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Production of the Enzyme Cyclodextringlycosyltransferase from *Bacillus firmus* Alkalophilic

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Abstract

Different culture media have been tested for the production of the enzyme CGTase (cyclodextringlycosyltransferase) from *Bacillus firmus* (strain #37). The concentration of different carbon and nitrogen sources have been varied and the enzyme activity, cell concentration, reducing sugars, total reducing sugars, soluble protein and pH have been followed during cultivation. Results indicate that higher concentrations of yeast extract and polypeptone lead to increased synthesis of CGTase, whereas when starch is substituted by glucose there is a drastic inhibition of CGTase production.

Introduction

Matioli [1] using modified Nakamura and Horikoshi [2] medium has isolated an alkalophylic microorganism (strain #37) that produces the enzyme cyclodextringlycosyltransferase (CGTase; *EC* 2.4.1.19), which converts starch to cyclodextrins (CDs), with a cell free culture medium activity in the range of 0.16 to 0.20 mmol β-CD/min.mL (U/mL), at pH 8.0, 50 °C. This microorganism has been characterized as *Bacillus firmus* and it is of the same species as those used for enzyme production studies by Gawande *et al*. [3], for branched CDs production, by Yim [4], and for production of extracellular proteases by Moon and Parulekas [5].

More recently, Kroumov [6] using Nakamura and Horikoshi [2] modified media by the addition of L-arabinose (LA) at various concentrations relative to soluble starch (S) (S : LA, 10 : 0, 10 : 1, 9 : 1, 5 : 5, and 1 : 9) has obtained the following free cell culture medium CGTase activities: 0.226; 0.300; 0.290; 0.280 and 0.210 U/ml, respectively. Therefore, by adding L-arabinose, the maximum gain in CGTase activity achieved was about 30%.

The objective of our work was to study the production of CGTase by *Bacillus firmus* (strain #37) in various culture media with different concentrations and sources of carbon and nitrogen.

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Materials and methods

Microorganism

The alkalophylic microorganism used was *Bacillus firmus* (strain #37) isolated by Matioli [1] using soil samples from cassava plantation.

Cultivation media and conditions of cultivation

The microorganism was first cultivated at 37 °C for a period of 48 h, in semi-solid medium containing (% w/v): soluble starch 1.0; polypeptone 0.5; yeast extract 0.5; K_2HPO_4 0.1; $MgSO_4.7H_2O$ 0.02; Congo red dye 0.01; sodium carbonate 1.0; and agar 1.5. Then, the microorganism was transferred to 300 mL of a pre-inoculum medium composed as above, except that it contained 2% w/v soluble starch and no dye, nor agar [7]. The pre-inoculum was kept under agitation for 48h at 37 °C and then, added at the rate of 5% by volume, to 750 mL of cultivation medium with the composition as given in Table 1. Hydrolyzed starch was produced with soluble starch and α-amylase (Termamyl 120L, from NOVO) at 95 °C, pH 6.5, reaction time 45 min, and then α-amylase was thermally deactivated. The cultivation media was maintained under agitation at 180 rpm and 37 °C in 2000 mL erlenmeyer flasks during the cultivation test, and 20 mL samples were collected at regular intervals for analysis.

Sample treatment

Collected samples were centrifuged at 7000 rpm during 25 min to separate the biomass from the supernatant and then the supernatant was used for the analysis of pH, cell concentration, reducing sugars, total reducing sugars, soluble protein, and enzymatic activity, according to the methodology described bellow.

Table 1. Composition of the media used for cultivation of *Bacillus firmus* (strain # 37) at 37 °C, initial pH 9–10, 180 rpm, for production of the CGTase enzyme, and maximum CGTase activity obtained

Components	Medium Composition (% w/v)							
	SM	G	HS	HS1	G1	HSY	HSGY	HSYP
Glucose	–	2.0	–	–	1.0	–	0.5	–
Soluble starch	2.0	–	–	–	–	–	–	–
Hydrolyzed starch	–	–	2.0	1.0	–	1.0	0.5	1.0
Polypeptone	0.5	0.5	0.5	0.5	0.5	0.5	0.5	2.0
Yeast Extract	0.5	0.5	0.5	0.5	0.5	2.0	2.0	2.0
K_2PO_4	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
$Mg_2SO_4.7H_2O$	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Na_2CO_3	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Cultivation time	64	96	120	204	72	96	120	120
Maximum activity (U/mL)	0.180	0.046	0.204	0.120	0.010	0.378	0.204	0.548

(Media description: SM = Standard medium; G = Glucose instead of starch; HS = Hydrolyzed starch instead of soluble starch; HS1 = Same as HS, but with half the amount of hydrolyzed starch; G1 = Same G, but half the amount of glucose; HSY = Same as HS1 but with 4 times the concentration of yeast extract; HSGY = Hydrolyzed starch and glucose instead of soluble starch; HSYP = Same as HSY but with 4 times the concentration of polypeptone)

Cell concentration

After the sample was centrifuged, cell biomass collected was washed with distilled water and centrifuged again at the same conditions as given above. Then, the biomass was resuspended in an equal volume of distilled water as the original sample and a light absorption measurement was carried out at 610 nm using water as the reference.

Reducing sugars (RS)

The concentration of reducing sugars was measured by the colorimetric method of DNS [8], as modified by Zanin and Moraes [9]. Total Reducing Sugars (TRS): Ten mL of the supernatant from sample centrifugation was transferred to a flat bottom flask containing 2.0 mL of concentrated HCl (38%) in 150 mL of distilled water, and boiled for 2 h 30 min to obtain the hydrolysis of starch present. At the end of this period, the solution was cooled, phenolphthalein was added, and NaOH 2M was added up to neutralization. The volume of the solution was completed to 200 mL and reducing sugars were measured by the colorimetric method of DNS according to Falcone and Marques [10].

Soluble proteins

Protein concentration was measured according to the colorimetric method of Bradford [11], using BSA as standard.

CGTase activity

Enzyme activity for CGTase was measured in relation to the initial rate of production of β-CD according to the method described by Hamon and Moraes [12], that can also be found in the work of Matioli [1]. CGTase activity was assayed with a 1% w/v maltodextrin solution, pH 8.0, in buffer Tris-HCl 50mM, CaCl2 5mM, at 50 °C, 30 min reaction time. One unit of enzyme activity is the amount of enzyme that produces 1 μmol β-CD/min.mL of enzyme solution.

Results and discussion

Results obtained for the different cultivation media are show in Table 1 and Figures 1 to 3. The data of the first three cultivation media (SM, G, and HS) show that the substitution of starch by glucose drastically reduces the activity of CGTase produced to levels lower than 30% of the standard cultivation media (SM), but the substitution of soluble starch by hydrolyzed starch does not cause a significant difference in enzyme production.

The cultivation media HS1 and G1 showed that with half of the carbon sources in media HS and G, reduction in hydrolyzed starch reduces CGTase activity, while reduction in glucose increases it. We concluded that while cultivation media SM, HS, and HS1, assure a certain production of CGTase, it seems nevertheless, that this production is limited by some nutritional deficiency. On the other hand, media G and G1, in addition to the nutritional deficiency, display also glucose repression of CGTase synthesis. These observations are in accord with Gawande *et al.* [3].

Based on the nutritional deficiency hypothesis and the work of Gawande *et al.* [3] and Lee and Chen [13], that showed higher enzyme production with increased amounts of yeast extract and peptone, we have tested the last three cultivation media (HSY, HSGY, HSYP), were yeast extract and polypeptone were increased in relation to the standard medium (SM). Figures 1 to 3 present the profiles for consumption of the carbon source, pH, microbial cell growth [$X = f(t)$], biosynthesis of CGTase [$A = f(t)$], and soluble protein [$P = f(t)$]. These data show that there are distinct phases of cell growth and these are associated with the profile of CGTase production. The results for medium HSY in Figure 1 suggested catabolic repression, although with higher CGTase activity than the standard medium (SM). For medium HSGY where hydrolyzed starch was reduced to 0.5% and glucose added to 0.5%, Figure 2, shows that although the profile for carbon source consumption was anticipated, and cell growth and CGTase production were better

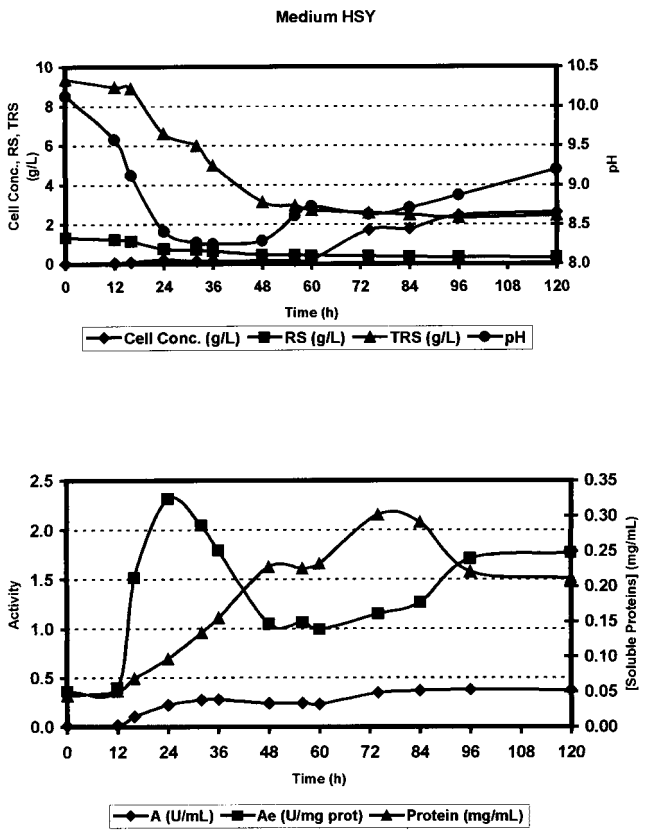

Figure 1. Evolution of growth variables for the cultivation of *Bacillus firmus* (strain #37) grown in the HSY medium (see Table 1), at 37 °C, 180 rpm.

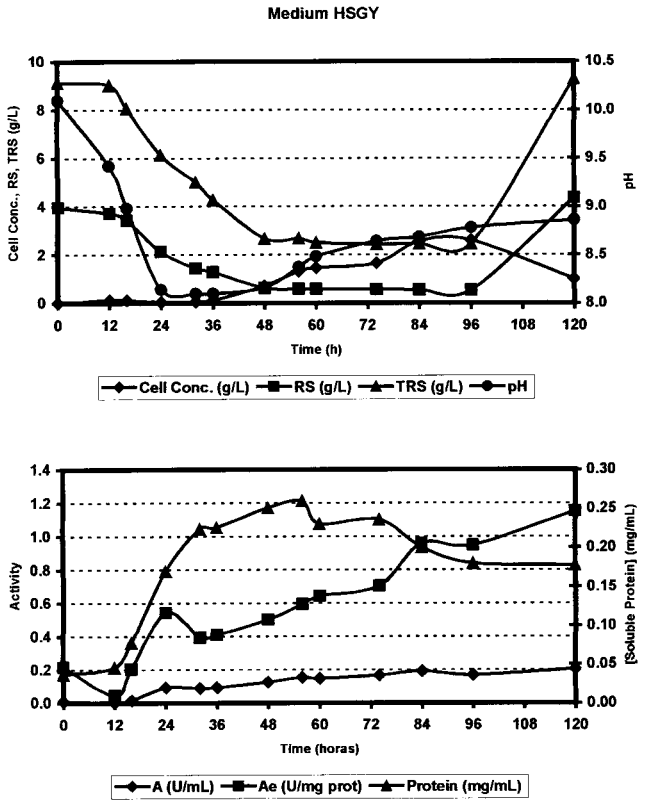

Figure 2. Evolution of growth variables for the cultivation of *Bacillus firmus* (strain #37) grown in the HSGY medium (see Table 1), at 37 °C, 180 rpm.

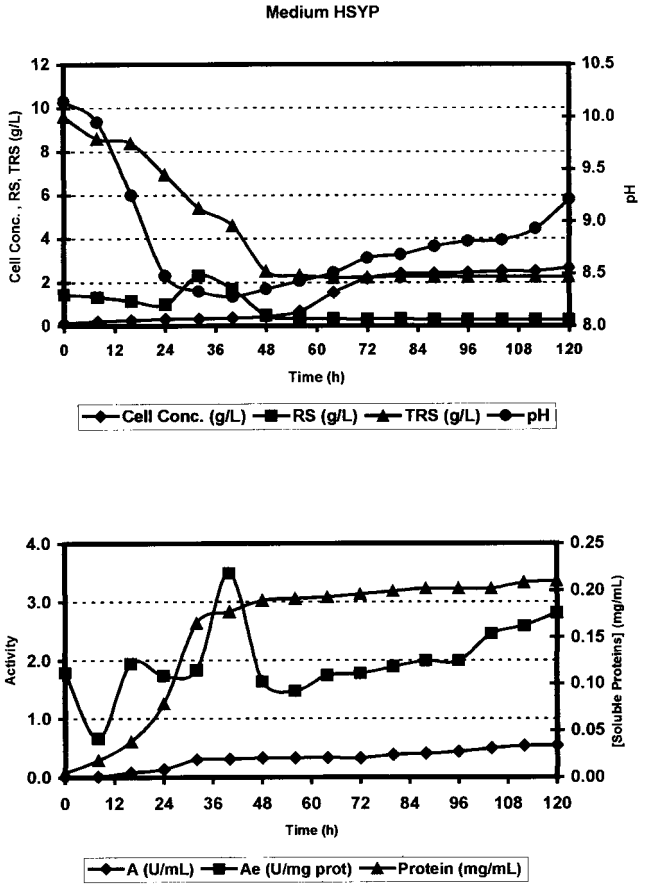

Figure 3. Evolution of growth variables for the cultivation of *Bacillus firmus* (strain #37) grown in the HSYP medium (see Table 1), at 37 °C, 180 rpm.

characterized, maximum CGTase activity obtained was still low, with glucose reducing the positive effect of higher yeast extract concentration that was shown for medium HSY.

For medium HSYP, Figure 3 shows that up to 48h, the first cell growth phase is associated with the consumption of the primary carbon source (hydrolyzed soluble starch), and there is a concomitant production of about 55% of the CGTase activity reaching 0.3 U/mL at 32 h. Thereafter, for the rest of this phase, CGTase production slows down considerably. A second growth phase is observed for the period of 48 to 72 h, where the secondary carbon sources are consumed, the value of the total reducing sugars are nearly constant, but there is rapid cell growth and CGTase activity continues to increase very slowly. A final growth phase occurs from 72 to 120 h, where cell growth becomes small again but CGTase activity augmentation resumes at a slightly increased rate, reaching about 0.55 U/mL within 120 h of cultivation.

Table 1 shows that for 120 h of cultivation, medium HSYP gives 0.55 U/mL of CGTase, and although these results have not yet been optimized in relation to other variables, such as dissolved oxygen, there was a great evolution from the standard medium case, where the CGTase activity obtained was about 0.2 U/mL. With this result there is a gain of about 4.8 times in the activity of the CGTase produced. If we compare the CGTase productivity for a period of 32 h of

cultivation, there is a gain of about 5.5 times in productivity, since with the standard medium it was obtained 0.0017 U/mL.h and the HSYP medium reaches a productivity of 0.0094 U/mL.h.

Conclusion

The different media tested for the cultivation of *Bacillus firmus* (strain #37) have shown that higher concentrations of yeast extract and polypeptone lead to an increase in the synthesis of CGTase, but when starch is substituted by glucose it was shown that glucose leads to a drastic inhibition of CGTase synthesis.

Acknowledgments

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