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STABILITY CELLULASES OF *BACILLUS* SP. SMIA-2 DURING STORAGE UNDER FREEZING AND IN THE PRESENCE OF ADDITIVES

ESTABILIDADE CELULAR DE Bacillus sp. SMIA-2 DURANTE O ARMAZENAMENTO SOB O CONGELAMENTO E NA PRESENÇA DE ADITIVOS

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Abstract

Enzymatic stability is undoubtedly one of the most important factors in biotechnology. In this sense, the stability of cellulases present in crude extracts of submerged cultures of Bacillus sp. SMIA-2 during freezing at 3 °C and in the presence of additives was studied. Cellulases were stable during storage for 154 days at 3 °C. The activity of avicellase and CMCase was maintained when crude extracts containing the enzymes were incubated in the presence of ethylene glycol (1M), glycerol (1%) and polyethylene glycol (0.01M) for 1 hour at room temperature.

Key-words: enzymatic stability, additives, *Bacillus* sp SMIA-2, cellulases.

1. Introdution

Cellulases and hemicellulases are enzymes that catalyze the hydrolysis of cellulose and hemicellulose, respectively, transforming these polymers into sugars of lower molecular weights (Peixoto, 2006). The economic feasibility of applying an enzyme in an industrial process is

determined by its stability during the process. In order to maintain the stability of the spatial conformation of the enzyme, chemical agents such as detergents, salts, solvents, polymers, polymers, and other compounds are generally added in addition to substrates and inhibitors that protect the active site (Illanes, 2008).

2. Material e methods

Microorganism: The bacterium Bacillus sp. SMIA-2, isolated by Souza and Martins (2001), from soil samples from the Campos dos Goytacazes region, Rio de Janeiro, Brazil was used in this work.

Culture medium: The culture medium for the production of the cellulases contained (g.L-1 distilled water): peptone, 1.0; KCl, 0.3; K2HPO4, 0.87; MgSO4, 0.5; NaCl, 10.0; and traces of metals (CaCl2, 2.2x10-3, ZnO, 2.5x10-3, FeCl3 · 6H2O, 2.7x10-2, MnCl2 · 4H2O, 1.0x10-2, CuCl2 ·2H2O, 8.5x10-4, CoCl2 .6H2O, 2.4x10-3, NiCl3.6H2O, 2.5x10-4, H3BO3, 3.0x10-4; Na2MoO4, 1.0x10-3). To this basal medium was added 0.8% (m / v) corn steep liquor (Sigma Aldrich) and 0.8% (w/v) sugarcane bagasse and 0.8% (w/v) of flour of the passion fruit peel. The pH of the culture medium was adjusted to 7.5 with 1.0 M NaOH and sterilized by autoclaving at 121 °C to 1 atm for 15 minutes.

Culture Conditions: The bacteria were streaked in Petri dishes containing TSY medium (g.L-1 distilled water): tryptone 20; NaCl 10; yeast extract 10 and agar 20. The plates were incubated in a QUIMIS oven (model Q 315 D26) at 50 °C for 18 hours. After this period, 5 ml of the basal culture medium were transferred to the plates to resuspend the cells which, with the aid of a sterile pipette, were subsequently sucked out. These cells were inoculated into 250 mL Erlenmeyer flasks containing 50 mL of the respective growth medium, incubated for an additional 18 hours at 50 °C in a Thermo Forma Orbital Shaker, Ohio, under 150 rpm shaking. This medium was called inoculum.

Enzyme Production: Samples containing the culture medium were centrifuged in a centrifuge (HERMLEZ 382K, Wehingen, Germany) at 4500 rpm for 30 minutes at 4 °C to obtain the cell-free supernatant, which was used for dosing the activity enzymatic activity.

Enzyme assays: Enzyme activity was determined by quantification of the release of reducing sugars from the hydrolysis of avicel (Avicelase) and carboxymethylcellulose substrates as described by Costa et al., 2017. A unit (U) of the enzyme was defined as 1 μmol of equivalent reducing sugar

released by the substrate per minute under the above described test conditions using a standard glucose curve at concentrations of 0.014 to 0.3 mg / mL.

Stability of enzymes during freezing: The activity of the cellulases present in the crude extract was determined at times 0, 3, 29, 34, 61, 92, 125 and 154 days of storage at 30 °C according to the procedure described above.

Influence of Additives on Cellulase Activity: The following additives were added to the crude extract containing the cellulases: 1M Ethylene Glycol, 0.01M Polyethylene Glycol, 0.01M Glycine and 1% Glycerol. Then, the activity of the enzymes was determined according to the procedure previously described.

3. Results and discussion

The stability of the cellulases present in the crude extract of the cultures of Bacillus sp. SMIA-2 as a function of the storage time at 3 °C is shown in Table 1. Avicellase and carboxymethylcellulose maintained their stable activities within 154 days of storage. To analyze the data, the statistical program used was SAS - Statistical Analysis System (2003), version 9.3. The results obtained in each experiment were performed in triplicate and submitted to analysis of variance (ANOVA) to compare the means of the different treatments at the same time, as between the means of the same treatment between different times by the Tukey test at the level of 5% significance.

Table 1– Relative activity of avicellase and carboxymethylcellulase (CMCase) present in the crude extract of *Bacillus* sp. SMIA-2 as a function of the storage time at 3ºC for 154 days. (100% avicellase activity = 1.20 U / mL and 100% CMCase activity = 0.50 U / mL).

Time (days)	Avicelase (%)	CMCase (%)
0	100,0 [a] ± 0,0007	100,0 [a] ± 0,0007
3	98,9 [a] ± 0,0028	99,0 [a] ± 0,0029
29	98,9 [a] ± 0,0027	99,0 [a] ± 0,0027
34	98,9 [a] ± 0,0028	99,0 [a] ± 0,0027
61	98,6 [a] ± 0,0026	98,6 [a] ± 0,0012
92	98,6 [a] ± 0,0012	98,6[a] ± 0,0014
125	98,2 [a] ± 0,0019	98,5 [a] ± 0,0018
154	98,0 [a] ± 0,0021	98,4 [a] ± 0,0017

[1]Means with equal letters in the same column do not differ significantly from $p \leq 0.05$, the Tukey test.

The stability presented by the cellulases of Bacillus sp. SMIA-2 was superior to the cellulases (carboxymethylcellulose) produced by the filamentous fungus Rhizopus sp grown in solid state fermentation (FES), using forage palm (Nopalea cochenillifera) as a substrate. The cellulose complex remained stable when stored at -18°C for up to 144 hours (Santos et al., 2014). According to Narra et al. (2014), the stability of a purified endoglucanase produced by Aspergillus terreus and stored at 4°C was studied and it was found that after 5 months there was a decrease of 3 to 5% of its initial activity.

Some polyols such as sorbitol, xylitol or glycerol are known to stabilize biological activity. Thus, the influence of the addition of some additives on the stability of the cellulases incubated at room temperature was studied and the results obtained are shown in Table 2. The activity of the avicellase did not differ significantly ($p \leq 0.05$) from the control when incubated for 1 hour in the presence of 0.01M polyethylene glycol and 1% glycerol. When incubated for 3 hours in the presence of all additives, the avicellase activity decreased significantly ($p \leq 0.05$) of the control. It should be noted that no significant difference ($p \leq 0.05$) in enzyme activity was observed for the control when incubated for 1 and 3 hours.

Table 2 - Relative activity of avicellase and carboxymethylcellulase present in the crude extract of *Bacillus* sp. SMIA-2 in the presence of stabilizing additives. (100% avicellase activity = 1.07 U / mL and 100% CMCase activity = 0.48 U / mL).

Treatment	Avicellase (%)		CMCase (%)	
	1 hour	3 hours	1 hour	3 hours
Control	100,0 ^{bA} ± 0,0029	100,0 ^{aA} ±0,0029	100,0 ^{aA} ± 0,0043	100,0 ^{aA} ± 0,0043
Ethylene glycol 1M	101,1 ^{aA} ± 0,0029	50,8 ^{eB} ± 0,0031	99,7 ^{aA} ± 0,0037	88,5 ^{cB} ± 0,0037
Glycerol 1%	100,1 ^{bA} ± 0,0029	57,8 ^{cB} ± 0,0029	98,7 ^{aA} ± 0,0043	87,9 ^{cB} ± 0,0043
PEG 0,01M	100,6 ^{abA} ± 0,0029	60,5 ^{bB} ± 0,0014	99,5 ^{aA} ± 0,0043	92,6 ^{bB} ± 0,0043
Glycine 0,01M	95,3 ^{cA} ± 0,0025	54,0 ^{dB} ± 0,0012	119,9 ^{bA} ± 0,0033	94,0 ^{bB} ± 0,0033

[1]Means with lower case letters in the same column do not differ significantly from $p \leq 0.05$, according to the Tukey test; Means with equal capital letters on the same line do not differ significantly at $p \leq 0.05$, according to the Tukey test.

Regarding CMCase, it was observed that with 1 hour of incubation at room temperature the activity of the enzyme showed no significant difference between the control, 1M ethylene glycol, 1% glycerol and 0.01M polyethylene glycol. Already, in the presence of 0.01M glycine there was a significant increase of activity in relation to the control. However, it was found that in the incubation of the CMCase for 3 hours there was a significant difference in all the stabilizing additives in relation to the control.

5. Conclusion

The stability of avicellase and CMCase secreted by Bacillus sp. GASIA-2 in submerged cultures containing sugarcane bagasse, corn maceration water and passionfruit meal, as a function of the storage time at 3º C did not show a significant difference ($p \leq 0.05$), which suggests that up to 154 days these enzymes have remained stable. The activity of avicellase and CMCase was maintained when crude extracts containing the enzymes were incubated in the presence of ethylene glycol (1M), glycerol (1%) and polyethylene glycol (0.01M) for 1 hour at room temperature. However, after 3 hours of incubation the enzymes showed a decrease in their initial activity for all the additives used.

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