometer (Model IL-227 Kasuaki) at an OD<sub>665</sub>. The activity results were expressed in UI ml<sup>-1</sup>, considering the free enzyme quantity of 1 µM glucose min<sup>-1</sup>, a unit (RAMÍREZ; COHA, 2003).

#### 4 STATISTICAL ANALYSIS

The data obtained was analyzed for normality using the Shapiro's Wilk test; the homoscedasticity using the Hartley test; and, the independence residuals by the graphic analysis. Later, the data was submitted to variance analysis through the F test ( $p \leq 0,05$ ). Showing statistical significance, the cellulolytic bacteria isolates effects were compared by the Tukey test ( $p \leq 0,05$ ); the controls were evaluated by the Dunnett test ( $p \leq 0,05$ ); and, the evaluation times, by the quadratic polynomial regression model ( $p \leq 0,05$ ), as it follows:  $y = y_o + ax + bx^2$ , were:  $y$  = variable response;  $y_o$  = response variable corresponding to the minimum point curve ;  $a$  = estimated highest value for the response variable;  $b$  = curve's declivity;  $x$  = evaluation time (hours). The time and doses comparison was realized by the multiple regression models, using the surface regression response procedure (PROC RSREG), with examination of the linear effects, quadratic and linear interactions of the independent variables (FREUND; LITTELL, 1991). The model's selection was based on the: (a) residue; (b)  $p$  value ( $p \leq 0,05$ ); (c) standard deviation; and (d) R2 and R2adj. Then, the polynomial equation of the second order to the response's variables data was adjusted:  $y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j$ , were  $y$  is the variable response;  $x_i, x_j$  are the entrance variables, that influenced the response variable  $y$ ;  $\beta_0$  is the interception;  $\beta_i$  is the linear effect;  $\beta_{ii}$  is the quadratic effect and  $\beta_{ij}$  is the interaction between  $x_i$  and  $x_j$ . The additional rotational canonical analysis to the surface response was applied for optimization, in which the variables levels ( $x_1$ , time;  $x_2$ , dose) (inside the experimental interval) were determinated to obtain the response of each dependent variable studied. The function's optimization responses, constitutes in the function response traduction ( $y_k$ ), from the origin towards the stationary points ( $x_0$ ). The function response was maximized when all the roots obtained negative values and was minimum when all the roots obtained positive values. If any of the roots presented positive and other negatives values, a saddle point was characterized (MYERS, 1971; KHURI; CORNELL, 1989).

## 5 RESULTS

### 5.1 BACTERIAL ISOLATES

Ten positive bacterial isolates from the three cities in two states of Brazil were obtained for the cellulose's degradation evaluation. As result, the bacterial isolates BC1, BC2, BC9 and BC10 were obtained from the rural open field of Canguçu, in the state of Rio Grande do Sul. Four isolates BC3, BC4, BC5, BC7 from the Amazon rainforest in Belém do Pará, state of Pará. And the two isolates;

BC6, BC8 from the familiar agro ecosystem, under the integration system Lavoura-Pecuária in Arroio do Padre, state of Rio Grande do Sul. From the ten bacterial isolates, only four (BC1, BC3, BC7, BC9) belongs to Gram positive bacteria and six (BC2, BC4, BC5, BC6, BC8, BC10) belongs to Gram negative bacteria Table 1.

Table 1 Morphologic characterization of the degradative cellulose bacteria

Bacteria	Gram stain	Bacteria morphology
BC1	Positive	Coco bacillus
BC2	Negative	Cocos
BC3	Positive	Coco bacillus
BC4	Negative	Cocos
BC5	Negative	Cocos
BC6	Negative	Cocos
BC7	Positive	Coco bacillus
BC8	Negative	Cocos
BC9	Positive	Cocos
BC10	Negative	Coco bacillus

## 6 CELLULOSE DEGRADATION ANALYSIS

### 6.1 CELLULOSE DEGRADATION DETERMINATION

For the filter paper's degradation variable, the significance difference only occurred for the native BC5 bacteria from the Amazonia rainforest. When each isolate was compared with the control 1 (dry weight), a difference in relation to the BC5 occurred, showing a higher percentage degradation with a value of 10,72 %, were probably the production of extracellular enzymes was more elevated. However, referring to control 2, there were no differences in all the isolates.

In general, the ten bacteria presented weights too closed in relation to the dried filter paper used as control 1 and the weight in control 2 filter paper, this can be associated to the low extracellular protein free production in the liquid medium, directly affecting the paper's degradation. Low percentages were identified as they were compared with the weight, for the dried filter paper as in the liquid medium containing saline solution as inoculum Table 2.

Table 2 Filter paper's degradation of the cellulolytic bacteria isolates

Bacteria	Degradation percentage (%)
0BC1	3,6 <sup>1/ ns β</sup>
BC2	3,6 ns β
BC3	0 ns β
BC4	7,14 ns β
BC5	10,71 * β
BC6	7,15 ns β
BC7	3,6 ns β
BC8	0 ns β
BC9	0 ns β
BC10	0 ns β

<sup>1/</sup> Averages ( $\pm$  Standard error) accompanied by the same letter does not differ between them by the Tuckey test ( $p \leq 0,05$ ) comparing the cellulolytic bacteria isolates. \* and ns Significance and no significance, respectively, in relation to control 1 (dry weight) by the Dunnet test ( $p \leq 0,05$ ).  $\beta$  No significance in relation to control 2 by the Dunnet test ( $p \leq 0,05$ )

## 6.2 CONFIRMATION OF THE CELLULOSE'S DEGRADATION IN SOLID MEDIUM

The cellulose's degradation confirmation was obtained from ten bacteria comparing the colony diameter, the bacteria with the highest development were CB6 with 6,55 mm and CB92, with 45 mm. However, the highest cellulose's degradation halo's diameter was for the CB6, with an average of 7, 85 mm. The method for the extracellular enzymes evaluation with solid medium, determined a direct relation between the halo's size and the microorganisms degradative capacity; therefore, it suggests that the enzyme's index  $\geq 2, 0$  mm allows the consideration of a microorganism as an enzyme potential producer in solid medium (LEALEM; GASHE, 1994). Nine bacteria showed enzymatic indexes highest than  $\geq 2,0$ , they were considered as extracellular enzyme potential producers Table 3. The BC4 isolate didn't showed colony growth and therefore, do not developed a degradation halo Fig1.

Table 3 Colony diameter (mm), degradation halo (mm) and cellulolytic bacteria enzymatic index

Bacteria	Colony diameter (mm)	Halo's diameter (mm)	Enzymatic Index (IE)
BC1	0,65 $\pm$ 0,12 de <sup>1/</sup>	1,55 $\pm$ 0,10 d	3,55 $\pm$ 0,33 b
BC2	0,97 $\pm$ 0,35 cde	3,35 $\pm$ 0,25 c	5,33 $\pm$ 0,90 a
BC3	1,87 $\pm$ 0,08 bc	4,10 $\pm$ 0,04 bc	3,20 $\pm$ 0,08 b
BC4	0,00 $\pm$ 0,00 e	0,00 $\pm$ 0,00 e	0,00 $\pm$ 0,00 c
BC5	1,75 $\pm$ 0,18 bc	3,80 $\pm$ 0,08 bc	3,25 $\pm$ 0,25 b
BC6	6,55 $\pm$ 0,16 a	7,85 $\pm$ 0,09 a	2,20 $\pm$ 0,03 b
BC7	1,80 $\pm$ 0,40 bc	4,25 $\pm$ 0,25 b	3,68 $\pm$ 0,48 ab
BC8	1,62 $\pm$ 0,02 bcd	3,92 $\pm$ 0,02 bc	3,42 $\pm$ 0,04 b
BC9	2,45 $\pm$ 0,14 b	4,42 $\pm$ 0,05 b	2,82 $\pm$ 0,10 b
BC10	1,55 $\pm$ 0,28 bcd	3,35 $\pm$ 0,43 c	3,23 $\pm$ 0,14 b

<sup>1/</sup> Average ( $\pm$  standard error) accompanied by the same letter do not differed



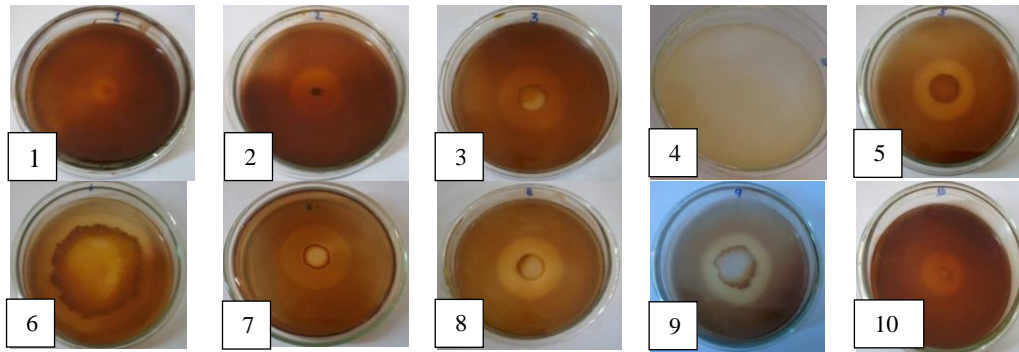


Fig. 1

Cellulose's activity through the substrate's degradation halo (CMC).  
1-BC1, 2-BC2, 3-BC3, 4-BC4, 5-BC5, 6-BC6, 7-BC7, 8-BC8, 9-BC9, 10-BC10.

### 6.3 EXTRACELLULAR ENZYMATIC PRODUCTION

#### *$\beta$ -1,4-exoglucanase's activity*

The glucose-cellobiose's activity data from the  $\beta$ -1,4-exoglucanase activity obtained from all the cellulolytic bacteria isolates adequately adjusted to the established regression model Table 4. During the optimization process for this variable, the auxiliary equations roots were positive and negative in their magnitudes, indicating that the stationary point was a saddle point for all the isolates Fig2.

For the BC10 isolate, the function response was maximal, all the roots obtained negative values and the predicted value in the maximal stationary point was of  $84,69 \text{ UI ml}^{-1}$  of glucose-cellobiose with 35,3 h and 0,88 ml Fig3 and table 4.

Fig2  $\beta$ -1,4 Exoglucanase's activity according to the best dose, best time and highest activity for each isolate (BC1 to BC10) according to the evaluation time (hours) and to the enzyme dose.

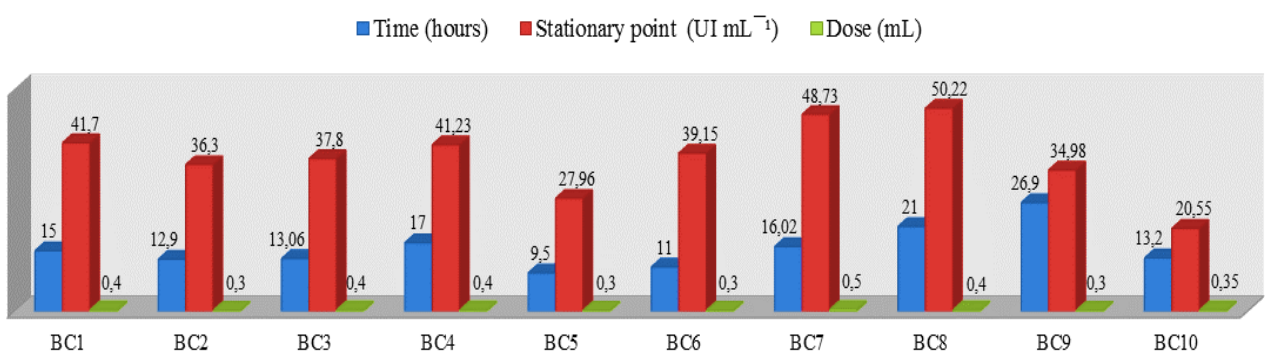
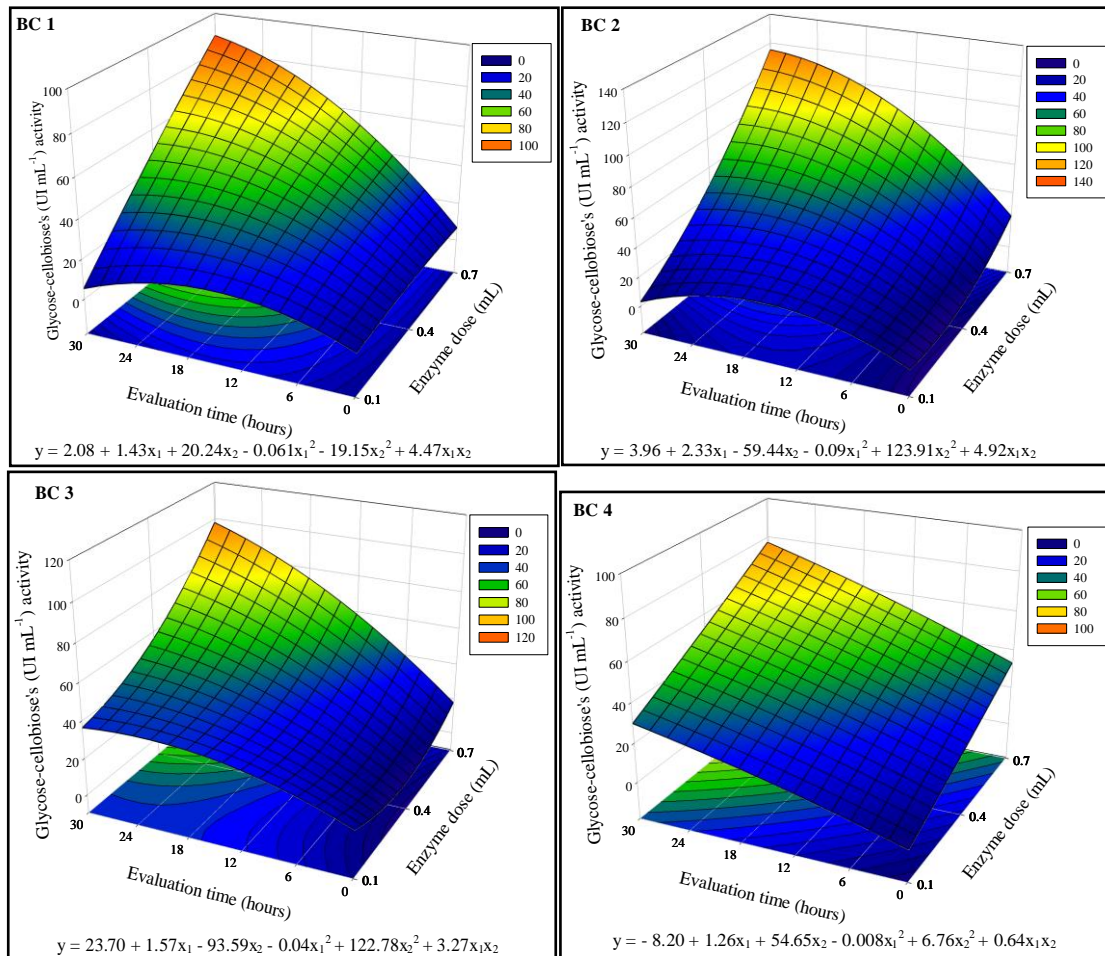


Fig3 Glycose-cellobiose's (UI mL<sup>-1</sup>) activity from the  $\beta$ -1,4-exoglucanase of cellulolytic bacteria isolates (BC1 to BC10) according to the evaluation time (hours) and to the enzyme dose.



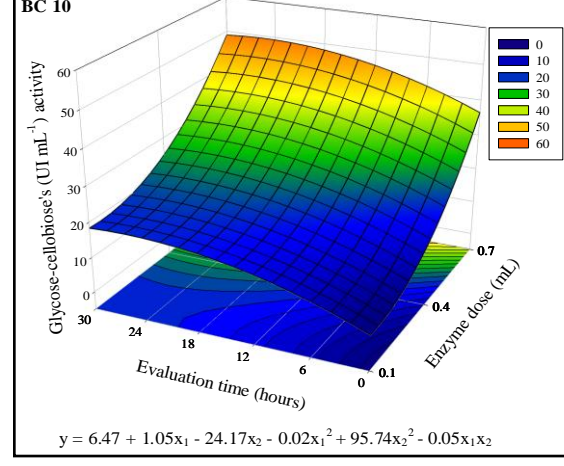
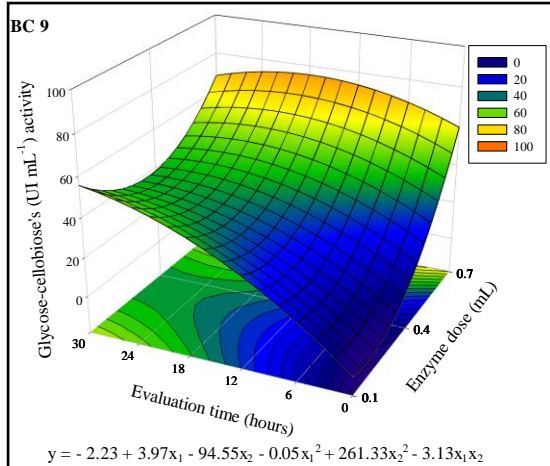
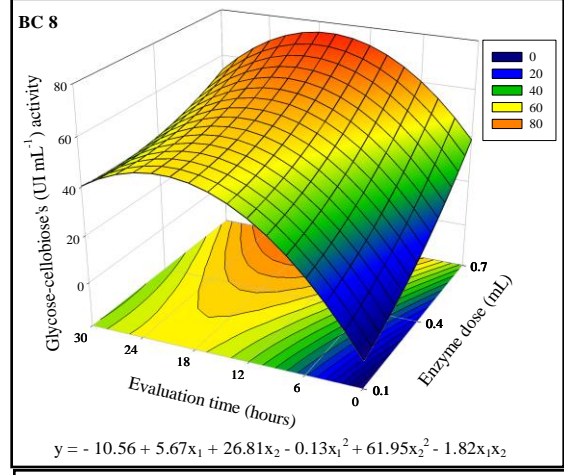
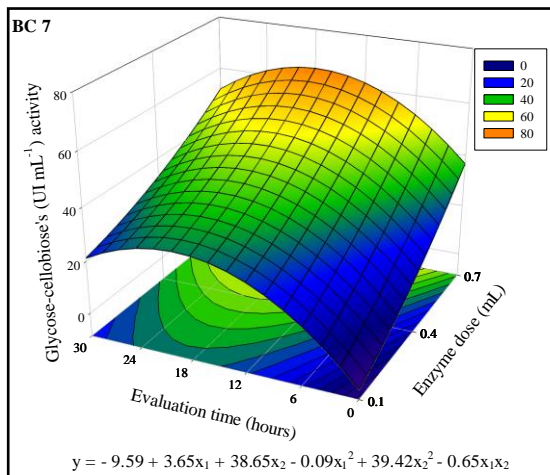
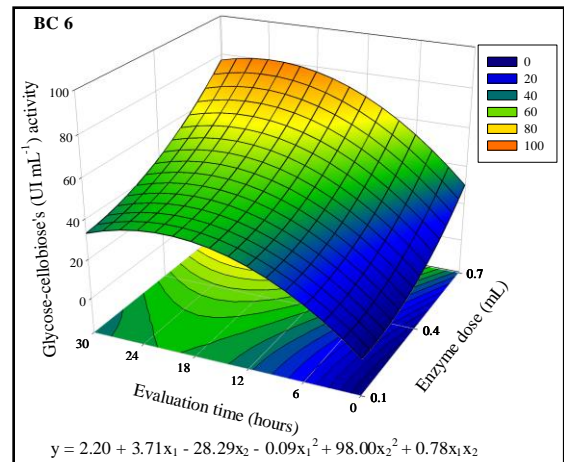
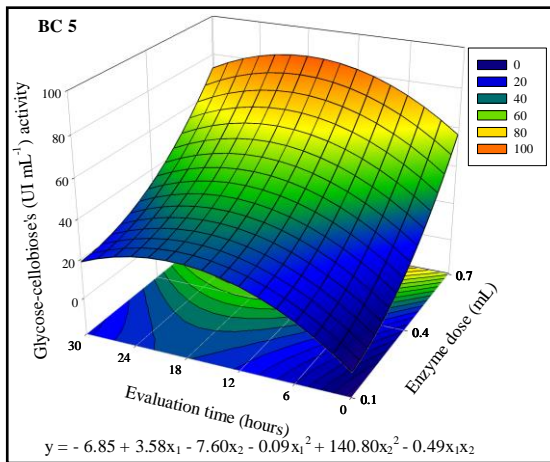


Table 4 Cellulolytic bacteria regression model adjusted values

Cellulolytic Bacteria	Value F	p-value	R <sup>2</sup>	R <sup>2</sup> adj
BC1	90,48	p = 0,004	0,85	0,82
BC2	6,99	p = 0,003	0,74	0,72
BC3	67,02	p = 0,0001	0,89	0,88
BC4	40,03	p = 0,001	0,83	0,80
BC5	43,48	p < 0,0001	0,60	0,59
BC6	46,98	p < 0,0001	0,65	0,62
BC7	9,57	p = 0,01	0,69	0,67
BC8	63,56	p < 0,0001	0,90	0,88
BC9	4,78	p = 0,01	0,68	0,66
BC10	36,56	p < 0,0001	0,86	0,84

### $\beta$ -1,4-endoglucanase's activity

The glucose-cellobiose's activity data from the  $\beta$ -1,4 endoglucanase activity in all cellulolytic bacteria isolates, adjusted adequately to the regression model shown in Table 5, also the roots from the equations were positive and negative in their magnitudes Fig4 and Fig5.

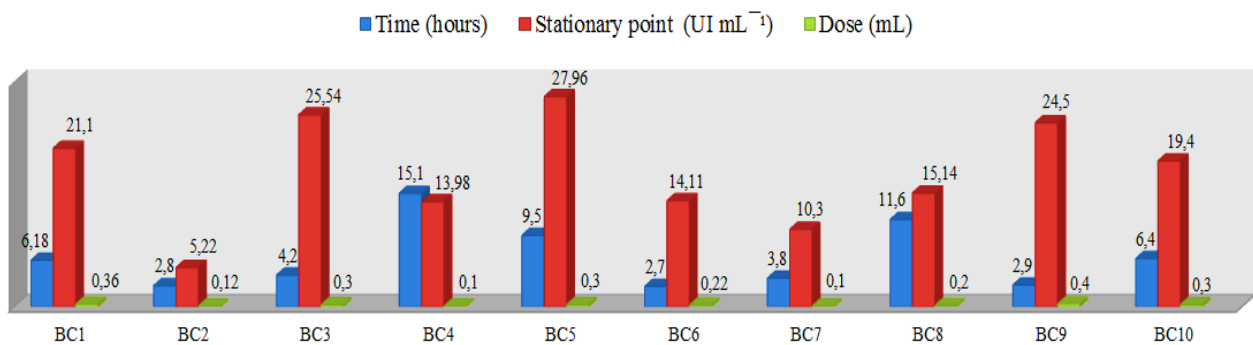
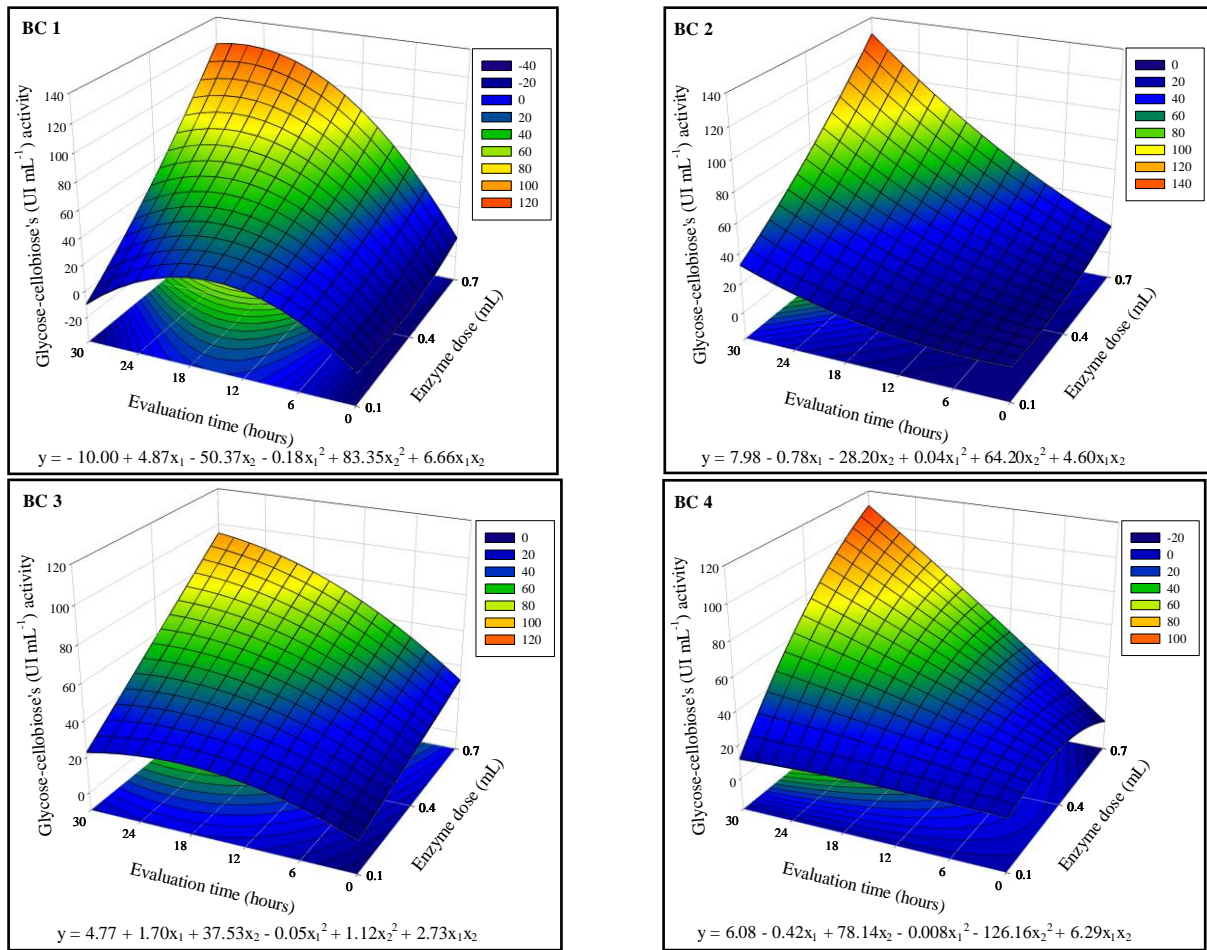
Fig4  $\beta$ -1,4 exoglucanase's activity according with the best dose, best time and highest activity for each isolate

Fig5 Glycose-cellobiose's (UI mL<sup>-1</sup>) activity from the  $\beta$ -1,4-endoglucanase of cellulolytic bacteria isolates (BC1 to BC10) according to the evaluation time (hours) and to the enzyme dose:



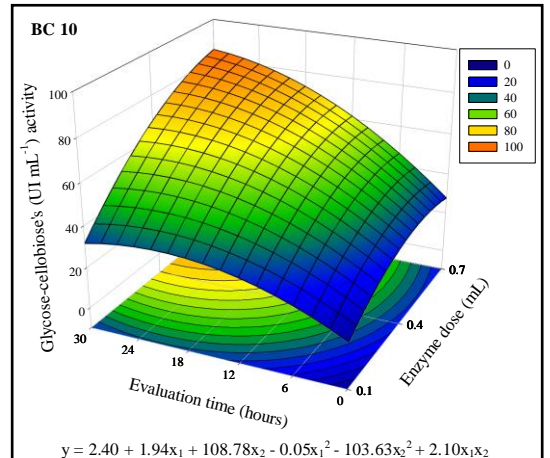
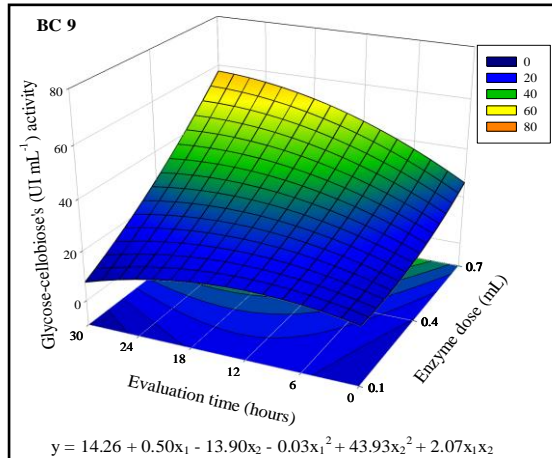
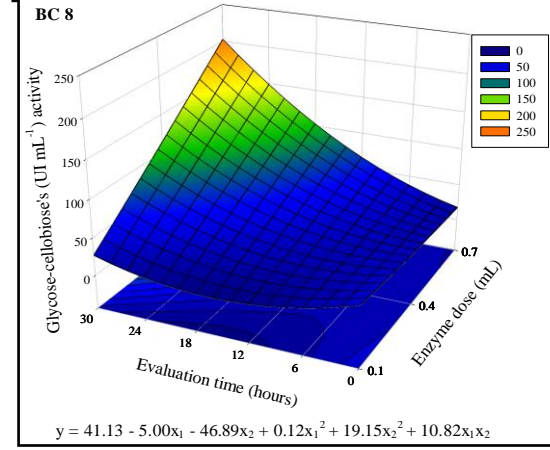
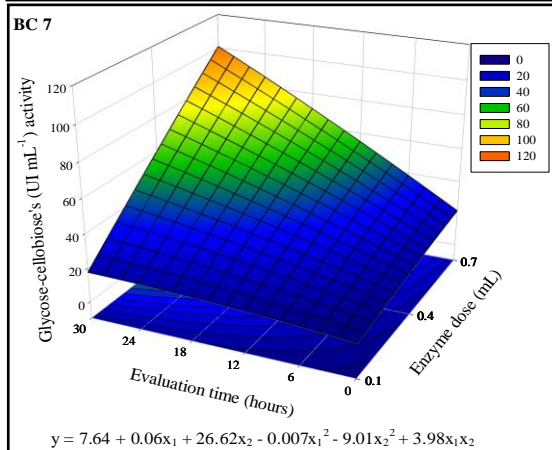
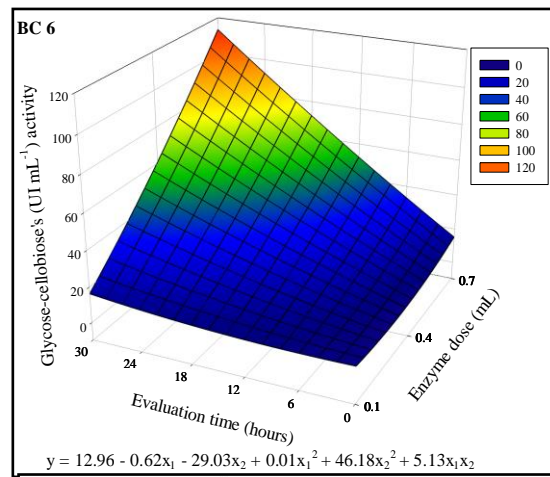
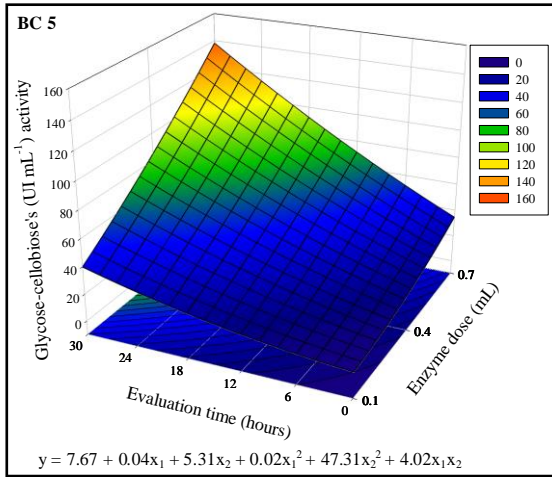


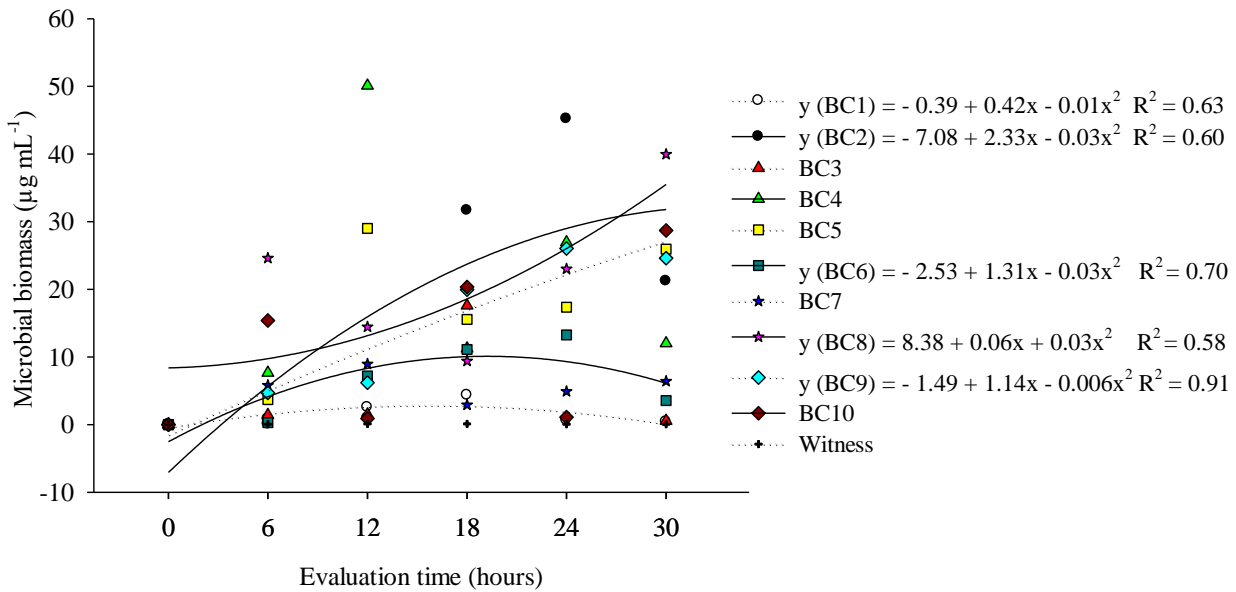
Table 5 Adjusted values to the cellulolytic bacteria regression model

Cellulolytic bacteria	Value F	<i>p</i> -value	R <sup>2</sup>	R <sup>2</sup> adj
BC1	7,08	<i>p</i> = 0,003	0,74	0,72
BC2	12,48	<i>p</i> = 0,0002	0,84	0,81
BC3	76,83	<i>p</i> = 0,0001	0,87	0,85
BC4	6,76	<i>p</i> = 0,003	0,74	0,73
BC5	3,35	<i>p</i> = 0,04	0,60	0,59
BC6	4,17	<i>p</i> = 0,02	0,63	0,60
BC7	9,57	<i>p</i> = 0,03	0,65	0,62
BC8	6,72	<i>p</i> = 0,003	0,74	0,72
BC9	5,96	<i>p</i> = 0,01	0,68	0,66
BC10	41,65	<i>p</i> < 0,0001	0,88	0,87

#### 6.4 MICROBIAL BIOMASS

The microbial biomass analysis results demonstrated significance in the interaction between the treatment isolates bacteria factors and the evaluation times ( $F = 34,55$ ;  $p < 0,0001$ ). In the isolate's comparison inside each time, significance differences were shown in the hour six of the evaluation time, the bacteria BC8 differed from the others with a highest average, followed by the BC10 and BC4 bacteria. While in the 12 h of the evaluation period, the BC4 bacteria differed from the others, followed by the BC5 and BC8 bacteria. In both the 18 and 24 h of the evaluation period, the BC2 bacteria was characterized by a higher average, but in the 18 h differed only from the BC1 and BC7, and in the 24 h presented difference in relation to all the others isolates. In the 30 h, the BC8 presented a higher average, differing from the others. Significance differences were shown for all the isolates in relation to the controls in the 12, 24 and 30 h of the evaluation time.

The microbial biomass data adequately adjusted to the regression quadratic polynomial model only for the isolates BC1 ( $F = 7,7461$  and  $p = 0,0111$ ), BC2 ( $F = 6,7476$  and  $p = 0,0162$ ), BC6 ( $F = 10,7765$  and  $p = 0,0041$ ), BC8 ( $F = 6,1425$  and  $p = 0,0208$ ) and BC9 ( $F = 45,2735$  and  $p < 0,0001$ ) Fig6.

Fig 6 Cellulolytic bacterial microbial biomass ( $\mu\text{g mL}^{-1}$ ) in function of the evaluation time (hours)

Increases are shown in the biomass values of 81,4, 122,0, 122,0 e 81,4 % respectively for 12, 18, 24 and 30 h periods for the BC1 bacteria, when compared at six hours of evaluation time. In the BC2 and BC6 bacteria at 18 h, the increases were superior to 150 % when compared at six hours of evaluation. Initially in the BC8, the lowest increases percentages being of 36,7 and 95,3 % for 12 and 18 h respectively were verified, when compared at 6 h. While the BC9 obtained 120,6 % of increase for the 12 h period of evaluation time, when compared at 6 h of evaluation.

## 7 DISCUSSION

The study of bacteria able to degrade cellulose can depend of several factors that influence the action mechanisms of the multienzymatic complex inside each specie. From the studying bacteria analysis of a macerated intestine of organisms with cellulose alimentation revealed that this can be more efficient, the analysis showed that the maximum and minimum rates of the filter paper's degradation were of 65,7 and of 55 %, respectively, estimated in the third day of incubation of 8 isolates (GUPTA, 2012). The documented result by Lu et al. (2004), demonstrated that the detected cellulose's degradation in four groups of mixed cultures varied between 19,4 to 26,3 %.

Similar results in the paper's degradation rates varied of 31 to 60 % after 10 days in mixed bacteria population by the gravimetric procedure (BICHET-HEBE, 1999). These results differed from this work considering that for the 10 days of incubation, the Whatman filter paper's degradation did not exceeded the 10 %, however they become comparative to the work by Silva et al. (2012), were it was demonstrated that after only 100 d of incubation of the filter paper, there was a loss of the cellulose's total mass, when the soil's isolates were evaluated with vinasse. There by the analysis



from these cellulose's enzymatic hydrolysis mechanisms, were from some aerobic bacteria that are bonded to the cells or to the substrate, were they can produce extracellular enzymes with different adherence ways in different substrate's configurations, meaning that a microorganism's population can adopt distinct strategies in the enzymatic production, like for example, the anaerobic and aerobic degradation relation, this symbiotic association may increase or not the degradation's efficacy (CUNHA-SANTINHO, 2003).

The results from this study for the cellulose degradation's verification in solid medium, were similar to the enzymatic index of 1,38 to 2,33 IE and 0,15 to -1,37 IE in the agriculture soil's cellulolytic aerobic bacteria and florestal soil, respectively (HATAMI et al. 2008), also they are comparative with the results of four bacteria EF1, RU3, RU4, RA2 isolated from *Macrotermes gilvus intestine*, being a termite specie. It was shown from the strain's cellulolytic activity that RA2 had the highest enzymatic index of 2,5 and the RU3 isolate the lowest of 0,75 (FERBIYANTO, 2015). In the other hand, Araújo et al (2015), studying 21 actinobacteria strains from the Parque Nacional de Ubajara, in the state of Ceará, verified that the inhibition halo diameters of the studied actinobacteria strains varied from 3,75 to 31,75, meanwhile the enzymatic index varied from 1,18 in the UB-03-R1 strain to 6,90 in the UB-05-R1 strain. These values can be compared with the evaluation of eight bacteria, obtained from four different invertebrates (termites, snail, caterpillar, and other). The reported enzymatic index varied between 9 and 9,81E and the degradation halo diameters were between 45 and 50mm (GUPTA, 2012) differing from this study results.

The analysis made for the exoglucanase enzyme showed different stationary points for each bacteria. Specific activities of the proteins from the production of  $\beta$ -1,4 exoglucanase by the *Streptomyces* sp CMC10 strain was of 2,61 UI mg<sup>-1</sup> in 72 h (RAMIREZ; COHA, 2003). Santos et al (2013), analyzed agro industrial residuals through fermentative process, which they obtained that the  $\beta$ -1,4 endoglucanase reach the index of 9,32 UI ml<sup>-1</sup>. However, *Streptomyces* sp cultivated in peptone contained solution and 1,0 % of Tween 80 in crystal cellulose (GEORGE, 2001), when cultivated it in paper powder containing medium with cellulose, yeast extract and Tween 80, showed a peak of 23 UI ml<sup>-1</sup>, meanwhile cultivated in wheat bran was of 8,5 UI ml<sup>-1</sup>. The enzymatic activities in the diverse microorganism's enzymatic complex are regulated by diverse environmental factors such as; the temperature, pH and salinity (VINOGRADOVA.; KUSHNIR, 2003), which can be involved with physicochemical factors in the soil and the genetic characteristics of the specie.

A similar study made by Ramirez and Cocha (2003), with *Streptomyces* sp. 7CMC10 and 11CMC1 strains, showed that the highest values for the two strains were of 20,14 UI mg<sup>-1</sup> and 9,55 UI mg<sup>-1</sup>, respectively, corresponding to proteins related to endoglucanases with activities in 72 h period. These results are similar to those found in the work of Corrêa Rorigues et al (2019) where the

values of enzymatic activity in actinobacteria from the amazon occurred between 96 hours and 120 hours of culture.

Results of 6 analyzed types of industrial celulasas (Youtell, RCconc, Lerkam, Yishui, R-10, and Sinopharm) shown that the enzymes activities vary in the glucose release and that the highest values were for 7,7 UI ml Sinopharm up to 69.90 UI ml Youtell (Yu, 2016) which are similar in the analysis of some bacteria of this work. In the cellulolytic enzymes study, the substrate's specificity and the specie's detailed knowledge can lead to more specific results, because the cellulase is a system that includes the endoglucanases, exoglucanases and  $\beta$ -D glycosidases that act hydrolyzing the crystal cellulose in a joint way (MULLINGS, 1985; CRIQUET et al., 2002; HELBERT et al., 2003; EVELEIGH et al., 2009; FARNET et al., 2010; DASHTBAN et al., 2010), which sometimes cannot be identified in inaccurate analysis.

The microbial biomass production process depends to the total reproductive capacity of the bacteria cells, which cannot be maintained for a long period of time. This is why the microbial population turns limited by the essential nutrient scarcity, or when an unfavorable ionic equilibrium develops (pH) or by the environmental toxic substances accumulation. Actinobacteria *Streptomyces* sp. 3 presented activity in the 116 h period and only had endoglucanase and cellobiase activity Fig3. It is worth mentioning that the actinobacteria *Streptomyces* sp. 3 have moderate biomass growth before the 116 h period, which can be related to the substrate degrade activity, not detected by the used methods.

When a constant mortality rate is established the culture begins to die exponentially, until finally sterility occurs. But, after most all of the cells had died, the mortality rate can show a decline marked by the fact that small number of cells continue to survive. The continuing growth in the nearby hours of this small survivors population can be attributed by the nutrients availability from cells that die and slowly decomposes, increasing again the population during the evaluation time.

## 8 CONCLUSION

The isolated cellulolytic bacteria from different soil types presented different cellulose's degradation index and efficiency, when compared with their realized enzymatic index in plates and the analysis of each one of the enzymes involved in the cellulose enzymatic complex. The most effectives isolates of each analysis were the BC5 for the Whatman filter paper's degradation, the BC2 in the enzymatic index analysis, the BC9 in the microbial biomass analysis, the BC8 in the  $\beta$ -1,4 exoglucanase's enzymatic activity, the BC5 in the  $\beta$ -1,4 endoglucanase's enzymatic activity.

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