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Isolation of Alkalophilic CGTase-Producing Bacteria and Characterization of Cyclodextrin-Glycosyltransferase

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ABSTRACT

One hundred and twenty five soil samples were collected from the regions of roots of corn, cassava, potato, bean, sugar cane, soya, and pumpkin. From these, 75 strains were isolated that produced a yellowish halo surrounding the colonies, due to a phenolphtalein-cyclodextrin (CD) complex, and these were selected as alkalophilic CGTase-producing bacteria. All the 75 strains were identified as Bacillus firmus by microscopy and biochemical tests. The activity of the CGTase's varied from 2^2 to 2^{10} dilutions, when assayed by CD-trichloroethylene (TCE)-complex precipitation. Strain 31 that produced the enzyme at the higher level was selected, and its enzyme was partially purified by starch adsorption (x 17) in a yield of 51%. Maximum enzyme activity occurred at pH 5.5 and 8.5. At pH 5.5, the optimum temperature was 60°C. On increased from 30°C to 85°C, the thermodynamic parameter for activation energy was 8.27 kcal.mol^{-1}. The enzyme was inhibited by Ca^{2+}, Mg^{2+}, Fe^{2+}, Cu^{2+}, Mn^{2+}, and Zn^{2+}.

Key words: Cyclodextrin glycosyltransferase, *Bacillus firmus*, characterization

INTRODUCTION

Cyclodextrin glycosyltranferase (CGTase EC 2.4.1.19) is a bacterial enzyme that converts starch and other 1,4-linked α-glucans to cyclodextrins (closed-ring structures composed mainly of 6, 7 and 8 glucosyl units, named α, β and γ-cyclodextrin, respectively) (French, 1957; Thoma and Stewart, 1965). Cyclodextrins have the ability to form inclusion complexes with organic and inorganic compounds, which have numerous applications in the food and pharmaceutical industries (Pszczola, 1988). Various CGTases have been purified by starch adsorption, precipitation with ammonium sulfate, ion exchange chromatography, etc (Ferrarotti et al., 1996; Salva et al., 1997; Matioli, et al., 1998; Akimaru et al., 1991).

We have now isolated new strains of CGTase-producing soil bacteria with high enzyme activity, and studied some of the characteristics of the partially purified enzyme.

MATERIAL AND METHODS

Microorganisms: Samples of soil (125) collected in the vicinity of roots of corn, cassava, potato, bean, sugar cane, soya, and pumpkin. These were analyzed, in order to isolate strains of alkalophilic

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CGTase-producing bacteria. Seventy-five strains of the bacteria were isolated as follows: samples of 1 g of soil were suspended in 10 ml of sterile distilled water. After soil sedimentation, 0.1 ml of the supernatant was spread on the surface of a plate containing soluble starch 2%, peptone 0.5%, yeast extract 0.5%, $MgSO_4.7H_2O$ 0.02%, K_2HPO_4 0.1%, phenolphthalein 0.03%, methyl orange 0.01%, and agar 1.5% at pH 10.5. The plates were incubated at 37 °C (24-72 h) and the colonies that were surrounded by a yellowish halo (Salva et al., 1997), were selected for further examination. CGTase activity was assayed as follows: the bacteria were grown for 6 days at 28 °C and 120 rpm in a New Brunswick rotatory shaker in a sterilized culture medium containing 2% soluble starch, 0.5% peptone, 0.5% yeast extract, 0.02% $MgSO_4.7H_2O$, 0.1% K_2HPO_4, pH 10.5, except for the exclusion of the dyes (Salva et al., 1997). The cells were removed by spinning, and the supernatant that contained the enzyme was assayed by CD-trichloroethylene (TCE) complex precipitation (Nomoto et al., 1984). The enzyme was successively diluted twice in sodium tetraborate buffer 50 mM, pH 8.5 to adjust the enzyme concentration from (1:2) to $(1:2)^n$ dilutions. The enzyme was incubated in the same buffer containing 2% soluble starch at 50 °C for 48 h. The activity of the resulting CGTase varied from 2^2 to 2^{10} dilutions. The culture was selected with a maximum CD-TCE activity of $1:2^{10}$ dilutions.

Enzyme assay: Dextrinizing activity was assayed using soluble corn starch as substrate and by measurement of the decrease in iodine-staining power (Salva et al., 1997). The reaction medium contained 0.1 ml of the enzyme solution, 0.5 ml of 1% starch solution, 0.4 ml of 0.1 M citrate buffer, pH 6.0, and incubated in a water-bath at 50 °C for 10 min. The reaction terminated with 0.5 ml of 1M HCl. 0.1 ml of 4 mM iodine in 30 mM potassium iodide was added and then diluted to 10 ml with water. The starch-iodine complex absorption was read at 620 nm.

Enzyme purification: Enzyme solution (900 ml) was mixed with 3% starch and ammonium sulfate at 20% saturation at 4 °C for 60 min with gentle stirring. The CGTase that was adsorbed on to the starch was collected by centrifugation (4000 x g for 20 min at 4 °C) and washed twice with 10 mM phosphate buffer pH 7.0. The enzyme was extracted with a 1 mM solution of α-cyclodextrin in 10 mM phosphate buffer, pH 7.0, under mechanical stirring at 37 °C for 30 min. This material was centrifuged (27000 x g for 10 min at 4 °C) and the supernatant dialyzed during 18 h against 10 mM phosphate buffer, pH 7.0 at 4°C (Ferrarotti et al., 1996).

Enzyme characterization: The enzyme activity was studied over the pH range of 4.5 – 10.5, using the following buffers: 0.2 M phosphate – 0.1 M citric acid, pH 4.5 - 7.5 and 0.1 M boric acid – 0.1 M potassium chloride, pH 8.0 – 10.5. Temperature values were selected over the range 30 – 85 °C. Analyses for the effect of cation concentrations on the enzyme activity were carried out with Ca^{2+}, Mg^{2+}, Fe^{2+}, Cu^{2+}, Mn^{2+}, and Zn^{2+}. Protein concentrations were determined by the method of Bradford (1976), using bovine serum albumin as standard.

RESULTS AND DISCUSSION

All the CGTase-producing strains (75) were identified as *Bacillus firmus* by microscopy and biochemical tests, according to Sneath et al. (1986). The isolated strains were motile, rod shaped gram positive, and formed spores. They were aerobic, +ve for catalase, -ve for Voges-Proskauer test, +ve for starch, -ve for citrate, +ve for gelatin, gave rise to normal growth in a medium containing 2 –5% NaCl, produce indol, -ve for production of H_2S and urease, and did not ferment L-arabinose and D-xylose. The CGTase activity was assayed by the method of Nomoto et al. (1984) as the relative amount of cyclodextrin-trichloroethylene complex that precipitated. The activity of CGTase varied from 2^2 to 2^{10} dilutions for all strains.

The effect of pH on CGTase activity was analyzed over the pH range of 4.5-10.5, under standard conditions (Fig. 1). Maximum enzyme activity occurred at pH 5.5 and 8.5. At pH 8.5, the enzyme activity was slightly higher than at pH 5.5. This was also shown by Salva et al., (1997) with *Bacillus circulans*.

A relatively single broad peak of optimum pH activity has been reported by other authors (Larsen et al., 1998; Liebl et al., 1992; Yong et al., 1996; Yim et al., 1997) suggested a different degree of ionization of the enzyme catalytic site in order to

produce the different cyclodextrins. The temperature optimum for the CGTase from *Bacillus firmus* was at ~ 60°C. Values have been reported over a broad range of temperature between 45°C and 70°C (Liebl et al., 1992; Sabioni and Park, 1992a,b; Salva et al., 1997; Yim et al., 1997). The thermodynamic parameter for activation energy, as calculated from Arrhenius equation, was 8.27 kcal.mol^{-1} (Fig. 2).

Table 1 - Effect of metal ion on CGTase activity

Metal ions	Enzyme activity (%) 2 mM	10mM
Control	100.00	100.00
$FeSO_4$	100.00	71.33
$CaCl_2$	72.03	58.74
$MgSO_4$	81.03	37.76
$MnSO_4$	90.21	18.88
$CuSO_4$	55.94	7.69
$ZnSO_4$	67.83	2.10

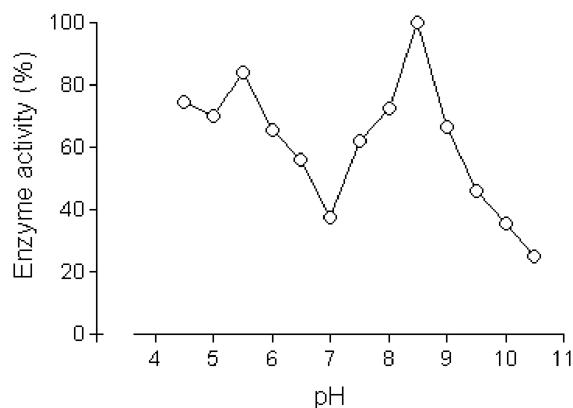

Figure 1 - Effect of pH on CGTase activity

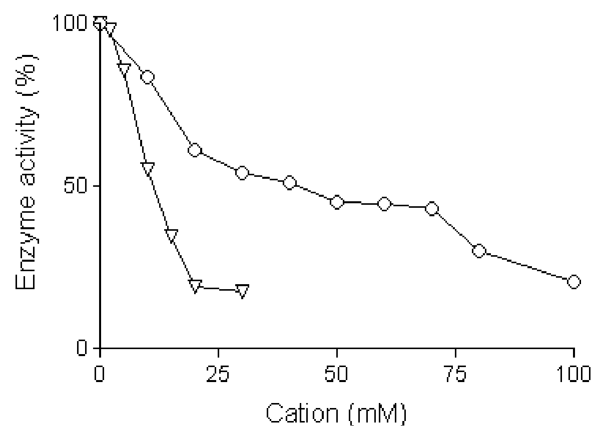

Figure 3 - Effect of calcium and magnesium on CGTase activity. o-o Ca^{2+}; ∇-∇ Mg^{2}

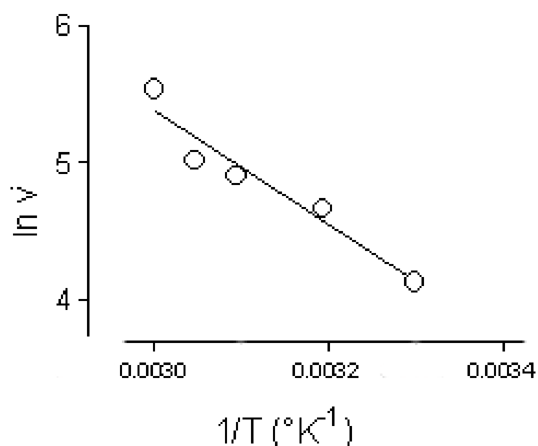

Figure 2 - Effect of temperature on CGTase activity. Arrhenius plot of ln activity (v) versus 1/absolute temperature (° K). The activity (v) was expressed as units.mg^{-1}

This high value reflected a high temperature sensitivity of the CGTase. De Pinto and Campbell (1968) demonstrated a value as high as 27.4 kcal.mol^{-1}. The effects of metal ions are illustrated in Table 1 and Fig. 3. CGTase was inhibited by Ca^{2+}, Mg^{2+}, Fe^{2+}, Cu^{2+}, Mn^{2+}, and Zn^{2+}. Magnesium was a better inhibitor than calcium. The enzyme is strongly inhibited by Cu^{2+} and Zn^{2+}. The effect of metal ions on CGTase activity seems to depend on the enzyme source (Fujita et al., 1990; Yim et al., 1997; Sabioni and Park, 1992b).

Table 2 - Purification of the CGTase from *Bacillus firmus* (strain 31)

Fraction	Total Volume (ml)	Activity (units ml^{-1})	Total units	Specific Activity (units.mg^{-1})	Purification (fold)	Yield (%)
Crude extract	875	0.195	170	1.47	1	100
Starch adsorption	180	0.48	86.4	25.45	17.31	50.8

Attempts to purify the enzyme by chromatography on DEAE-cellulose were unsuccessful. Table 2 showed that there was a loss of 50% of enzyme activity during purification. Larsen et al. (1998) observed a loss of 67% of enzyme activity due to the ammonium sulfate precipitation step. Comparable losses were shown by Ferraroti et al. (1966) (66%) and Salva et al. (1997) (50%). These results suggested that the ammonium sulfate precipitation step should be avoided in the purification scheme.

RESUMO

Foram coletadas 125 amostras de solo de raízes de milho, mandioca, batata, feijão, cana-de-açúcar, soja e abóbora. Destas, 75 cepas foram isoladas por desenvolverem um halo amarelo ao redor das colônias, devido a formação do complexo fenoftaleína-ciclodextrina (CD) sendo selecionadas como bactérias alcalofílicas produtoras de CGTase (EC 2.4.1.19).Todas as 75 cepas foram identificadas como *Bacillus firmus* por microscopia e testes bioquímicos. Quando ensaiada por precipitação com tricloroetileno a atividade da CGTase variou de diluição de 2^2 a 2^{10}. A cepa 31 foi selecionada devido a elevada produção de enzima, sendo esta parcialmente purificada por adsorção em amido (17 vezes) com uma recuperação de 51%. A máxima atividade enzimática ocorreu em pH 5,5 e 8,5. Em pH 5,5, a temperatura ótima foi de 60°C. Sob o aumento de 30°C a 85°C, o parâmetro de energia de ativação foi de 8,27 kcal.mol^{-1}. A enzima foi inibida por Ca^{2+}, Mg^{2+}, Fe^{2+}, Cu^{2+}, Mn^{2+} e Zn^{2+}.

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