Original Article
Food Science and Technology

ISSN 0101-2061 (Print)
ISSN 1678-457X (Online)

DOI: https://doi.org/10.1590/fst.104122

Challenges and alternatives for the production of cyclodextrins from the CGTase enzyme from recombinant *Bacillus subtilis* WB800

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Abstract

Cyclodextrins (CDs) have the ability to encapsulate numerous molecules and have applicability in several industrial areas, however, their cost has made their use difficult. To seek alternatives that may enable the use of DCs, the present study evaluated the efficiency of the ultrafiltration process in a continuous system to produce CDs from the enzyme cyclomaltodextrin glucanotransferase (CGTase) from recombinant *Bacillus subtilis* WB800. The possibility of using the crude enzyme as an alternative means of producing CDs was also evaluated. All strategies evaluated in this research proved to be promising for the production of CDs, with the production of β-CD being the most efficient (average of 15 mmol/L) using crude recombinant enzyme and a temperature of 50 °C. Therefore, the results obtained can contribute to the reduction of stages and costs of production of CDs, favoring their industrial application.

Keywords: cyclodextrins; CGTase recombinant; continuous system; ultrafiltration.

Practical Application: This study presents innovative alternatives to produce cyclodextrins from the recombinant enzyme.

1 Introduction

Cyclodextrins (CDs) are cyclic oligosaccharides with expressive applicability in several industrial segments, such as the food, pharmaceutical, cosmetics, chemical sectors, among others. CDs are formed by 6 (α-CD), 7 (β-CD), and 8 (γ-CD) glucose units, joined by α-1,4 glycosidic bonds, obtained from the transglycosylation reaction of starch, which is catalyzed by the enzyme cyclomaltodextrin glucanotransferase (CGTase) (Ogunbadejo & Al-Zuhair, 2021; Cid-Samamed et al., 2022).

Due to the nonpolar characteristic of the CD cavity, inclusion complexes with a wide range of organic and inorganic molecules are capable of formation, modifying the physicochemical properties of the guest molecule, making it possible to increase its stability and solubility. (Del Valle, 2004; Brewster & Loftsson, 2007; Fenelon et al., 2015). Such skills explain the growing interest in the development of innovative biotechnological processes that can enable the industrial use of CDs (Astray et al., 2010; Cid-Samamed et al., 2022).

The research group of the present study obtained promising results when they evaluated the production of CDs from genetically modified bacteria and ultrafiltration systems. (Fenelon et al., 2018; Gimenez et al., 2019). Therefore, it is highly relevant to evaluate new biotechnological alternatives to optimize the production of CDs, especially in Brazil, which has substrate availability and still does not produce the molecule on an industrial scale.

In view of the above, the present study aimed to evaluate strategies for the production of CDs from the CGTase of the recombinant B. subtilis WB800, using a continuous production system associated with ultrafiltration for the semi-purified and purified enzyme, and alternative production means for the crude enzyme.

2 Materials and methods

2.1 Materials

The materials used were ethanol, soluble starch, commercial corn starch, tryptone, yeast extract, sodium carbonate, sodium chloride, and agar. β-cyclodextrin and HPLC grade acetonitrile were purchased from Sigma-Aldrich Ltda, São Paulo, Brazil. The antibiotics used were Kanamycin Sulfate and Hygromycin B., from *Streptomyces hygroscopicus*. All other reagents used were of analytical or chromatographic grade.

2.2 Methods

Cultivation and growth of the recombinant B. subtilis WB800 bacterium

For the cultivation and growth of the bacteria, 2xYT medium (liquid and solid) was used, composed of 1.6% tryptone, 1% yeast

Received 14 Oct., 2022
Accepted 07 Dec., 2022
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extract and 0.5% NaCl and 1.5% agar for the solid medium, both supplemented with the antibiotics hygromycin (100 μg/mL) and kanamycin (25 μg/mL*). B. subtilis* WB800 containing the recombinant plasmid pWB980-CGTase was grown in a Petri dish containing 2xYT solid medium supplemented with kanamycin (25 μg/ml) and hygromycin (100 μg/mL) for 12 h at 37 °C. Then, an isolated colony was added to 5 mL of 2xYT liquid medium supplemented with antibiotics and placed in a shaker at 37 °C overnight at 100 rpm. Subsequently, a pre-inoculum (50 mL) was prepared, also supplemented with both antibiotics. In this pre-inoculum, 0.5 mL of the previously activated enzyme was added to the liquid medium, and it was incubated in a shaker at 37 °C for 24 h at 100 rpm.

Production means and obtaining the crude extract of recombinant CGTase

For the production of recombinant *B. subtilis* CGTase, the methodology proposed by Fenelon et al. (2015) was used, with modifications. 2xYT medium supplemented with kanamycin (25 μg/mL) was used and 250 mL of liquid medium was prepared. 5 mL aliquots of the pre-inoculum were transferred to the production medium, which was incubated at 30 °C, at 100 rpm, for 5 days. 5 mL aliquots were collected every 24 hours to determine enzymatic activity. After the period of production of recombinant CGTase, the entire contents were centrifuged at 8,000 rpm, 4 °C for 10 min. The pellet composed of bacteria and insoluble compounds was discarded and a sample of the supernatant containing the enzyme was separated and named crude extract.

Obtaining semipurified and purified recombinant CGTase

The procedure for obtaining semi-purified recombinant CGTase was carried out through ultrafiltration processes, according to the methodology described by Fenelon et al. (2015). To obtain purified recombinant CGTase, the technique of biospecific affinity chromatography (CAB) was used, according to the methodology described by Moriwaki et al. (2009). An aliquot of each sample was used to determine the enzyme activity and protein concentration.

CDs production assays by CGTase of recombinant B. subtilis WB800

For the assays for the production of CDs by the CGTase of semi-purified and purified B. *subtilis* WB800, the reaction medium used was: substrate corn starch 5% (w/V), ethanol 10% (V/V), Tris-HCl 50 buffer mmol/L (pH 8.0) 20% (V/V), CaCl2 solution 5 mmol/L 10% (V/V) and purified water q.s.p. 100% (Fenelon et al., 2015). The media were previously sterilized in an autoclave at 121 °C for 15 min.

Production of CDs in a continuous ultrafiltration system with CGTase from semipurified and purified recombinant *B. subtilis* WB800

Production was carried out continuously with 5% corn starch (w/V) substrate, in the presence of 10% ethanol (V/V), in a glass jacketed reactor coupled to a Hollow Fiber TE-0198 ultrafiltration module equipped with 50,000 NMWL exclusion threshold column. This system provided a constant separation of the CDs and other inhibitory products formed in the reaction medium and, at the same time, the retention of the recombinant CGTase, which returned to the reactor. The system was operated with a volume of 800 mL of the reaction medium and followed the parameters optimized by Matioli et al. (2001). The pH was controlled and maintained at 8.0 and the temperature at 50 °C. The concentration of semi-purified and purified recombinant CGTase was adjusted to obtain 0.1 U/mL of reaction medium (Fenelon et al., 2018). After the first 12 h of reaction, the continuous system was put into operation. Pump power was adjusted to 15%, resulting in an average flow of 4.5 mL/min, which was maintained until the drastic reduction of recombinant CGTase activity.

Production of CDs in alternative media

The production of CDs by the CGTase of *B. subtilis* WB800 was also studied using alternative media, which were evaluated to verify the efficiency of the recombinant CGTase in the production of CDs directly in the enzyme production step. The media used were: CD production media described in item 2.2.4 (medium 1A and 1B) and enzyme production media described in item 2.2.1, plus 5% corn starch substrate (medium 2A and 2B). All media were kanamycin supplements (25 μg/mL).

- **Medium 1A (with the presence of the microorganism):** 50 mL of the pre-inoculum **without centrifugation**, 5% corn starch substrate (w/V), 50 mmol/L Tris-HCl buffer (pH 8.0) 20% (V/ V), 5 mmol/L 10% CaCl2 solution (V/V) and purified water q.s.p 100%.
- **Medium 1B (only with the presence of the enzyme):** 50 mL of **centrifuged** pre-inoculum, 5% corn starch substrate (w/V), 50 mmol/L Tris-HCl buffer (pH 8.0) 20% (V/ V), 5 mmol/L 10% CaCl2 solution (V/V) and purified water q.s.p. 100%.
- **Medium 2A (with the presence of the microorganism):** 50 mL of pre-inoculum **without centrifugation**, 5% (w/v) corn starch substrate, 1.6% tryptone, 1% yeast extract, 0.5% NaCl and purified water q.s.p. 100%.
- **Meio 2B (only with the presence of the enzyme):** 50 mL of **centrifuged** pre-inoculum, 5% corn starch substrate (w/v), 1.6% tryptone, 1% yeast extract, 0.5% NaCl and purified water q.s.p. 100%.

For this evaluation, the tests were divided into two stages: 30 and 50 °C. All media were incubated in shakers at 100 rpm for 5 days. 5 mL aliquots were collected every 24 h for chromatographic analysis.

2.3 Analytical methods

Determination of enzyme activity, determination of protein concentration, and chromatographic determination of CDs

The protein concentration of CGTase from *B. subtilis* WB800 was determined by the method of Bradford (1976). Enzyme

activity was determined according to the production of β-CD, quantified in a spectrophotometer at 550 nm (Matioli et al., 1998). The concentrations of α-CD, β-CD, and γ-CD were determined by HPLC using a Waters 2695 liquid chromatograph (Milford, MA, USA) equipped with a Waters 2414 refractive index detector and a Microsorb-MV 100 NH2 column. Acetonitrile and water solution (60:40) were used as mobile phase and flow rate of 1 mL/min at room temperature. Standard solutions and samples were filtered using 0.45 μm membrane. Analytical curves were constructed for α-CD, β-CD, and γ-CD in different concentration ranges.

2.4 Statistical analysis

The assays were performed in triplicate and the results of enzymatic activity were evaluated using analysis of variance (ANOVA) at a 5% significance level.

3 Results and discussion

3.1 Enzymatic activity of CGTase from recombinant B. subtilis WB800

After 5 days of production, the enzymatic activity of the crude extract was determined, which resulted in 1.60 μmol of β-CD/min/mL. Results of activities and total protein of semipurified and purified recombinant CGTases are described in Table 1.

In the work carried out by Gimenez et al. (2019), which used the same recombinant CGTase of the present research, the value of the enzymatic activity for the purified enzyme was 157.78 μmol of β-CD/min/mL, and the specific enzymatic activity of 114.92 U/mg. Comparing the results of Gimenez et al. (2019) with the present study, it is possible to observe that the specific enzymatic activity, both for the semipurified and the purified enzyme, was significantly lower. Therefore, to verify the role of the recombinant bacterium in the production of the enzyme, new assays of activation and growth of the recombinant *B. subtilis* WB800 microorganism were carried out and the results obtained were similar to those shown in Table 1, inferring that some elements may have negatively influenced the activity or in the process of secretion of the enzyme in the production medium.

Thus, and according to Zhao et al. (2020), the plasmid pWB980, the same one used in the present study, is a promising expression vector in Bacillus due to its high copy number and high stability. However, the low rate of transformation of recombinant plasmids in wild-type cells may limit their application. Furthermore, the authors describe that plasmid stability consists of structural and segregation stability. Thus, it is hypothesized that the plasmid used for the cloning of the recombinant *B. subtilis* WB800 bacterium may have suffered some interference in its structural stability during the storage time, implying the segregation structure of the recombinant enzyme.

Table 1. Enzyme activity, total protein, and specific activity of semipurified and purified recombinant CGTase.

Fraction	Enzyme activity (μmol β-CD/min/mL)	Total Protein (mg/mL)	Specific Activity (U/mg)
Semipurified	10.40 ± 0.02	4.31 ± 0.01	2.40 ± 0.04
Purified	8.90 ± 0.01	0.34 ± 0.03	25.62 ± 0.02

3.2 Production of CDs in continuous ultrafiltration system for 120 h

The production of CDs using a continuous system associated with the ultrafiltration process was carried out from the semi-purified and purified enzymes. Continuous production was maintained for 120 h (5 days). Aliquots of the ultrafiltrate were collected every 12 hours to determine the concentration of CDs produced. The production of β-CD in the first 12 h, without ultrafiltration, was 17.16 mmol/L. After this period, the continuous process with ultrafiltration was started and, after 24 h, it was possible to observe a decrease in the concentration of CDs produced (9.83 mmol/L of β-CD). The production of CDs was approximately constant for 120 h, which was terminated with 7.14 mmol/L of β-CD (Figure 1).

The production of α-CD and γ-CD in the first 12 h was 0.78 mmol/L and 0.09 mmol/L, respectively, and decreased throughout the 120 h of the assay. With the results obtained, a greater selectivity was observed for the production of β-CD.

Figure 2 shows the production of CDs using the purified recombinant CGTase and, unlike the production from the

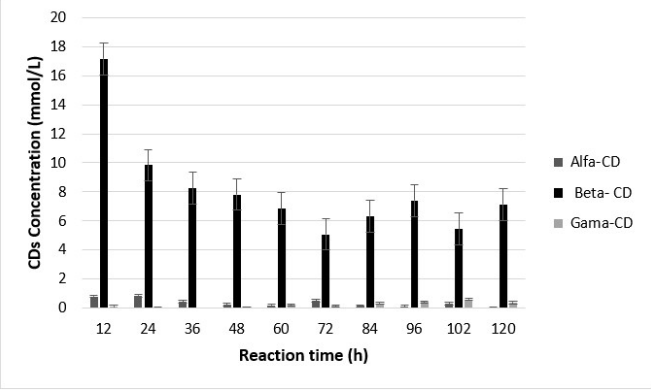

Figure 1. Production of CDs in a continuous ultrafiltration system for 120 h, using semi-purified recombinant CGTase, 5% (w/V) corn starch substrate, 10% (V/V) ethanol, pH 8.0.

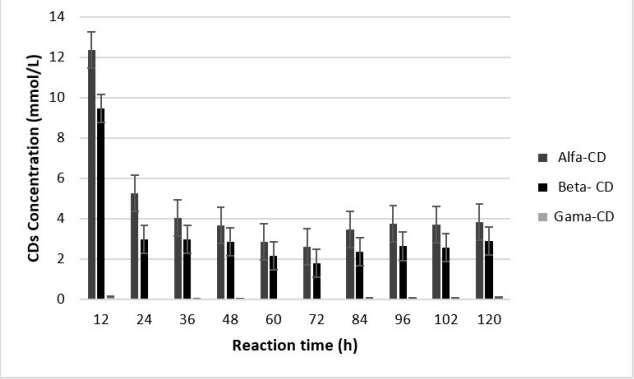

Figure 2. Production of CDs in a continuous ultrafiltration system for 120 h, using purified recombinant CGTase, 5% (w/V) corn starch substrate, 10% (V/V) ethanol, pH 8.0.

semi-purified enzyme, the 12 h batch showed more significant production of α-CD and β-CD, that is, 12.35 mmol/L and 9.46 mmol/L, respectively. Similar behavior was verified in the other batches. It is possible to suggest that the purification of the enzyme eliminates compounds that prevent or inhibit the production of α-CD by binding to the active site of the enzyme responsible for the production of this CD. A reduction in the production of total CDs of around 30% was also observed, while the expectation was the opposite. Therefore, it is possible to suggest that compounds eliminated during purification may be important to maintain or increase enzyme activity. Another viable possibility is to alter the protein structure of the enzyme during purification.

Koga et al. (2020) also used the ultrafiltration system with the commercial enzyme Toruzyme® and evaluated the production of CDs in eight batches of 72 h. The authors obtained a maximum production of α-, β- and γ-CD equal to 24.75 mmol/L, 20.59 mmol/L, and 1.66 mmol/L, in the first batch, and a production of 13.51 mmol/L of α-CD and 7.96 mmol/L of β-CD in the last batch. The Toruzyme® enzyme is marketed as an α, β-CGTase, that is, it produces similar amounts of α- and β-CD, requiring a subsequent process of separation of these CDs.

In view of the results obtained in the present study, it is possible to suggest that, even with the low enzymatic activity observed previously (item 3.1), the continuous process associated with ultrafiltration is a promising strategy for the production of CDs, since the production of α- and β-CD, although it decreased after 24 h of production, it was constant throughout the 120 h of the assay, without the need to add more enzyme to the reaction medium during the time of production of the CDs. It is also worth noting that the use of a semi-purified enzyme, in addition to resulting in a more economical process because it does not have expenses with the purification of the enzyme, produces much more β-CD in relation to other CDs, not requiring separation and purification.

3.3 Production of CDs in alternative media

A new challenge for this research was to obtain CDs directly from the production medium of the recombinant CGTase enzyme. The media used were selected based on previous studies, which showed good results in the production of CDs and growth of the recombinant CGTase enzyme (Fenelon et al., 2015; Hao et al., 2017).

Research has also shown that corn starch is one of the most efficient substrates for the production of CDs (Fenelon et al., 2015). Therefore, to analyze the behavior of the bacteria in the production of CDs simultaneously with the step of obtaining the recombinant CGTase enzyme, 5% corn starch (w/v) substrate was added to all tested media. In addition, all media were supplemented with the antibiotic kanamycin (25 μg/mL), to provide selectivity, since only the recombinant bacterium is resistant to kanamycin.

Figure 3 presents the results obtained in the first step, which used an incubation temperature equal to 30 °C, which is the ideal temperature for the growth of the recombinant CGTase enzyme.

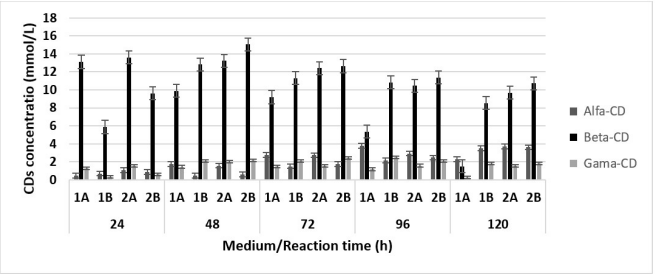

Figure 3. Chromatographic determination of CDs using CD production medium with 5% (w/v) corn starch substrate with microorganisms (1A medium) and enzyme only (1B medium), and recombinant bacteria growth medium (2xYT) with the addition of 5% of the starch substrate with microorganisms (2A medium) and only with enzyme (2B medium). All media were incubated at 30 °C, 100 rpm, for 5 days.

With the results obtained, it was verified that all the evaluated media showed considerable production of CDs, even without the enzyme going through the semi-purification and/or purification step. The 48 h time showed the highest production of CDs, especially β-CD, mostly in the 2xYT medium, regardless of the presence of the microorganism or just the enzyme (13.26 and 15.06 mmol/L of β- CD, respectively). Also, Gimenez and collaborators (2019) evaluated different means of production of recombinant CGTase and observed that the 2xYT medium was the most efficient for the growth of the bacteria and the production of the enzyme. Thus, the results obtained in this research corroborate those obtained in previous research.

Furthermore, it was possible to observe that the concentration of β-CD began to progressively decrease, especially in medium containing microorganisms (1A medium). This event may be related to the fact that the microorganism is producing other enzymes that act in the degradation of the recombinant CGTase or, still, due to the possibility of the microorganism consuming the DCs over time. Similar behavior was observed by Fenelon et al. (2018), who evaluated the strategy of producing CDs in 12 h repetitive batches with the semi-purified non-recombinant enzyme and observed a maximum production value equal to 12.6 mmol/L in the first batch and the following batches verified that the production of β-CD progressively decreased until reaching values below 50% of the initial capacity. The authors also evaluated the continuous production strategy with ultrafiltration and until 36 h the production of β-CDs remained high (15.3 mmol/L), however, the β-CD yield gradually decreased throughout the assay.

Figure 4 shows the results obtained when using an incubation temperature equal to 50 °C, which is the ideal temperature for the growth of the recombinant CGTase enzyme.

The temperature of 50 °C was more efficient for the production of CDs, especially for β-CD, which showed an average production of approximately 15 mmol/L throughout the entire assay. The production of α-CD was also more significant compared to the previous trial. The CD production medium (Medium 1) showed a slight drop in CD yield, while the 2xYT medium maintained a continuous yield. In addition, and similar to the production at 30 °C, medium 1A was the one that showed the highest production of β-CD at the initial time (24 h) and

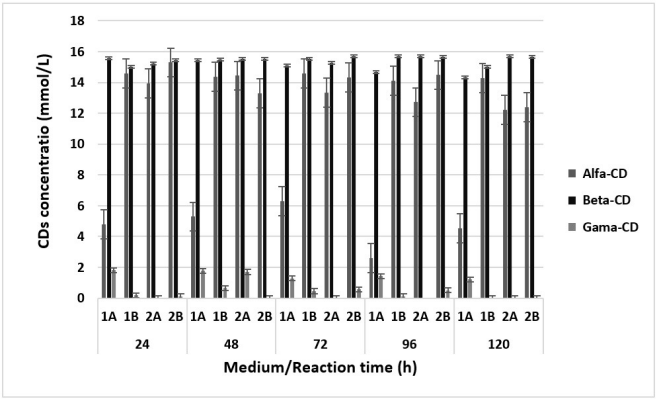

Figure 4. Chromatographic determination of CDs using CD production medium with 5% (w/v) corn starch substrate with microorganisms (1A medium) and enzyme alone (1B medium), and recombinant bacteria growth medium (2xYT) with the addition of 5% of the starch substrate with microorganisms (2A medium) and only with enzyme (2B medium). All media were incubated at 50 °C, 100 rpm, for 5 days.

the one that showed the lowest yield in the final period (120 h), which it also suggests the production of other enzymes that may be degrading the produced CDs.

Gimenez et al. (2019) evaluated optimal conditions for the production of CDs for CGTase from B. *subtilis* WB800 and compared it with the production of strain 37 of *B. firmus* and found that the catalytic properties of the recombinant CGTases were equivalent, that is, the yield of production was similar for the two lines. The authors showed a β-CD yield of approximately 13 mmol/L in 24 h of production, which is lower than that observed in the present study.

4 Conclusion

Although the recombinant B. *subtilis* WB800 CGTase enzyme showed low enzymatic activity, possibly due to interference in its structural stability during storage, it was possible to conclude that the use of the continuous production system associated with the ultrafiltration process proved to be a beneficial alternative to optimize CD production. Alternative media plus corn starch was an interesting strategy, especially for the production of β-CD, which is currently the most used and commercially available. In addition, the usage of the crude enzyme is a promising alternative, as it contributes to the reduction of costs and steps in the production of CDs and, consequently, can favor its industrial application.

Acknowledgements

The authors are grateful for the support and financial contribution of Organs Brazilian agencies CAPES, CNPq, Fundação Araucária, and Finep for the development of this study.

References

Astray, G., Mejuto, J. C., Morales, J., Rial-Otero, R., & Simal-Gándara, J. (2010). Factors controlling flavors binding constants to cyclodextrins and their applications in foods. *Food Research International*, 43(4), 1212-1218. http://dx.doi.org/10.1016/j.foodres.2010.02.017.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72(1-2), 248-254. http://dx.doi.org/10.1016/0003-2697(76)90527-3. PMid:942051.

Brewster, M. E., & Loftsson, T. (2007). Cyclodextrins as pharmaceutical solubilizers. *Advanced Drug Delivery Reviews*, 59(7), 645-666. http://dx.doi.org/10.1016/j.addr.2007.05.012. PMid:17601630.

Cid-Samamed, A., Rakmai, J., Mejuto, J. C., Simal-Gandara, J., & Astray, G. (2022). Cyclodextrins inclusion complex: preparation methods, analytical techniques, and food industry applications. *Food Chemistry*, 384, 132467. http://dx.doi.org/10.1016/j.foodchem.2022.132467. PMid:35219231.

Del Valle, E. M. M. (2004). Cyclodextrins and their uses: a review. *Process Biochemistry*, 39(9), 1033-1046. http://dx.doi.org/10.1016/S0032-9592(03)00258-9.

Fenelon, V. C., Aguiar, M. F., Miyoshi, J. H., Martinez, C. O., & Matioli, G. (2015). Ultrafiltration system for cyclodextrin production in repetitive batches by CGTase from *Bacillus firmus* strain 37. *Bioprocess and Biosystems Engineering*, 38(7), 1291-1301. http://dx.doi.org/10.1007/s00449-015-1369-8. PMid:25656697.

Fenelon, V. C., Miyoshi, J. H., Mangolim, C. S., Noce, A. S., Koga, L. N., & Matioli, G. (2018). Different strategies for cyclodextrin production: ultrafiltration systems, CGTase immobilization, and use of a complexing agent. *Carbohydrate Polymers*, 192, 19-27. http://dx.doi.org/10.1016/j.carbpol.2018.03.035. PMid:29691012.

Gimenez, G. G., Costa, H., Lima, Q. A. No., Fernandez, M. A., Ferrarotti, S. A., & Matioli, G. (2019). Sequencing, cloning, and heterologous expression. of cyclomaltodextrin glucanotransferase of *Bacillus firmus* strain 37 in *Bacillus subtilis* WB800. *Bioprocess and Biosystems Engineering*, 42(4), 621-629. http://dx.doi.org/10.1007/s00449-018-02068-4. PMid:30604010.

Hao, J. H., Huang, L.-P., Chen, A.-T., Sun, J. J., Liu, J. Z., Wang, W., & Sun, M. (2017). Identification, cloning, and expression analysis of an alpha-CGTase produced by stain Y112. *Protein Expression and Purification*, 140, 8-15. http://dx.doi.org/10.1016/j.pep.2017.07.015. PMid:28757468.

Koga, L. N., Fenelon, V. C., Miyoshi, J. H., Moriwaki, C., Wessel, K. B. B., Mangolim, C. S., & Matioli, G. (2020). Economic model for obtaining cyclodextrins from commercial CGTase. *Brazilian Journal of Pharmaceutical Sciences*, 56, 1-14. http://dx.doi.org/10.1590/s2175-97902020000118993.

Matioli, G., Zanin, G. M., & De Moraes, F. F. (2001). Characterization of cyclodextrin glycosyltransferase from *Bacillus firmus* strain no. 37. *Applied Biochemistry and Biotechnology*, 91-93(1-9), 643-654. http://dx.doi.org/10.1385/ABAB:91-93:1-9:643. PMid:11963893.

Matioli, G., Zanin, G. M., Guimarães, M. F., & Moraes, F. F. (1998). Production and purification of CGTase ofalkalophylic Bacillus isolated from Brazilian soil. *Applied Biochemistry and Biotechnology*, 70(1), 267. http://dx.doi.org/10.1007/BF02920143. PMid:18575996.

Moriwaki, C., Mazzer, C., Pazzetto, R., & Matioli, G. (2009). Production, purification, and performance improve of cyclodextrin glycosyl transferases to cyclodextrins production. *Quimica Nova*, 32(9), 2360-2366. http://dx.doi.org/10.1590/S0100-40422009000900024.

Ogunbadejo, B., & Al-Zuhair, S. (2021). MOFs as potential matrices in cyclodextrin glycosyltransferase immobilization. *Molecules (Basel, Switzerland)*, 26(3), 680. http://dx.doi.org/10.3390/molecules26030680. PMid:33525568.

Zhao, X., Xu, J., Tan, M., Zhen, J., Shu, W., Yang, S., Ma, Y., Zheng, H., & Song, H. (2020). High copy number and highly stable Escherichia coli–Bacillus subtilis shuttle plasmids based on pWB980. *Microbial Cell Factories*, 19(1), 25. http://dx.doi.org/10.1186/s12934-020-1296-5. PMid:32028973.