0273-2289/06/129–132/234–246/$30.00

Evaluation of Solid and Submerged Fermentations for the Production of Cyclodextrin Glycosyltransferase by *Paenibacillus campinasensis* H69-3 and Characterization of Crude Enzyme

Heloiza Ferreira Alves-Prado,[1,2] Eleni Gomes,[1] and Roberto Da Silva*,[1]

[1]UNESP-State University of São Paulo, Biochemistry and Applied Microbiology Laboratory, Rua Cristóvão Colombo no. 2265, 15054-000, São José do Rio Preto, SP, Brazil, E-mail: dasilva@ibilce.unesp.br; and [2]UNESP-State University of São Paulo, Biology Institute, Rio Claro, SP, Brazil

Abstract

Cyclodextrin glycosyltransferase (CGTase) is an enzyme that produces cyclodextrins from starch by an intramolecular transglycosylation reaction. Cyclodextrins have been shown to have a number of applications in the food, cosmetic, pharmaceutical, and chemical industries. In the current study, the production of CGTase by *Paenibacillus campinasensis* strain H69-3 was examined in submerged and solid-state fermentations. *P. campinasensis* strain H69-3 was isolated from the soil, which grows at 45°C, and is a Gram-variable bacterium. Different substrate sources such as wheat bran, soybean bran, soybean extract, cassava solid residue, cassava starch, corn starch, and other combinations were used in the enzyme production. CGTase activity was highest in submerged fermentations with the greatest production observed at 48–72 h. The physical and chemical properties of CGTase were determined from the crude enzyme produced from submerged fermentations. The optimum temperature was found to be 70–75°C, and the activity was stable at 55°C for 1 h. The enzyme displayed two optimum pH values, 5.5 and 9.0 and was found to be stable between a pH of 4.5 and 11.0.

Index Entries: Cyclodextrin glycosyltransferase; *Paenibacillus campinasensis;* submerged fermentation; solid-state fermentation.

Introduction

Cyclodextrin glycosyltransferase (CGTase; EC 2.4.1.19) is an enzyme used for cyclodextrin production from starch by an intramolecular

*Author to whom all correspondence and reprint requests should be addressed.

transglycosylation reaction. Cyclodextrins (CDs) are cyclic malto-oligosaccharides made up of D-glucose residues linked by α-1,4 bonds. The most common CDs are α-CD, β-CD, and γ-CD types, containing six, seven and eight D-glucose residues, respectively *(1–3)*. CD cavities have hydrophobic interiors and hydrophilic exteriors, which allows hydrophobic molecules or the hydrophobic portion of a molecule to become included within the inner cavity and form an inclusion complex. Because of their ability to form inclusion complexes with organic molecules, CDs and their derivatives have become increasingly useful in pharmaceutical, analytical chemistry, agricultural, cosmetics, food, and biotechnology applications. CDs can be used to capture flavors or odors, to stabilize volatile compounds, to improve the solubility of hydrophobic substances and to protect substances against undesirable modifications *(1–6)*.

A number of microorganisms produce extracellular CGTases. This production occurs mainly through members of the genus *Bacillus*, especially aerobic alkalophilic types *(7–19)*. Other reported mesophilic and thermophilic CGTase producers include: *Klebsiella* sp. *(20,21)*, *Brevibacterium* sp. *(22)*, and *Paenibacillus* sp. *(23)*, *Thermoanaerbacter* sp. *(24,25)*, *Thermoanaerobacterium* sp. *(26)* and hyperthermophilic archae-bacteria *Thermococcus* sp. *(27)*.

The CGTase production is typically conducted by submerged fermentation (SmF) utilizing starch as carbon source and other nutrients. However, solid-state fermentation (SSF) has been exploited extensively on the industrial scale in recent years. The growing conditions in SSF approximate the natural habitat of some microorganisms more closely than those in liquid culture, so these microorganisms are able to grow and excrete large quantities of enzyme *(28,29)*. Both types of fermentation present advantages and disadvantages, the choice will depend on operational limitations, microbial performance and its enzyme production. In most literature reports, the CGTase productions were conducted in SmF with mesophilic microorganisms. Horikoshi *(30)* and Starnes *(24)* reported that industrial CGTase production became possible because of the high level of the enzyme produced by a mesophilic alkalophilic *Bacillus* sp. Mesophilic microorganisms usually produce enzymes with low-thermal stability. In the CD production process thermally stable α-amylase is added during the liquefaction step which is carried out at temperatures between 95°C and 105°C. Thermally stable CGTases would make it possible to increase the temperature for CGTase action, and decrease the cost of CD production. The high production costs of CGTase and CDs are considered a limiting factor for CD application on an industrial scale. Research into the reduction of CGTase production costs is important to enable the economic commercial scale use of CDs, and finding a thermophilic CGTase producing microorganism with high-thermal stability is of commercial interest.

This study reports the isolation of a novel CGTase from *Paenibacillus campinasensis* strain H69-3 isolated from a soil sample in São José do Rio Preto, SP, Brazil. The production of CGTase using submerged and SSF was studied, and the properties of the crude CGTase determined.

Material and Methods

Medium

The selective medium used as reported by Park et al. *(31)* was made up of soluble starch 10 g/L, peptone 5 g/L, yeast extract 5 g/L, K_2HPO_4 1 g/L, $MgSO_4 \cdot 7H_2O$ 0.2 g/L, Na_2CO_3 10 g/L (separately sterilized), phenolphthalein 0.3 g/L, orange methyl 0.1 g/L, and agar 15 g/L, pH 10.0. The colonies surrounded by a yellowish halo were selected for examination on a medium made up of the same ingredients without phenolphthalein, orange methyl and agar *(7)*. Stock cultures were maintained in this culture medium with the addiction of agar 15 g/L at 4°C in a slant tube (maintenance medium) *(8)*.

Microorganism Isolation

Approximately 1 g of shaded soils from market-garden and crop soils were added to flasks with 5-mL culture medium. The samples were incubated at 45°C for 24 h and then inoculated on Petri dishes containing selective medium for CGTase detection. The plates were incubated at 45°C for a period of 144 h. Positive colonies for CGTase enzyme were transferred to tubes containing maintenance medium.

Submerged Fermentation

Two methods based on the culture medium proposed by Nakamura and Horikoshi *(7)*, were used to analyze the SmFs. The culture medium was made up of soluble starch 10 g/L, peptone 5 g/L, yeast extract 5 g/L, K_2HPO_4 1 g/L, $MgSO_4 \cdot 7H_2O$ 0.2 g/L, and Na_2CO_3 5 g/L (separately sterilized), pH 9.6. For the first method, peptone and yeast extract were not included in the culture medium, exchanging the soluble starch for other carbon sources at 10 g/L concentration. These sources were: cassava bran, corn starch, cassava starch, corn flour, wheat bran, soy bran, soy extract, soy, and soy flour. The second method used the same culture medium concentration was only soluble starch was substituted for sweet potato starch, corn starch, cassava starch, cassava bran, and corn flour. The culture medium proposed by Nakamura and Horikoshi *(7)* was used as the enzyme production control.

The preinoculum was prepared using 125-mL Erlenmeyer flasks containing 20 mL of the culture medium and a microbial mass produced in an agar slant tube. Microbial growth was carried out using a rotary shaker at 45°C and 150 cycles/min for 24 h. SmFs were carried out in 125-mL

Erlenmeyer flasks containing 20 mL of culture medium for CGTase production analysis and cellular growth. Shake flasks were inoculated with 0.5-mL cellular suspension obtained from a 24-h preinoculum. The Erlenmeyer flasks were incubated in a rotary shaker at 45°C and 150 cycles/min, for 96 h. After fermentation, the volume of all flasks was centrifuged at 10,000*g* at 5°C for 15 min. The supernatant liquid, free of cells, was utilized to determine enzymatic activities. The centrifuged cell mass was washed with saline solution and the microbial biomass was quantified by absorbance at 640 nm. All experiments were performed once.

Solid-State Fermentation

Various substrates, such as wheat bran, soy bran, soy flour, corn flour, cassava bran, cassava starch, corn starch, and soluble starch, were used to measure CGTase production in SSFs. In the previous article *(32)*, describing CGTase production from *Bacillus* sp., it was shown that the use of a mixture, made up of a substrate and wheat bran, presented better results than the use of only one substrate each time. Thus, a mixture was prepared, with a 1 : 1 proportion, with each substrate and wheat bran. These media were prepared using 250-mL Erlenmeyer flasks containing a total of 5 g of solid material (2.5 g of wheat bran and 2.5 g of another substrate). After sterilization, this solid material was incubated with 2.5 mL of preinoculum and 2.5 mL of Na_2CO_3 solution 0.5%. The initial humidity was 70%. The flasks were incubated in an oven at 45°C and in accordance with the literature; SSF lasted 72 h *(29,32,33)*. Growth was detected through visualization and the typical smell. The enzyme was extracted by adding 20 mL of distilled water to the solid media. These solid media were gently cleaved using a glass rod, and then shaken at 100 cycles/min for 1 h at room temperature. The suspension was filtered under vacuum using Whatman filter paper followed by centrifugation at 10,000*g* at 5°C for 15 min. The enzymatic activities were determined using the supernatant liquid.

Enzymatic Assay Determination

Two methods were used for CGTase determination because CD formation involves both dextrinization and cyclization. The iodine method was applied to determine dextrinization whereas the phenolphthalein method was applied to determine CD formation. Dextrinizing was determined in accordance with Fuwa *(34)* and Pongasawasdi and Yagisawa *(35)* with slight modifications. A sample of 0.1 mL of appropriately diluted enzyme was added to 0.3 mL of 0.5% soluble starch prepared in 0.1 *M* acetate buffer, pH 5.5 and incubated at 55°C for 10 min. The enzyme reaction was stopped by the addition of 4 mL of 0.2 *M* HCl solution. Then, 0.5 mL of iodine solution prepared with 0.03% I and 0.3 KI was added to the reaction mixture. The absorbance was measured at 700 nm and a decrease in absorbance was verified, when compared with a control tube with heat inactivated enzyme.

One unit of enzyme activity was defined as the quantity of enzyme that reduces the blue color of starch–iodine complex by 10%/min.

The method of Mäkelä et al. *(36)* was slightly modified to determine the cyclization reaction. A sample of 0.1 mL of appropriately diluted enzyme was added to 0.8 mL of 1% soluble starch prepared in 0.1 *M* acetate buffer, pH 5.5 and incubated at 55°C for 10 min. The enzyme reaction was stopped by the addition of 4 mL of 0.25 M Na_2CO_3 solution and 0.1 mL of 1 m*M* phenolphthalein solution was added to the reaction mixture. The absorbance was measured at 550 nm and a decrease in absorbance was compared with a control tube with inactive enzyme at 100°C for 30 min. One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol of β-CD/min.

Protease Activity

For the protease activity determination, a mixture made up of 0.2 mL of enzyme solution and 0.8 mL of the 1% (p/v) casein solution was prepared in acetate buffer 50 m*M*, pH 5.6. After incubation at 37°C for 60 min, the reaction was stopped by the addition of 1 mL of 10% trichloroacetic acid (TCA). The mixture was centrifuged at 6000*g* for 5 min and the absorbance of the supernatant was determined at 280 nm. A control tube was prepared by adding the enzymatic solution to the reaction mixture after the addition of TCA solution. One protease activity unit was defined as the quantity of enzyme needed to produce 1 μmol of tyrosine/min *(37)*.

Protein Determination

Protein concentration was estimated according to the Hartree-Lowry method, using bovine-serum albumin as standard *(38)*.

Results and Discussion

Microorganism Isolation

Eighty strains of CGTase producers were isolated. Only three strains presented high-CGTase activity and grew at 45°C. These strains were isolated from the soil in shady sites in cassava crop fields, in São José do Rio Preto. The strains were designated H69-2, H69-3, and H69-5 *(32)*. Strain H69-3 presented the highest CGTase activity (Table 1) and was selected for additional characterization.

The strain designated H69-3 was identified by the *Centro Pluridisciplinar de Pesquisas Químicas, Biológicas e Agrícolas* (CPQBA, Unicamp, Campinas, SP, Brazil). The partial sequence of 16S rRNA revealed 99% homology with the 16S rRNA sequence of *P. campinasensis* strain 324 *(39)*. Based on these results the strain was classified as *P. campinasensis* strain H69-3. The 16S rRNA sequence has been deposited in the Gene Bank database under the accession number DQ153080.

Table 1
CGTase Production After 60 h of Fermentation and Origin of Isolated Strains

Strains	H69-2	H69-3	H69-5
Soil crop	Cassava	Cassava	Cassava
Dextrinizing activity (U/mL)	42.3	85.75	73.15
Phenolphthalein activity (U/mL)	0.81	1.34	0.88

Submerged Fermentations

SmFs were evaluated using two different media compositions. The first consisted of medium without peptone and yeast extract using different substrates. The culture medium of Nakamura and Horikoshi *(7)* containing yeast extract and peptone was used as a control. It was observed that the microorganism presented a protein content and CGTase activity lower than the control medium in which, after the 48-h fermentation, there was significant protein content and CGTase production. Because of the lack of peptone and yeast extract in the culture medium, there was no significant biomass production, indirectly observed by the protein content, for all media used. Because of this fact, fermentation was extended to 96 h, with no change in the results observed. For each assay, the mean values of enzymatic activities are shown in Fig. 1.

According to Fig. 1, CGTase production was lower compared with the control medium. The medium with wheat bran showed the most significant CGTase production, followed by the medium with soy bran. Surprisingly, CGTase activity was not detected by the phenolphthalein method probably owing to low-enzyme activity. Based on these results, it was inferred that peptone and yeast extract are important nutrients for microbial growth and CGTase production. It is likely that the supply of nitrogen and/or amino acids by the substrates was insufficient for the complete development of the *P. campinasensis* strain H69-3.

For the second experiment, only the starch source was changed, keeping the usual concentrations of peptone and yeast extract. With this composition, growth was complete, showing good CGTase production (Fig. 2). In this case, it was possible to determine significant CGTase activity using both methods for activity determination. The most significant CGTase production was obtained for the medium containing cassava bran as a substrate. The medium with cassava starch also presented significant activity, higher than the control medium. These two substrates have the same vegetal origin, the cassava plant. As the microorganism was isolated from soil of the cassava crop, we can infer that the natural habitat of the microorganism may be a good indicator of the kind of enzyme that it is able to produce. This result also suggests the nutritional importance of yeast extract and peptone for CGTase production of the *P. campinasensis* strain H69-3 studied under SmF.

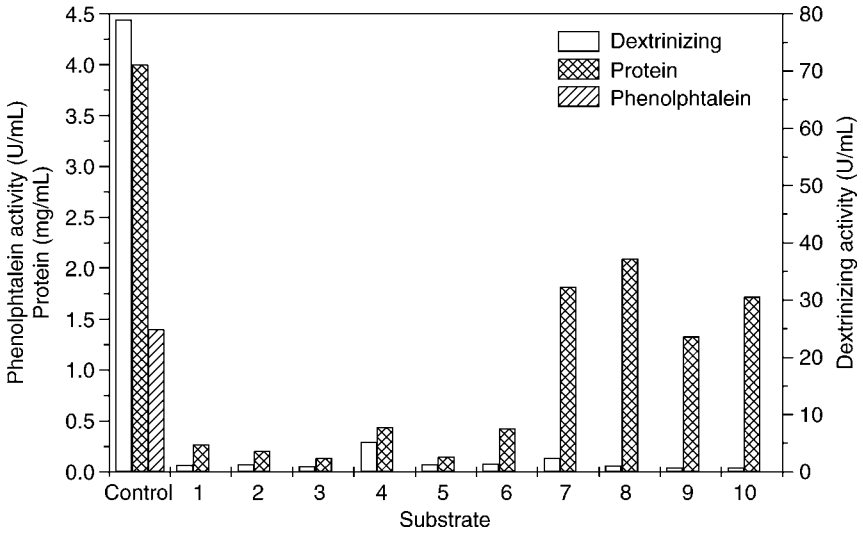

Fig. 1. Cyclodextrin glycosyltransferase production from *Paenibacillus campinasensis* strain H69-3 in submerged fermentation after 96 h. The culture media was without peptone and yeast extract. The control medium was based on Nakamura and Horikoshi *(7)*. Substrates: soluble starch (1), cassava bran (2), cassava starch (3), wheat bran (4), corn starch (5), corn flour (6), soy bran (7), soy extract (8), soy (9), and soy flour (10).

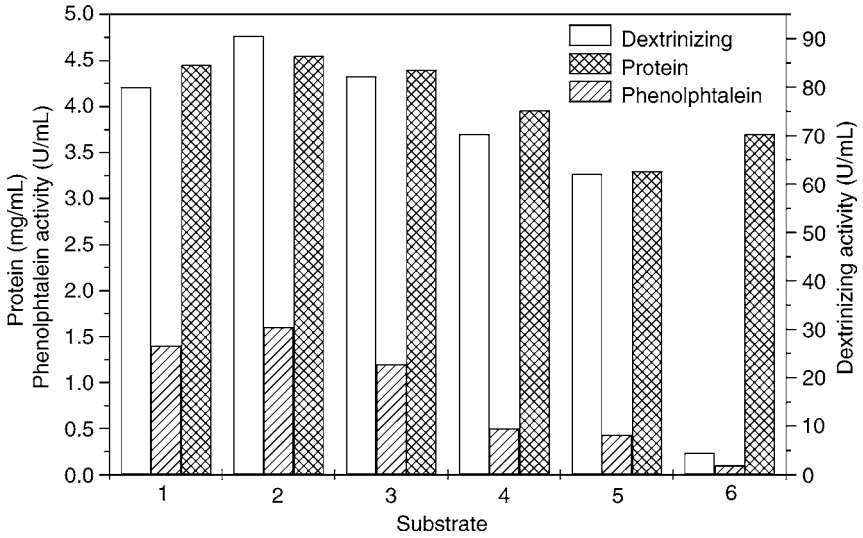

Fig. 2. Cyclodextrin glycosyltransferase production obtained from *Paenibacillus campinasensis* strain H69-3 using the submerged fermentation after 48-h cultivation. Substrate: soluble starch (1), cassava bran (2), cassava starch (3), sweet potato starch (4), corn starch (5), and corn flour (6).

Solid-State Fermentation

For the composition of culture media, the use of traditional commercial sources of carbon (C) and nitrogen (N) leads to high costs for industrial enzyme production *(32,33,40,41)*. So research that investigates enzyme

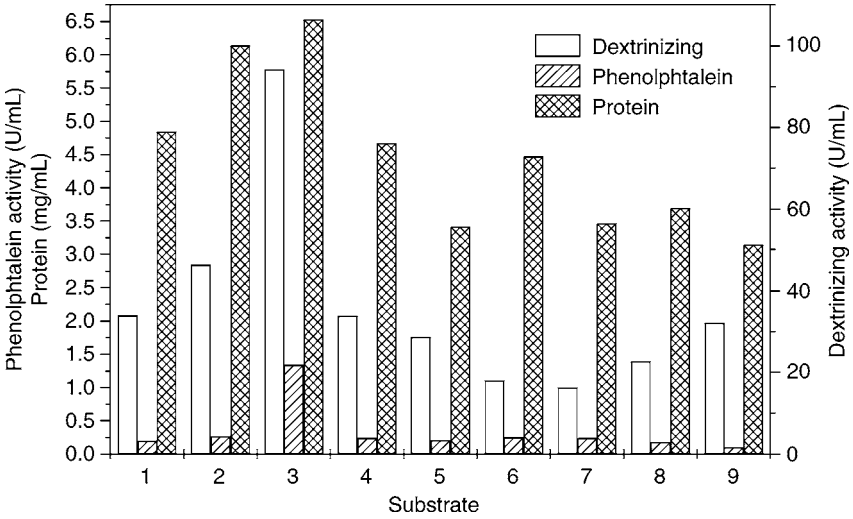

Fig. 3. Cyclodextrin glycosyltransferase production from *Paenibacillus campinasensis* strain H69-3 using solid-state fermentation after 72-h cultivation. Substrate: wheat bran (1), soy bran (2), wheat bran + soy bran (3), wheat bran + soy flour (4), wheat bran + corn flour (5), wheat bran + cassava bran (6), wheat bran + cassava starch (7), wheat bran + corn starch (8), and wheat bran + soluble starch (9).

production using low-cost agroindustrial residues is important to make the use of commercial enzymes economically feasible. In this case, commercial sources of carbon and nitrogen were replaced by low-cost agroindustrial residues *(3,7,28,33)*. Nine different media compositions with solid substrates were analyzed and all demonstrated microbial growth, measured as protein content. Only the medium made up of wheat bran and soy bran showed CGTase production similar to the levels obtained for SmF. The other media presented a CGTase production lower than the medium mentioned. Figure 3 shows that if the soy bran or wheat bran substrates are used separately, the resulting enzymatic activity is lower than the activity obtained with a mixture of these substrates. In this case, the enzymatic activity is increased about two- or threefold. Thus, it can be inferred that some nutrients of wheat bran or soy bran would not be available to the microorganisms if both bran types were not mixed. Few studies have reported CGTase production obtained from solid substrates. This is probably related to the fact that CGTase producing microorganisms belong to the bacilli gender and SSF is a more common characteristic of filamentous fungi. SSF is generally defined as the growth of microorganisms on solid material in the absence or near-absence of free water, which is physiologically more favorable to the cultivation of filamentous fungi *(42)*. Even so, Ramakrishna *(29)* and Alves-Prado *(32)* obtained CGTase activity, using SSF, similar to activities using SmF. These researchers used mesophilic species: *Bacillus cereus* and *Bacillus* sp. subgroup *alkalophilus*, respectively.

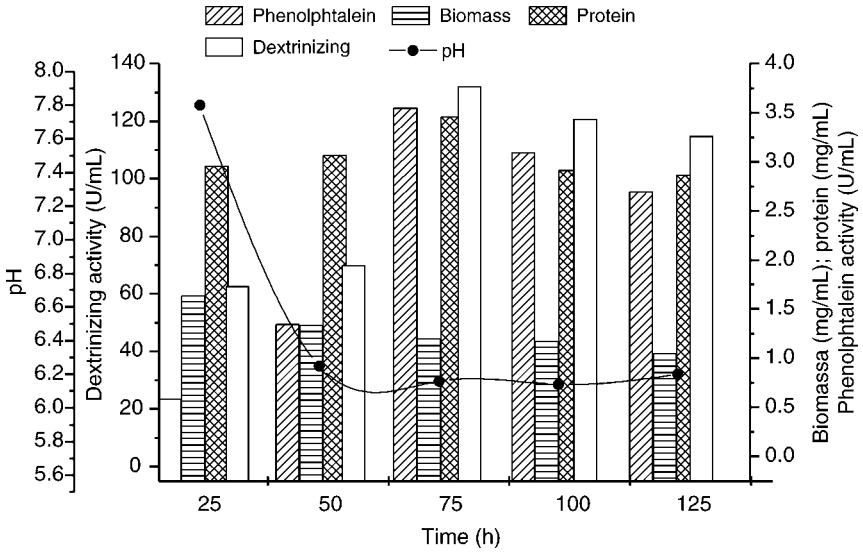

Fig. 4. Cyclodextrin glycosyltransferase production from *Paenibacillus campinasensis* strain H69-3 using a control medium based on Nakamura and Horikoshi *(7)*.

Protease activity was analyzed using all media compositions shown in this article and detected in almost all solid-state media (data not shown). A possible explanation for this detection result is the high level of protein available in these media. There was also protease activity for the second composition of SmF and the highest production level was obtained by using cassava bran which, coincidentally, also presented the largest CGTase activity level. Because of protease presence during CGTase production, the time of these fermentations must not be extended because protease production can interfere with CGTase production. Ramakrishna *(29)* noticed a reduction in CGTase activity that was dependent on the culture time of the media analyzed in their article, suggesting that this reduction was an effect of protease.

Time-Course of Enzyme Production in SmF

Figure 4 shows the CGTase activity, protein content, biomass, and pH variation produced by *P. campinasensis* strain H69-3 as a function of fermentation time, using the control medium proposed by Nakamura and Horikoshi *(7)*. In earlier studies (data not shown), it was indicated that *P. campinasensis* strain H69-3 grew best in 0.5% Na_2CO_3 and at an initial pH of 9.6. During fermentation, the pH of the medium was lowered and stabilized at pH 6.0. The CGTase producers reported in the literature usually show maximum production at approx 40–60 h of fermentation *(3)* and the results shown here are in agreement with these studies. The CGTase activity determined by the dextrinizing and phenolphthalein methods showed an increase during growth in the exponential phase, which occurred in the first 24 h. CGTase activity peaks were (132 U/mL) and (3.5 U/mL) for

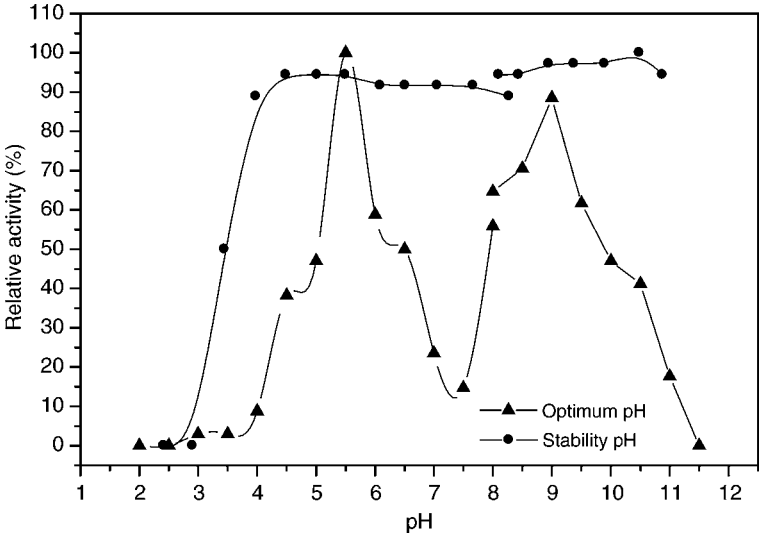

Fig. 5. Effects of pH on CGTase activity (—▲—) and CGTase stability (—●—) from *Paenibacillus campinasensis* strain H69-3. The buffers used were: MacIlvaine (pH 2.5–8.0) and glycine-NaOH (pH 8.0–11.5).

dextrinizing and phenolphthalein methods, respectively, during 72-h fermentation. After these peaks, CGTase activity slowed down and stabilized after approx 80 h of fermentation, coinciding with the growth in the stationary phase. Other authors show that *Bacillus cereus*, *Bacillus stearothermophilus* ET1 and other alkalophilic *Bacillus* sp. CGTase producers reveal a CGTase production peak during the growth stationary phase, but for *B. cereus* this peak occurs after 16–20 h of fermentation. This was probably owing to a high concentration of the initial inoculum at 5% *(3,7,18,43)*.

Effects of pH on Activity and Stability of Enzyme

For characterization of the CGTase from *P. campinasensis* strain H69-3, crude CGTase was produced under SmF for 50 h using the methods of Nakamura and Horikoshi *(7)*. The crude CGTase was characterized through the dextrinizing method under standard activity conditions. The enzyme activity was measured at varying pH values ranging from 2.5 to 11.5 at 70°C. Optimal activity was seen at two peaks of optimum pH 5.5 and 9.0 (Fig. 5), a characteristic also shown by other CGTases. This may suggest that there are two proteins with distinct catalytic activity or that the same enzyme is capable of acting at different pH values. This has also been reported for *Bacillus circulans* no. 79 *(44)*, *Bacillus amiloquefaciens* AL35 *(9)*, *Bacillus firmus (14–16)*, alkalophilic *Bacillus* sp. *(7,8)*, and *Thermoanaerobacterium thermosufurigenes* EM1 *(26)*. The pH stability was determined by incubating the crude CGTase at different pH values, ranging from 2.5 to 12.0 for 24 h at 25°C. Then, the residual activity was measured as a standard activity condition. As shown in Fig. 5, the crude CGTase presented a wide range of pH stability from 4.5 to 11.0.

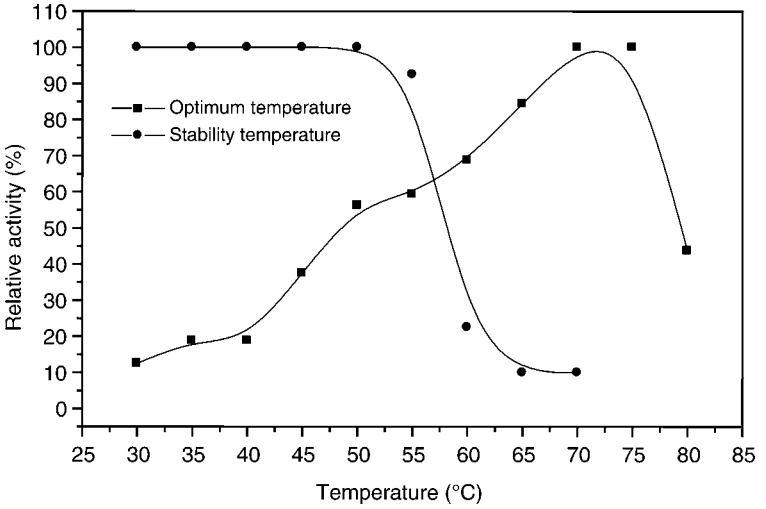

Fig. 6. Temperature effects on Cyclodextrin glycosyltransferase activity and stability from *Paenibacillus campinasensis* strain H69-3. (—■—) optimum temperature; (—●—) thermal stability.

Effect of Temperature on Enzyme Activity and Stability

The activity of crude CGTase was measured at different temperatures, 30–80°C at pH 5.5. Figure 6 shows that CGTase was optimally active at 70–75°C. The effect of temperature on the stability of crude CGTase was also investigated. The enzyme was incubated for 1 h at various temperatures (30–70°C) followed by the measurement of residual activity under standard assay conditions. CGTase activity was maintained up to 55°C, indicating a good thermal stability (Fig. 6).

CGTase from *P. campinasensis* strain H69-3 presented high-optimum temperature and thermal stability. These values are lower than some results obtained with thermophilic microorganisms, for example: *B. stearothermophilus* ET1 *(18)* which was reported to have an optimum temperature of 80°C and to be thermally stable at 60°C, *B. stearothemophilus* R2 *(19)*, with was thermally stable at 70°C after 30-min heat treatment; *Thermoanaerobacter* sp. and *Thermoanaerobacterium thermosufurigenes* EM1, with optimum temperatures of 80°C and 95°C, respectively, and a thermal stability up to 70°C for both enzymes *(25,26)*. However, *P. campinasensis* strain H69-3 showed higher CGTase activity than many other microorganisms reported, indicating a potential industrial application of this CGTase in processes in which thermal stability is required. This enzyme could be used after starch gelatinization without cooling the solution to temperatures lower than 60°C.

Conclusions

The importance of adding yeast extract and peptone to *P. campinasensis* strain H69-3 SmFs for the production of CGTase has been demonstrated. The

substrate sources with high-starch contents showed higher CGTase production. *P. campinasensis* strain H69-3 showed good growth using SSF when combined with wheat bran and soy bran. In this case, the CGTase production levels were similar to that obtained for SmF. Crude CGTase activity was stable up to 50°C, and the optimum temperature was in the range of 70–75°C. The optimum pH values were found to be 5.5 and 9.0, and CGTase activity was stable between 6.5 and 10.5. These results suggest that CGTase from the *P. campinasensis* strain H69-3 presents potential for industrial applications.

Acknowledgment

The authors are grateful to the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) for its financial support.

References

1 Bender, H. (1986), *Adv. Biotechnol. Process* **6,** 31–71.
2 Szejtli, J. (1997), *J. Mater. Chem.* **7,** 575–587.
3 Tonkova, A. (1998), *Enzyme Microb. Technol.* **22,** 678–686.
4 Szejtli, J. (1982), *Starch/Stärke* **34,** 379–385.
5 Pszczola, D. E. (1988), *Food Technol.* January, 96–100.
6 Allegre, M. and Deratani, A. (1994), *Agroo Food Ind. Hi Technol.* January/February, 9–17.
7 Nakamura, N. and Horikoshi, K. (1976), *Agric. Biol. Chem.* **40,** 1785–1791.
8 Nakamura, N. and Horikoshi, K. (1976), *Agric. Biol. Chem.* **40,** 935–941.
9 Yu, E. K. C., Aoki, H., and Misawa, M. (1988), *Appl. Microbiol. Biotechnol.* **28,** 377–379.
10 Tomita, K., Kaneda, M., Kawamura, K., and Nakanishi, K. (1993), *J. Fermen. Bioeng.* **75,** 89–92.
11 Bovetto, L. J., Backer, D. P., Villette, J. R., Sicard, P. J., and Bouquelet, S. J. -L. (1992), *Biotechnol. Appl. Biochem.* **15,** 48–58.
12 Sabioni, J. G. and Park, Y. K. (1992), *Starch/Stärke* **44,** 225–229.
13 Yim, D. G., Sato, H. H., Park, Y. H. E., and Park, Y. K. (1997), *J. Ind. Microbiol. Biotechnol.* **18,** 402–405.
14 Matioli, G., Zanin, G. M., Guimarães, M. F., and Moraes, F. F. (1998), *Appl. Biochem. Biotechnol.* **70–72,** 267–275.
15 Gawande, B. N., Singh, R. K., Chauhan, A. K., Goel, A., and Patkar, A. (1998), *Enzyme Microb. Technol.* **22,** 288–291.
16 Martins, R. F. and Hatti-Kaul, R. (2002), *Enzyme Microb. Technol.* **30,** 116–124.
17 Alves-Prado, H. F., Gomes, E., and DaSilva, R. (2002), *Bol. SBCTA.* **36,** 43–54.
18 Chung, H. J., Yoon, S. H., Lee, M. J., et al. (1998), *J. Agric. Food Chem.* **46,** 952–959.
19 Kabavainova, L., Dobreva, E., and Miteva, V. (1999), *J. Appl. Microbiol.* **86,** 1017–1023.
20 Bender, H. (1977), *Arch. Microbiol.* **111,** 271–281.
21 Gawande, B. N. and Patkar, A. Y. (2001), *Enzyme Microb. Technol.* **28,** 9, 10.
22 Mori, S., Hirose, S., Oya, T., and Kitahata, S. (1994), *Biosc. Biotechnol. Biochem.* **58,** 1968–1972.
23 Larsen, K. L., Duedhal-Olisen, L., Christensen, H. J. S., Mathiesen, F., Pedersen, L. H., and Zimmermann, W. (1998), *Carbohyd. Res.* **310,** 211–219.
24 Starnes, R. L. (1990), *Cereal Foods World* **35,** 1094–1099.
25 Zamost, B. L., Nilsen, H. K., and Starnes, R. L. (1991), *J. Ind. Microbiol.* **8,** 71–82.
26 Wind, R. D., Libl, W., Buitelaar, R. M., et al. (1995), *Appl. Environ. Microbiol.* **61,** 1257–1265.
27 Tachibana, Y., Kuramura, A., Shirasaka, N., et al. (1997), *Appl. Environ. Microbiol.* **65,** 1991–1997.
28 Raimbault, M. (1998), *Eletr. J. Biotechnol.* **1,** 174–188.

29 Ramakrishna, S. V., Saswathi, N., Sheela, R., and Jamuna, R. (1994), *Enzyme Microb. Technol.* **16,** 441–444.
30 Horikoshi, K. (1996), *FEMS Microbiol. Rev.* **18,** 259–270.
31 Park, C. S., Park, K. H., and Kim, S. H. (1989), *Agric. Biol. Chem.* **53,** 1167–1169.
32 Alves-Prado, H. F., Gomes, E., and DaSilva, R. (2002), *Brazilian J. Food Technol.* **5,** 189–196.
33 Dias, A. A. M., Andrade, C. M. M. C., and Linardi, V. R. (1992), *Rev. Microbiol.* **23,** 189–193.
34 Fuwa, H. (1954), *J. Biochem.* **41,** 583–603.
35 Pongsawasdi, P. and Yagisawa, M. (1987), *J. Fermen. Technol.* **65,** 463–467.
36 Mäkelä, M. J., Korpela, T. K., Puisto, J., and Laakso, S. V. (1988), *Agric. Food Chem.* **36,** 83–88.
37 Sarath, G. (1996), In: *Proteolytic Enzymes a Practical Approach*. Beynon, R. J., Bond, J. S., (ed.), Oxford University Press, New York, pp. 25–55.
38 Hartree, E. F. (1972), *Anal. Biochem.* **48,** 422–427.
39 Yoon, J. -H., Yim, D. K., Lee, J. -S., et al. (1998), *Internat. J. System. Bacteriol.* **48,** 833–837.
40 Bailey, J. E. and Ollis, D. F. (1986), *Biochemical Engineering Fundamentals.* 2nd edition, McGraw-Hill International Editions, New York.
41 Damaso, M. C. T., Andrade, C. M. M. C., Pereira, N. Jr, (2000), *Appl. Biochem. Biotechnol.* **84,** 821–834.
42 Pandey, A. (1992), *Process Biochem.* **27,** 109–117.
43 Jamuna, R., Saswathi, N., Sheela, R., and Ramakrishna, S. V. (1993), *Appl. Biochem. Biotechnol.* **43,** 163–176.
44 Salva, T. J. G., Lima, V. B., and Pagan, A. P. (1997), *Rev. Microbiol.* **28,** 157–164.