CYCLODEXTRIN GLYCOSYLTRANSFERASE PRODUCTION BY NEW *BACILLUS* SP. STRAINS ISOLATED FROM BRAZILIAN SOIL

Vivian Menocci[1]; Antonio José Goulart[1]; Paulo Roberto Adalberto[1]; Olga Luisa Tavano[1]; Daniela Parreira Marques[1]; Jonas Contiero[2]; Rubens Monti[1]*

[1]Departamento de Alimentos e Nutrição, Faculdade de Ciências Farmacêuticas, Universidade Estadual Paulista, Araraquara, SP, Brasil; [2]Departamento de Bioquímica e Microbiologia, Instituto de Biociências, Universidade Estadual Paulista, Rio Claro, SP, Brasil

ABSTRACT

Three strains of *Bacillus sp*. (BACRP, BACNC-1 and BACAR) were isolated from soil adhered to cassava husk. CGTase specific activity for the three isolated strains was higher when cultivated at 40ºC. Potato starch, cassava starch, maltodextrin and glucose were used as carbon source and growth temperatures varied from 25 to 55ºC. The three isolates presented higher CGTase specific activity when cultivated with potato starch at 40ºC. Isolated BACRP and BACAR presented specific activity of 4.0×10^{-3} and 2.2×10^{-3} U/mg prot at pH 7.0, respectively, when cultivated in mediums added with NaCl 2%; at pH 10,0 their activities were of 3.4×10^{-3} and 3.0×10^{-3} U/mg prot, respectively, in the same concentration of NaCl. On the other hand, the isolated BACNC-1 presented activity specific of 2.4×10^{-3} U/mg prot when cultivated at pH 7.0 added of NaCl 1%, and at pH 10.0 the specific activity was of 3.4×10^{-3} U/mg prot without NaCl addition. This work also showed the presence of cyclodextrins formed during fermentation process and that precipitation with acetone or lyophilization followed by dialysis was efficient at removing CDs (cyclodextrins), thus, eliminating interference in the activity assays. The enzyme produced by the BACAR strain was partially purified and β-CD was liberated as a reaction product.

Key words: *Bacillus,* cyclodextrin glycosyltransferase, CGTase production

INTRODUCTION

Cyclodextrin glucanotransferase (1,4-α-D-glucana: 1,4-α-glucanotransferase, EC 2.4.1.19) converts starch into nonreducing cyclic maltooligosaccharides, called cyclodextrins (CDs). The most common are α-CD, β-CD and γ-CD, with 6, 7 and 8 glucose units, respectively. Cyclodextrins are macrocyclic carbohydrates, and all three types are non-hygroscopic crystalline substances. In aqueous solution, their macro-rings are cylindrical, with a hydrophilic external surface and a hydrophobic internal cavity, allowing for the accommodation of organic and inorganic molecules in their interior (4,26). CDs were initially studied by Villiers in 1891 (25) and several published works exist, including reviews regarding elements derived from CDs, complexation, catalytic reactivity and photochemical studies (8,26,29,30). Such molecules have also been used in the food, agriculture, pharmaceutical, cosmetic and other industries (5,22,23,24). The CGTase enzyme is produced by different species of microorganisms and the most commonly cited in the literature is the *Bacillus* genera (1,4,15). According to Van der Veen and Uitdehaag (29), CGTases catalyze the following reactions:

a) hydrolysis reaction of the a link (1-4), described as:

G(n) or cyclic G(x) → maltooligosaccharides
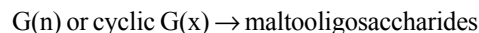
b) cyclization reaction to form CD rings (a CGTase specific reaction), described as:

*Corresponding Author. Mailing address: Deppartamento de Alimentos e Nutrição, Rodovia Araraquara-Jaú, Km 1, CEP 14801-902, Araraquara, SP, Brasil. Tel.: +55 16 3301-6934. E-mail: montiru@fcfar.unesp.br

$$G(n) \leftrightarrow \text{cyclic } G(x) + G(n\text{-}x)$$

c) disproportionation reaction, which is a glucotransferase reaction, where a maltooligosaccharide is hydrolyzed and transferred to another receiving linear oligosaccharide, described as:

$$G(n) + G(m) \rightarrow G(n\text{-}x) + G(m\text{+}x)$$
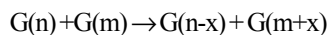
d) coupling reaction, which represents reverse cyclization, in which the CD ring is hydrolyzed and transferred to a receiving linear oligosaccharide, described as:

$$\text{cyclic } G(x) + G(m) \; G(m\text{+}x)$$

Most CGTases, especially the alkalophilic bacteria type, convert starch into β-CD rings as a main product, but in a mixture of different proportions of CDs (7). Moreover, some Brazilian researchers have reported the isolation and production of CGTases by *Bacillus* sp. (2,3,9,15,16,17,19,21). This work reports the isolation of new *Bacillus* sp. strains from the soil of organic cassava culture and the production and partial characterization of the CGTase enzyme released by one of the strains.

MATERIAL AND METHODS

Isolation of CGTase producing soil bacteria

Soil adhered to husks of amylaceous roots (cassava) of the cities of Araraquara, São Carlos and São José do Rio Preto, São Paulo State, Brazil, was used to isolate CGTase producing bacteria. The samples were collected in plastic bags and transported to the laboratory for isolation. For every 10g of cassava husk containing adhered soil, 20mL of sterile saline solution was added (0.85% NaCl). The mixture was maintained at 35ºC for 15 min under agitation. The solutions were then diluted 10, 100, 1,000, and 10,000 times and inoculated on Petri plates containing Nakamura and Horikoshi medium (18), composed of 1% soluble starch, 0.5% peptone, 0.5% yeast extract, 0.1% KH_2PO_4, 0.02% $MgSO_4.7H_2O$; 3.0% agar; 0.03% phenolphthalein, 1% $NaCO_3$, at pH 10.0.

Maintenance of the microorganisms

After isolation, the CGTase producing strains were maintained in Nakamura and Horikoshi liquid medium (18) without starch and phenolphthalein through periodic transplantation, incubated at 35ºC and, after growth, maintained at 4ºC.

Growth in liquid medium for CGTase production

The isolated strains were grown in 2.5 mL of Nakamura and Horikoshi liquid medium (18) at 35ºC, under agitation at 130 rpm for 12h. Then they were transferred to 20 mL of the same medium and incubated for another 20 h. After growth, the culture was centrifuged at $8735g$ for 20min at 4ºC and the supernatant was used to determine the CGTase activity. This procedure was used to evaluate the effect of pH of the medium, carbon source (cassava starch, potato starch, maltodextrin and glucose), temperature (25-55ºC) and NaCl concentration (none, 1% and 2%) on growth and production of the enzyme.

Influence of Sodium ion production of CGTase

Liquid medium described by Nakamura and Horikoshi (18) was added of 1% Na_2CO_3 to raise the pH to 10. In order to verify the effect of sodium ion on the CGTase production strains were grown in the same medium replacing Na_2CO_3 by NaCl, at pH 7.0.

Qualitative analysis of CGTase

Qualitative analysis of the enzyme was carried out applying aliquots of 10 µL of the culture supernatant on Petri plates containing solid medium with 1% potato starch (Sigma) as substrate and 3mM of phenolphthalein. The plates were incubated at 37ºC for 20 h and the CGTase activity was indicated by the presence of colorless halos.

Enzymatic assays

CGTase activity was determined by the Makela's method (14). The supernatant obtained was incubated in 1% potato starch in 2.0 ml of 50 mM Tris buffer pH 8.0, with 1.0 mL of 50 mM $CaCl_2$ and 2.0 mL of milli-Q water. At periods of 0, 10, 20 and 30 min, 0.5 mL aliquots were removed and incubated at 100ºC for 3 min to inactive the enzyme, followed by an ice bath for a further 2 min. After this treatment, 1.2 mL of 3 mM phenolphthalein solution previously prepared in 600 mM Na_2CO_3 was added. CGTase activity was determined at λ 550 nm. One CGTase activity unit was defined as the amount of enzyme capable of producing 1 µmol of β-CD per minute under the assay conditions. Specific activity was expressed in units per milligrams of protein.

Determination of proteins and β-CD

Proteins determination was done according to Hartree (11), using bovine serum albumin as standard, with an extinction coefficient of $2.12mg^{-1}mL^{-1}cm^{-1}$. The molar extinction coefficient for β-CD was of $6.47 \; \mu mol^{-1} \; mL^{-1}cm^{-1}$, as experimentally determined at our laboratory.

Electrophoreses in polyacrylamide gels (PAGE) and enzyme identification

Samples obtained from DEAE-Trisacryl and SP-Trisacryl columns were submitted to 10% nondenaturing PAGE at pH 8.9, according to Davis (6). One gel was dyed for proteins and another was placed on a Petri plate containing agar and phenolphthalein. Through the colorless halos formed on the plate with phenolphthalein, it was possible to determine which

bands corresponded to CGTase, by comparing with the gel dyed for proteins.

Partial purification of CGTase by ion exchange chromatography

Ion exchange chromatography was carried out using DEAE-Trisacryl (1.3 x 13.5 cm) columns, equilibrated and eluted with 5mM sodium phosphate pH 8.9. Columns with SP-Trisacryl (1.3 x 13.5 cm) were equilibrated and eluted with 5 mM Tris-HCl buffer pH 8.2. In both columns, fractions of 2.2 mL were automatically collected and a NaCl gradient (0-0.5M) was applied to release the adhered proteins. Protein concentration of the fractions was determined by a spectrophotometer at λ 280 nm and the enzymatic activities were determined as previously described.

RESULTS AND DISCUSSION

Isolation and identification of the soil bacteria

The methodology described permitted the isolation of three bacterial strains that produced a yellow halo in the plates containing phenolphthalein in the medium, indicating the dye encapsulation and, therefore, the presence of cyclodextrin glycosyltransferase enzyme. The strains were identified as BACRP, BACNC-1, and BACAR. They were Gram-positive, catalase and motility-positive, thus belonging to the *Bacillus* genera. Spores production, verified through growth on minimum medium, confirmed that the strains were *Bacillus* sp. (pictures not presented). Another strain named BACNC-2 was isolated and classified as alkalophilic *Bacillus licheniformis*, and described by Bonilha *et al.* (3).

Determination of the optimum temperature for CGTase enzyme production

The results presented in Fig. 1 show that at 40ºC, higher production of proteins occurred for BACRP, at 55ºC for BACNC-

1 and at 30ºC for BACAR. However, the greatest CGTase specific activity for the three isolated strains was obtained when growth occurred at 40ºC. Bonilha *et al.* (3) reported that the greatest activity of BACNC-2 was obtained when culture occurred at 37ºC. Martins and Hatti-Kaul (21) showed that *B. agaradherens* LS-3C produced CGTase when cultivated at 37ºC and Larsen *et al.* (12) showed that the CGTase production by *Paenibacillus* also occurred at 37ºC.

Optimum pH and NaCl concentration for CGTase production

Results presented in Table 1 indicate that in the amount of protein and CGTase specific activity when the pH differed according to the pH of the medium suggesting that the isolated strains belong to different species. Results at pH 7.0 plus 1.0% NaCl showed that a higher amount of protein was produced by BACRP and BACAR while for the BACNC-1 this occurred at pH 7.0 plus 2.0% NaCl. However, BACRP and BACAR presented higher specific activity when grown in medium at pH 7.0 plus 2.0% NaCl. At pH 10.0, a different behavior was verified BACRP produced a higher amount of proteins at pH 10.0 plus 2.0% NaCl; the same occurred with BACNC-1 at pH 10.0 plus 1.0% NaCl; and BACAR at pH 10.0 without NaCl. Regarding the specific activities at pH 10.0, the highest activity for BACRP and BACAR occurred at pH 10.0 plus 2.0% NaCl and at pH 10.0 without the addition of NaCl for BACNC-1. These results suggest that the work involved different microorganisms and that the pH and the addition of NaCl interfered in CGTase enzyme production. These results are similar to those reported by Bonilha *et al.* (3), where *B. licheniformis* grew and produced CGTase in a culture medium at pH adjusted to 7.0, when 0.6% NaCl was added, and also similar to the results presented by Tsai *et al.* (28), where *Bacillus* Ya-B presented growth at pH 7.0 in culture mediums supplemented with 1% NaCl. Tachibana *et al.* (27) reported that a *Thermococcus* sp. isolate produced
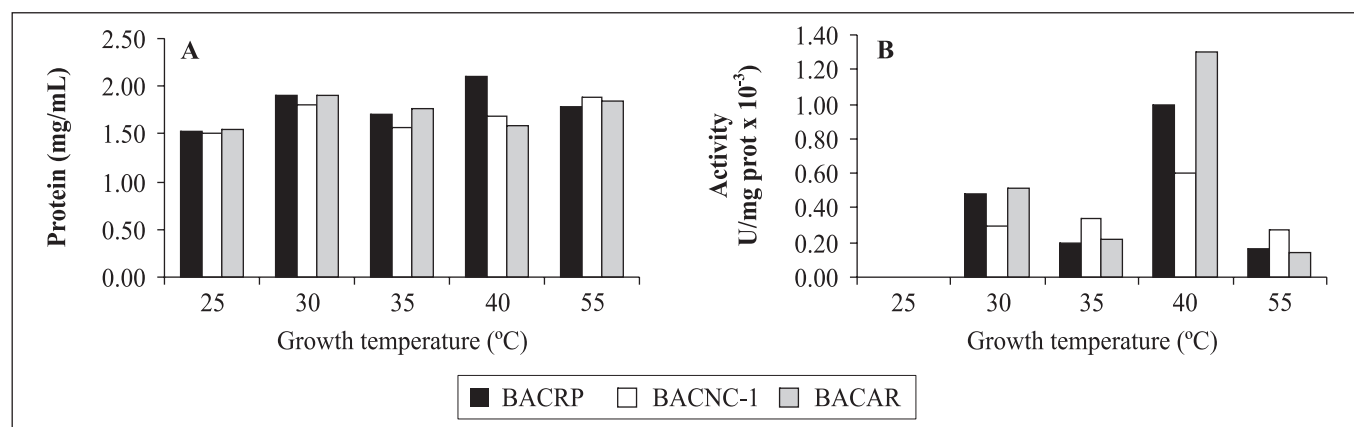

Figure 1. Protein concentration and CGTase specific activity of three strains of *Bacillus* sp. grown at different temperatures. Results are expressed as an average of three determinations.

CGTase when cultivated at pH 7.0 with the addition of 2% NaCl to the culture medium.

Carbon source and best starch concentration

Fig. 2 shows the results of the effect of different carbon sources on the protein production and CGTase specific activity of the three isolated *Bacillus* sp. strains. The carbon source that provided the greatest specific activity for BACRP and BACAR was potato starch; for BACNC-1, potato and cassava starch provided similar activities, while cassava starch was the second best for BACRP and slightly inferior to maltodextrin for BACAR, while maltodextrin was the third best carbon source for BACRP and BACN-1. These data are coincident with that obtained by Bonilha *et al.* (3) who verified that potato starch provided the greatest specific activity. Researches using different carbon sources in bacterial culture mediums for CGTase production have shown that each microorganism behaves in a different way. According to Gawande *et al.* (10) maximum CGTase production was obtained when corn starch was added to the culture medium for *Bacillus firmus*. Rosso *et al.* (20) reported that *Bacillus circulans* DF9R produced a higher amount of CGTase when the culture medium used cassava starch and rice starch as the main carbon sources. Fig. 3 shows the effect of the potato starch concentration on CGTase production and specific activity, and indicates that a 4% concentration of this carbon source provided better specific activity for BACRP and BACNC-1, while for BACAR, a 2% concentration was better. Therefore, differences in carbon source used for CGTase production can be verified for different strains of *Bacillus* sp.

Cyclodextrin detection in the culture filtrates

The formation of transparent halos was verified after the addition of 10 mL of each isolated culture on Petri plates containing Nakamura and Horikoshi medium (18) plus 1% starch,

Table 1. Protein concentration and CGTase specific activity in culture filtrates of three strain of *Bacillus* sp. at pH 7.0 and 10.0 with or without NaCl.

pH	[NaCl]	Protein (mg/mL)			Specific activity (U/mg prot)		
		BACRP	BACNC-1	BACAR	BACRP	BACNC-1	BACAR
7.0	-	1.73	2.36	2.21	4.1×10^{-4}	ND*	7.0×10^{-4}
7.0	1%	**2.44**	2.26	**2.36**	2.3×10^{-3}	**2.4x10^{-3}**	1.9×10^{-4}
7.0	2%	1.77	**2.48**	2.15	**4.0x10^{-3}**	1.4×10^{-3}	**2.2x10^{-3}**
10.0	-	1.55	1.95	**2.32**	2.8×10^{-3}	**3.4x10^{-3}**	2.1×10^{-3}
10.0	1%	1.62	**2.02**	1.83	1.8×10^{-3}	2.7×10^{-3}	2.7×10^{-3}
10.0	2%	**1.68**	1.75	1.93	**3.4x10^{-3}**	1.9×10^{-3}	**3.0x10^{-3}**

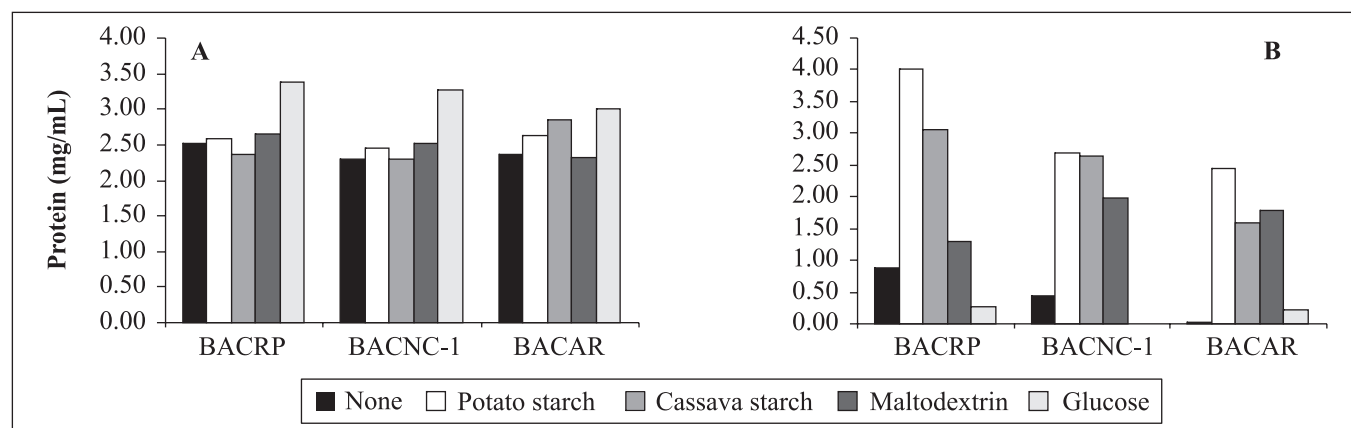Note: *Non-detectable. Results are expressed as an average of three determinations.

Figure 2. Effect of carbon sources on CGTase specific activity on three strains of *Bacillus* sp. grown at 40º C; BACRP at pH 7.0 plus 2% NaCl; BACNC-1 at pH 10.0 without NaCl; BACAR at pH 10.0 plus 2% NaCl. Results are expressed as an average of three determinations.
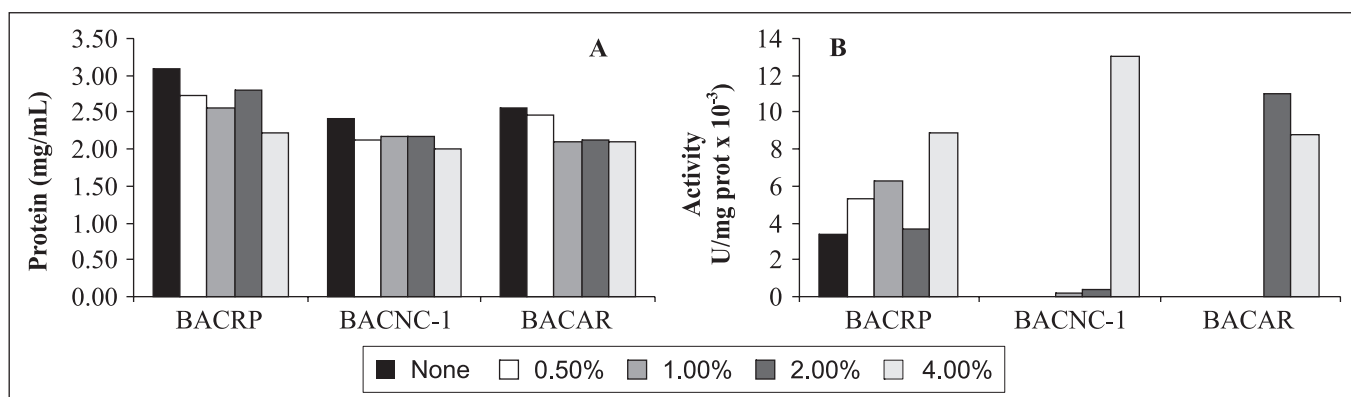
Figure 3. Effect of potato starch concentration on CGTase production and specific activity of three strains of *Bacillus* sp. grown at 40ºC. Results are expressed as an average of three determinations.

phenolphthalein and agar. When the cultures were centrifuged at 8735*g* to remove the microorganisms and the supernatants were applied on plates containing the same medium without starch, transparent halos were also observed. These results clearly show that during microorganism growth, CGTase enzyme production and cyclodextrins formation occurred simultaneously. These cyclodextrins interfere in the activity values, thus overestimating the results. In this work, the culture filtrates were concentrated with acetone or lyophilized and dialyzed to eliminate the cyclodextrins, eliminating interferences in the activity determinations. Table 2 shows the results of protein determination and CGTase specific activity in samples concentrated by these two methods. The data were compared to the dosages found in culture filtrates not submitted to the concentration treatment. The results indicate that concentration with three volumes of acetone at 4ºC was better for BACRP and

BACNC-1, while lyophilization was better for BACAR. However, lyophilization can also be used for concentration of proteins of the culture filtrates of BACRP and BACNC-1.

Partial purification of CGTase

CGTase was partially purified using the lyophilized and dialyzed BACAR culture filtrate. A 5.0 mL aliquot was applied to DEAE-Trisacryl columns. The results shown in Fig. 4 were reproducible in three repetitions. In sample 1, corresponding to fractions 4 to 11 of the eluate, high CGTase activity was verified, while the other two samples (sample 2, corresponding to fractions 12 to 21 and sample 3, corresponding to fractions 22 to 50) presented no catalytic activity. Next, a NaCl gradient was applied and another sample of proteins was eluted (sample 4, corresponding to fractions 104 to 116), which also showed no enzymatic activity. These results show that the CGTase enzyme

Table 2. Effect of treatment on protein concentration and CGTase specific activity in cultures of three strains of *Bacillus* sp.

Strains	Treatment	Protein (mg/mL)	Activity (U/mg prot.)
BACRP	None	2.32	2.0×10^{-3}
	Acetone	11.52	**4.6×10^{-3}**
	Lyophilization	21.60	3.5×10^{-3}
BACNC-1	None	2.29	9.0×10^{-4}
	Acetone	12.09	**2.6×10^{-3}**
	Lyophilization	15.76	1.7×10^{-3}
BACAR	None	2.06	1.4×10^{-3}
	Acetone	10.13	3.4×10^{-3}
	Lyophilization	14.21	**6.0×10^{-3}**

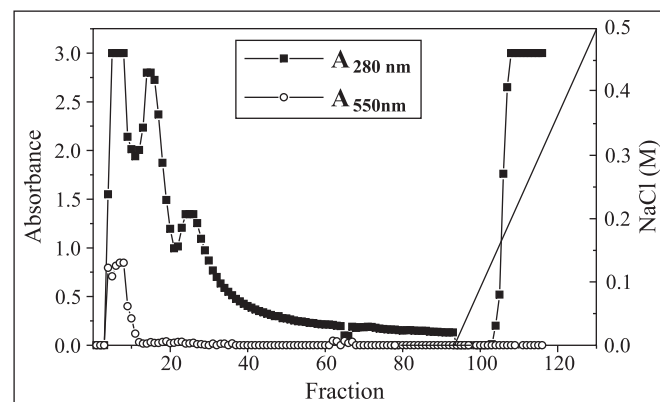Note: Results are expressed as an average of three determinations.

Figure 4. Chromatogram of BACAR culture filtrates in DEAE-Trisacryl (1.3 x 13.5 cm), equilibrated and eluted with 5mM sodium phosphate buffer pH 8.9. A_{280nm}: Absorbance of proteins. A_{550nm}: Absorbance of enzymatic activity.

of this microorganism did not interact with the DEAE-Trisacryl resin, but was eluted at the onset of chromatography. Identical aliquots were submitted to SP-Trisacryl chromatography. The results shown in Fig. 5 indicate that in sample 5 (fractions 2 to 11) a large amount of protein presenting activity was eluted, and that in sample 6 (fractions 107 to 111), eluted with saline gradient, a small amount of protein with enzymatic activity was also liberated when the samples were submitted to PAGE, it was possible to identify the protein bands corresponding to CGTase, as shown in Fig. 6. A colorless halo was observed in samples 1 (DEAE-Trisacryl), 5 and 6 (SP-Trisacryl), while sample 6 did not produce a well defined halo, probably due to a small quantity of enzyme. These electrophoresis results confirm the chromatographic results. The results also show that the chromatography in DEAE-Trisacryl did not favor the complete purification of CGTase, but permitted the separation of several protein contaminants that presented higher migration than CGTase in the polyacrylamide gel. An identical situation occurred with chromatography in SP-Trisacryl. With these results it was possible to show that the enzyme produced by this microorganism, was not adsorbed by the positively charged resin, whereas in negatively charged resin, part of the enzyme is eluted and another part is adsorbed. The results presented in Fig. 6B reveal that sample 1, corresponding to peak 1 of the DEAE-Trisacryl, and samples 5 and 6 corresponding to peaks 1 and 2 of the SP-Trisacryl, presented CGTase activity, indicating partial purification of cyclodextrin glycosyltransferase enzyme, and permitted the understanding of its migration behavior in nondenaturing gel. Studies regarding CGTases are very important due to the possibilities of immobilizing these enzymes and their use in several industrial sectors, such as the production of foods with higher aggregated value, pharmaceutical products and in agriculture (23,24).
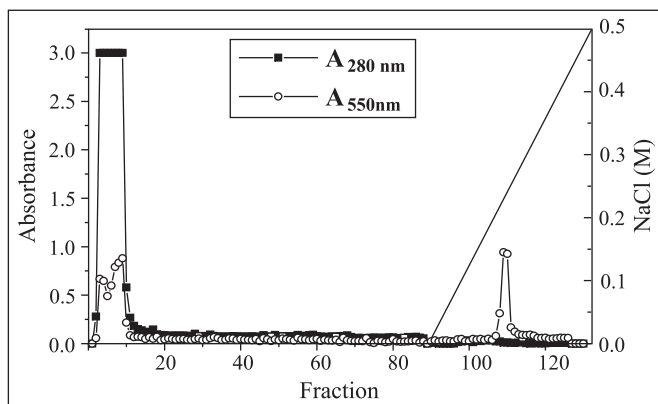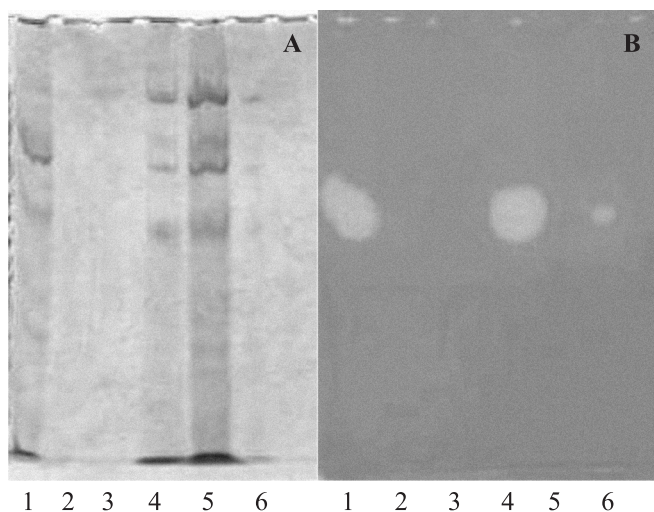

Figure 6. A) 10% PAGE of the samples collected from the DEAE-Trisacryl (samples 1 to 4) and SP-Trisacryl (samples 5 and 6) columns. B) The halos present on plate with agar indicates the CD formation produced by BACAR enzyme and encapsulation of phenolphthalein.

Determination of the type of CD produced by CGTase

In the 1980s, several works were published on the development of colorimetric tests for measuring CGTase activity (13). In this work, the type of CD produced with the partially purified enzyme was determined using Petri plates containing 1% starch plus 4% agar and the dyes 3 mM phenolphthalein, 0.035 mM methyl orange, 5 mM bromocresol green, and 0.035 mM Congo red. When aliquots of the eluate from the SP-Trisacryl column (sample 5) were applied to these plates, the presence of halos in the plates with phenolphthalein and Congo red suggests that the partially purified enzyme in this work is a β-CGTase, due to the liberation of a higher amount of β-CD. Absence of halos in the plates containing methyl orange and bromocresol green dyes indicates that sample 5 does not contain α or γ-CGTase. However, the presence of these enzymes in the culture filtrate cannot be rulled out because Makela's method detects α and γ-CD, besides β-CD.

Figure 5. Chromatogram of BACAR culture filtrates in SP-Trisacryl (1.3 x 13.5 cm), equilibrated and eluted with 5mM Tris-HCl buffer pH 8.2. A_{280nm}: Absorbance of proteins. A_{550nm}: Absorbance of enzymatic activity.

RESUMO

Produção de ciclodextrina glicosiltransferase por novas cepas de *Bacillus* sp. isoladas de solo brasileiro

Três linhagens de *Bacillus sp* (BACRP, BACNC-1 e BACAR) foram isoladas a partir de solo aderido em casca de mandioca. Foram utilizados amido de batata, amido de mandioca, maltodextrina e glicose como fonte de carbono, e temperaturas de crescimento de 25-55°C, sendo que os três isolados apresentaram maior atividade específica de CGTase quando cultivados com amido de batata a 40°C. Em pH 7,0 os isolados BACRP e BACAR apresentaram atividade específica de 4,0x 10^{-3} e 2,2x10^{-3} U/mg prot, respectivamente, quando cultivados em meios acrescidos de 2% de NaCl; em pH 10,0 suas atividades foram de 3,4x10^{-3} e 3,0x10^{-3} U/mg prot na mesma concentração de NaCl. Por outro lado, o isolado de BACNC-1 apresentou atividade específica 2,4x10^{-3} U/mg prot quando cultivado em pH 7,0 acrescido de 1% de NaCl, e em pH 10,0 sua atividade específica foi de 3,4x10^{-3} U/mg prot sem adição de NaCl. Também foi demonstrada neste trabalho que ciclodextrinas são formadas durante o processo fermentativo, e que a precipitação com acetona ou liofilização seguida de diálise foram eficientes na remoção destas CDs, eliminando sua interferência nos ensaios enzimáticos. A enzima produzida pela cepa BACAR foi purificada parcialmente liberando β-CD como produto da reação.

Palavras-chave: *Bacillus*, ciclodextrina-glicosiltransferase, produção de CGTase.

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