0273-2289/98/70–72—0267$10.25

Production and Purification of CGTase of Alkalophylic *Bacillus* Isolated from Brazilian Soil

GRACIETTE MATIOLI,[1] GISELLA M. ZANIN,[1] MANOEL F. GUIMARÃES,[2] AND FLÁVIO F. DE MORAES*,[1]

[1]*State University of Maringá, Chemical Engineering Department, Av. Colombo, 5790 - BL. E46 - 09 87020-900, Maringá, PR, Brazil;*
[2]*Federal University of Paraná, P. O. Box 19046, Biochemistry Department, 81531-990-Curitiba - PR, Brazil*

ABSTRACT

Alkalophylic bacilli that produce cyclodextringlycosyltransferase (CGTase) were isolated from Brazilian soil, with a scheme of two plating steps. In the first step, the bacterial isolate forms a halo in the cultivation medium that contains γ-cyclodextrin (CD) complexing dyes. The CGTase of an isolate was purified 157-fold by biospecific affinity chromatography, with β-CD showing a mol wt of 77,580 Daltons. It produces a γ- to β-CD ratio of 0.156 and a small amount of α-CD, using maltodextrin 10% as substrate, at 50°C, pH 8.0 and 22 h reaction time, reaching 21.4% conversion of the substrate to cyclodextrins. In the second screening step, the isolates chosen give larger halos with β-CD complexing dyes, and smaller halos with β-CD complexing dyes, leading to a 30% improvement in γ-CD selectivity, although at lower total yield for cyclodextrins (11.5%).

Index Entries: Cyclodextringlycosyltransferase; CGTase; cyclodextrin; alkalophylic bacillus, screening.

INTRODUCTION

Cyclodextrins (CDs) are cyclic oligosaccharides formed by D-glucosyl residues linked by α,1–4 bonds. The most common are the α-, β-, and γ-CD, containing 6, 7, and 8 glucosyl residues, respectively. They are produced by reacting liquefied starch with the enzyme cyclodextringlycosyltransferase

* Author to whom all correspondence and reprint requests should be addressed.
e-mail: flavio@cybertelecom.com.br

(CGTase). Normally, a mixture of CDs is formed and the concentration ratio depends on the enzyme source. Frequently, β-CD is produced in a greater amount, in some cases α-CD, but γ-CD is very seldom produced in high yields. Depending on which cyclodextrin, α-, β-, or γ-CD, is the main product, the enzyme is called an α-, β-, or γ-CGTase, respectively. Only two nonrecombinant strains are known to produce relatively high yields of γ-CD, those of Englbrecht et al. *(1)*, and Mori et al. *(2)*. However, these research groups have put an extreme effort to select those strains that were isolated from so many CGTase-positive microorganisms.

The CD rings are highly hydrophilic externally, and relatively hydrophobic internally *(3)*. In aqueous solution, this structure thermodynamically favors the inclusion of nonpolar molecules that can be fitted entirely or partially inside the ring *(4)*. This encapsulation at the molecular level has been amply used for protecting labile molecules against chemical or physical damage in diverse industrial products, such as food, drugs, agrochemicals, cosmetics, and perfumes *(5)*.

More recently, the pharmaceutical industry has shown renewed interest in encapsulation with γ-CD, because this CD can accommodate large drug molecules. This has stimulated research to aim at better ways of producing larger quantities of γ-CD. Three different routes have been taken, as follows:

1. The development of superior processes for separating γ-CD from other CDs in the reaction mixture *(6)*.
2. Prevention of γ-CD destruction by reversible reactions. This is achieved through the formation of a stable γ-CD complex with an appropriate substance *(7)*.
3. Alternatively, the search for new strains whose CGTase enzyme kinetically favors the production of γ-CD at the beginning of the reaction *(1,2)*. However, it is worthy of note that, at equilibrium, reached after a long time, β-CD will always be in a greater concentration *(8)*.

This article presents a scheme of plate screening, with two steps that help in selecting new bacterial isolates that produce CGTase with higher γ-CD selectivity. Results of the application of this scheme to regional soil samples, as well as data on production of cyclodextrins by the CGTase-producing isolates, are shown.

MATERIALS AND METHODS

First Screening Step

The first screening technique to isolate microorganisms that produce CGTase was based on the Nakamura and Horikoshi screening medium II *(9)*, for alkalophylic microorganisms. The medium was supplemented with dyes to detect CGTase-positive isolates. The isolates produce a clear halo

around the colonies, since the complexation of these dyes with CDs changes their light absorption characteristics. Dyes that were appropriate for complexing with γ-CD were Congo red and xylene cyanole FF. These dyes have been used by Hamaker and Tao with Luria-Bertoni neutral media (pH 7.0) containing 1% starch to detect γ-CD production by recombinant *Escherichia coli* cells *(10)*. The final composition of the modified plate medium was (w/v): soluble starch (1%), peptone (0.5%), yeast extract (0.5%), K_2HPO_4 (0.1%), $MgSO_4 \cdot 7H_2O$ (0.02%), Na_2CO_3 (1%), Congo red (0.01%), xylene cyanole FF (0.001%), and agar (1.5%). The pH of this medium was 10.3. A drop of solution containing either α-, β-, or γ-CD, deposited over a plate containing this medium, produced a clear halo only with γ-CD.

Microorganisms were isolated from soil samples collected from farm fields used for cultivation of wheat, corn, potato, and cassava. Each sample was suspended in dH_2O and screening plates containing the above modified medium were inoculated with appropriate volumes of these suspensions. Plates were incubated at 37°C for 24 h, after which high-enzyme-producing isolates, associated with larger halos, were chosen.

Second Screening Step

In this case, a second plating medium was used with the isolates of the first screening step. For the second plate medium, the dyes were changed, to be specific for detecting the presence of β-CD. These dyes are phenolphthalein mixed with methyl orange, and have been used by Park et al. *(11)*.

CGTase Activity

The enzymatic activity of the CGTase produced by the bacterial isolates was determined through the cultivation of these microorganisms in liquid cultures containing a medium with the same composition as the plate medium, except the dyes, and agar. Cultures were grown at 37°C up to 5 d, after which cell-free supernatant was used to assay for CGTase activity. Assay tubes with 0.5 mL of the CGTase-containing sample were mixed with 0.5 mL of substrate solution: soluble starch 1% (w/v), Tris-HCl buffer, pH 8.0 (0.01 *M*), and $CaCl_2$ (5 m*M*). Enzyme samples and substrate solution were warmed to 50°C before mixing, and left to react at this temperature for 20 min. Reactions were stopped by boiling the tubes for 5 min, and the CDs produced were measured by colorimetric assays as described below. One unit of CGTase is the amount of enzyme that produces one μmol of β-CD/min/mL of enzyme in these conditions. CGTase enzyme activity was also assayed by the method of successive dilutions of the supernatant. The amount of CD produced in this case was assayed through the precipitation with (trichloroethylene) (TCE) *(12)*.

β-CD concentration was measured, based on the discoloration of phenolphthalein solutions at 550 nm, which occurs after complexation with β-CD, following the method of Vikmon *(13)* as modified by Hamon and

Moraes *(14)*. γ-CD concentration was determined based on increased absorption at 620 nm of bromocresol green solutions upon complexation with γ-CD, according to the method of Kato and Horikoshi *(15)*, as modified by Hamon and Moraes *(14)*. Soluble proteins were determined by the method of Bradford *(16)*, using BSA as standard protein.

Enzyme Purification

Selected microorganisms were cultivated in 5-L cultures (medium described in CGTase Activity) for 5 d at 37°C, with agitation. Each culture was centrifuged at 8800/*g* for 15 min to pellet cells. The cell-free supernatant was mixed with ammonium sulfate (80% saturation) and left in a refrigerator overnight. A second centrifugation at 8800/*g* for 30 min was conducted, and the precipitate was dissolved in Tris-HCl buffer, pH 8.0 (0.01 *M*). The enzyme was further purified by biospecific affinity chromatography column using Sepharose 6B gel, and both β-CD *(17)* and γ-CD *(1)* were independently tried as affinants. Purified CGTase was concentrated by ultrafiltration, dialyzed 5 × with Tris-HCl buffer, pH 8.0 (0.01 *M*), to remove molecules smaller than 30 kD, and stored in a refrigerator for later use.

Mol Wt Determination

The mol wt of the CGTase was determined according to Weber and Osborn *(18)*, by SDS-PAGE, using a mol wt reference kit (Pharmacia, Uppsala, Sweden) of six standard proteins with mol wt in the range of 14,400 to 94,000 Daltons. The relation between log mol wt and relative mobility was established, and the mol wt of the CGTase was determined through this relation.

Cyclodextrin Production Tests

A batch reactor test was used for the production of cyclodextrins with the purified CGTase. Conditions were: 50°C, about 1 mg/L of pure enzyme (80.1 U), and the substrate solution contained maltodextrin 10% (w/v) (Dextrin 10, Fluka [Buchs, Switzerland] article 31412), Tris-HCl buffer, pH 8.0 (0.01 *M*), and 5 m*M* $CaCl_2$. The test was run for a period of 24 h, and samples were taken at regular intervals and boiled for 5 min, after which they were assayed for β- and γ-CD with the colorimetric methods above described. α-CD was measured by HPLC, using C-18 hydrophobic interaction column and water–methanol (92–8%) eluent *(19)*.

RESULTS AND DISCUSSION

First Screening Step

Application of the first screening step has screened for 57 strains that produce CGTase. The cell-free supernatant of isolate number 37, up to a dilution of 2^8, was able to produce CDs in sufficient amount to be precipi-

tated by TCE. This isolate has shown the highest enzyme activity in this group. Media presented in Table 1 were tested with isolate 37. The highest activity observed in the cell-free supernatant occurred with medium A, which contained peptone and yeast extract as source of nitrogen, reaching 0.1155 μmol of β-CD/(min·mL). Therefore, isolate 37 has shown a divergent behavior in comparison with the alkalophylic bacillus isolated by Nakamura and Horikoshi *(9)* which produces much higher CGTase activity in medium B, which contains corn-steep liquor as source of nitrogen.

Enzyme Purification

CGTase enzyme from isolate 37 was purified as described in Materials and Methods, giving a electrophoretically homogeneous enzyme solution with 0.171 mg of protein/mL, activity of 13.7 μmol of β-CD/(min·mL) and 157-fold purification. The specific activity of this enzyme was 80.1 μmol of β-CD/(min·mg of enzyme). This value is very close to the specific activity determined by Hamon and Moraes *(14)* for the CGTase enzyme from alkalophylic bacillus 1-1 described by Schmid et al. *(23)*. The purification obtained in this work is superior to that obtained by László et al. *(17)*, who obtained 120-fold, and also, greater than that obtained by Saha et al. *(24)*, who purified the enzyme by agarose and Sephacryl 200 column, and obtained 129-fold purification.

Enzyme Mol Wt

The mol wt of the CGTase enzyme of isolate 37 was determined by SDS-PAGE, and found to be 77,580 Daltons. This value is within the usual range of mol wt obtained for CGTases from different microorganisms (66,000 to 80,000 Daltons).

Production of Cyclodextrins

The production of CDs from maltodextrins upon using purified CGTase of isolate 37, in the 24 h batch test, is shown in Fig. 1. As can be seen, β-CD concentration, at any time, is higher than γ-CD concentration, indicating that this enzyme is a β-CGTase enzyme. β-CD concentration reached about 16 m*M*, and the ratio of γ- to β-CD concentration was 0.156; that is, a maximum of 2.5 m*M* of γ-CD was obtained. Production of α-CD was very low, reaching only 0.085 m*M*. This lower value would reduce separation requirements in an industrial production process of CDs. Moreover, total conversion of the substrate, maltodextrin at 10% (w/v), reached 21.4% after a period of 22 h, with 1 mg/mL of CGTase enzyme of isolate 37, at 50°C and pH 8.0.

Second Screening Step

The second screening step was applied to the isolates already screened through the first step. The idea of this new screening step is based on the fact that, although the first screening technique spotted the colonies that

Table 1
Production of CGTase by Isolate 37 with Alternative Liquid Cultivation Media

Medium component composition % w/v	Ref.	*9*	*9*	*20*	*21*	*22*	*2*
	Medium	A	B	C	D	E	F
Soluble starch		2	1	2	2	6	2[a]
Peptone		0.5	–	–	–	0.5	–
Yeast extract		0.5	–	–	0.5	0.15	1.0
Corn-steep liquor		–	5[b]	2	–	–	–
$MgSO{\cdot}7H_2O$		0.02	0.02	0.02	0.02	0.02	0.02
K_2HPO_4		0.1	0.1	0.1	0.1	0.6	0.1[a]
Na_2CO_3		1	1	1	1	1	1
Total protein in the cell-free supernatant (μg/mL)		1488	1616	1343	1372	1442	1376
CGTase protein in the cell-free supernatant (μg/mL)		1.44	0.42	0.58	1.06	0.44	1.16
CGTase activity (μmol β-CD/min mL)		0.1155	0.0333	0.0463	0.0849	0.0355	0.0929

[a] Mori et al. *(2)* used half these values.
[b] In v/v.

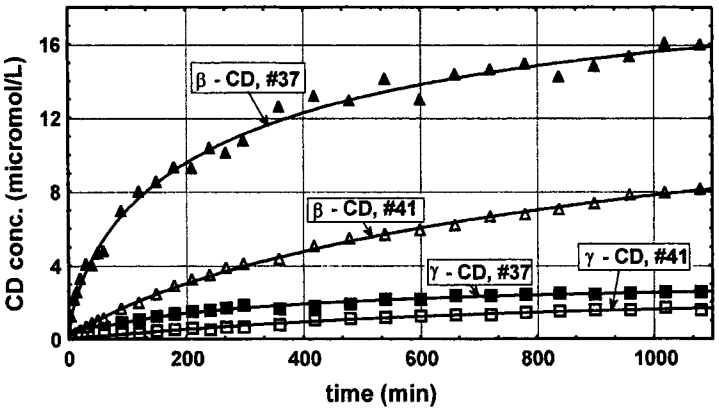

Fig. 1. Production of cyclodextrins by the CGTase from isolates 37 and 41, using maltodextrins 10% (w/v) as substrate, pH 8.0, at 50°C, and about 1 mg/mL of pure enzyme.

produce γ-CD, they could still be large producers of β-CD. Through the detection of small halos in the second plate medium, this technique aims at selecting isolates that would produce γ-CD at higher selectivity, that is, at higher ratios of γ- to β-CD.

Two new isolates, 41 and 47, were chosen to work with. Production level of CDs by the purified enzyme of isolate 41 is also shown in Fig. 1, for comparison with the results of isolate 37. Both β-CD and γ-CD production by the CGTase of isolate 41 are proportionally smaller than that of isolate 37, but the ratio of γ- to β-CD concentration produced by this isolate is higher, and reached 0.204. Therefore, isolate 41 produces γ-CD at about 30% higher selectively than isolate 37, although with lower total CD yield (11.5%). The γ-CD selectivity improvements, so far, are modest. The production of CDs by the purified enzyme of isolate 47 was very similar to that of isolate 41, and the ratio of γ- to β-CD concentration was 0.187.

Although the purification by affinity chromatography of the CGTase enzyme of isolates 37, 41, and 47 was attempted in two different columns, one containing β-CD as affinant and the other γ-CD, purification was achieved only with the β-CD column, showing that these CGTase are in fact β-CGTase enzymes.

Sabioni and Park *(21,25)* have exclusively used the phenolphthalein screening technique of Park et al. *(11)*, with Brazilian soil from Campinas-SP, and isolated an alkalophylic β-CGTase strain of *Bacillus lentus*, which formed α-, β-, and γ-CD in the proportion of 1:67:1.6, respectively. The ratio of γ- to β-CD in this case is much lower: 0.015. This confirms that the application of the screening scheme with two steps proposed in this work helps to isolate CGTase with higher selectivities for γ-CD. Since the γ-CGTase isolated by Mori et al. *(2)* can reach a γ- to β-CD ratio of 1.4 with 10% starch *(26)*, there is still much scope for further improvements

of the screening scheme tested in this work. The development of such techniques would facilitate the search for γ-CGTase enzyme producers with superior selectivity, and this will be of great interest to the pharmaceutical industry.

CONCLUSIONS

The isolation of alkalophylic microorganisms that secrete CGTase enzyme has become relatively easy with the application of the screening scheme presented in this paper.

The second screening step introduced was able to screen for isolates whose CGTase leads to slightly better selectively for γ-CD. A 30% higher selectivity over step one was achieved, although at lower overall yield for cyclodextrins.

New, improved schemes should still be developed for facilitating the isolation of strains that are good producers of γ-CGTase, since only two are known presently, and these were obtained by highly laborious and time-consuming procedures.

The interest of the pharmaceutical industry in using γ-CD for complexing large drugs warrants further research in this area.

ACKNOWLEDGMENTS

The authors thank Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação Coordenacão de Aperfeiçoamento de Pessóal de Nível Superior (CAPES), Programa de Apoio ao Desenvolvimento Científico e Tecnológico (PADCT), and the State University of Maringá for financial support.

REFERENCES

1. Englbrecht, A., Harrer, G., Lebert, M., and Schmid, G. (1990), in *Minutes of the 5th International Symposium on Cyclodextrins*, Duchêne, D. ed., Editions. de Santé, Paris, pp. 25–31.
2. Mori, S., Hirose, S., Oya, T., and Kitahata, S. (1994), *Biosci. Biotechnol. Biochem.* **58**, 1968–1972.
3. Duchêne, D., Debruères, B., and Brétillon, A. (1984), *Labo-Pharma-Probl. Tech.* **32**, 843–850.
4. Szejtli, J. (1988), in *Cyclodextrin Technology*, Szejtli, J. ed., Kluwer, Dordrecht, The Netherlands, pp. 79–185.
5. Lee, J. H., Choi, K. H., Choi, J. Y., Lee, Y. S., Kwon, I. B., and Yu, J. H. (1992), *Enzyme Microb. Technol.* **14**, 1017–1020.
6. Mattsson, P., Mäkelä, M., and Korpela, T. (1988), in *Proceedings of the Fourth International Symposium on Cyclodextrin*, Huber, O. and Szejtli, J., eds., Kluwer, Dordrecht, The Netherlands, pp. 65–70.
7. Schmid, G., Huber, O. S., and Eberle, H. J. (1988). in *Proceedings of the Fourth International Symposium on Cyclodextrin*, Huber, O. and Szejtli, J, eds., Kluwer, Dordrecht, The Netherlands pp. 87–92.

8. French, D. (1957), *Adv. Carbohydr. Chem.* **12,** 189–260.
9. Nakamura, N. and Horikoshi, K. (1976), *Agric. Biol. Chem.* **40,** 753–757.
10. Hamaker, K. and Tao, B. Y. (1993), *Starch/Stärke* **45,** 181, 182.
11. Park, C. S., Park, K. H., and Kim, S. H. (1989), *Agric. Biol. Chem.* **53,** 1167–1169.
12. Nomoto, M., Shew, D. C., Chen, S. J., Yen, C. W. L., and Yang, C. P. (1984), *Agric. Biol. Chem.* **48,** 1337, 1338.
13. Vikmon, M. (1981), in *The First International Symposium on Cyclodextrin.* Szejtli, J. ed., D. Riedel, Dordrecht, The Netherlands, pp. 69–74.
14. Hamon, V. and de Moraes, F. F. (1990), in *Etude Preliminaire a L'immobilsation de L'enzyme CGTase WACKER.* Laboratoire de Tecnologie Enzymatique. Université de Tecnologie de Compiègne.
15. Kato, T. and Horikoshi, K. (1984), *Anal. Chem.* **56,** 1738–1740.
16. Bradford, M. (1976), *Anal. Biochem.* **72,** 248.
17. László, E., Bánky, B., Seres, G., and Szejtli, J. (1981), *Starch/Stärke* **33,** 281–283.
18. Weber, K. and Osborn, M. (1969), *J. Biol. Chem.* **244,** 4406–4412.
19. Chatjigakis, A. K., Cardot, P. J. P., Coleman, A. W., and Parrot-Lopez, H. (1993), *Chromatographia* **36,** 174–178.
20. Sabioni, J. G. (1991), PhD Thesis, Universidade Estadual de Campinas, Campinas-SP, Brasil.
21. Sabioni, J. G. and Park, Y. K. (1992), *Revista de Microbiologia* **23,** 128–132.
22. Tulfahi, A. (1991), PhD Thesis, Université de Technologie de Compiègne, Compiègne, France.
23. Schmid, G., Englbrecht, A., and Schmid, D. (1988), in *Proceedings of the Fourth International Symposium on Cyclodextrins,* Huber, O and Szetjtli, J., eds., Kluwer, Dordrecht, Netherlands, pp. 71–76.
24. Saha, B. C., Freer, S. N., and Bothast, R. J. (1994), *Appl. Environ. Microbiol.* **60,** 3774–3780.
25. Sabioni, J. G. and Park, Y. K. (1992), *Starch/Stärke* **44,** 225–229.
26. Mori, S., Goto, M., Mase, T., Matsuura, A., Oya, T., and Kitahata, S. (1995), *Biosci. Biotechnol. Biochem.* **59,** 1012–1015.