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PRODUCTION OF THE ENZYME CYCLODEXTRIN GLYCOSYLTRANSFERASE USING DIFFERENT FERMENTATION TECHNIQUES

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Cyclodextrins produced by cyclodextrin glycosyltransferase (CGTase) are widely used in the pharmaceutical industry to improve the solubility of drug substances as well as protect them against oxidation. The use of this enzyme in the cosmetics industry is also significant. CGTase is an enzyme that belongs to the α-amylase family, which is part of the group of non-Leloir glycosyltransferases. Enzyme-catalysed transglycosylation reactions may involve cyclization, coupling and disproportionation processes. The enzyme CGTase is mostly used to produce cyclodextrins (CDs). CGTase can produce α-, β- and γ-CDs during transglycosylation reactions, depending on the number of glucopyranose units involved (6, 7 or 8). The enzyme CGTase can also be used for enzymatic bioconversion, e.g., in the development of alternative sweeteners, where the bitter aftertaste of the product is reduced during the enzymatic bioconversion of steviol glycosides, thereby obtaining an even sweeter and more advantageous material. In our research, the enzyme CGTase was produced using different fermentation techniques to compare the activity and amount of CGTase produced by each process and optimize the subsequently planned scale-up. In our studies, the strain DSM 13 of *Bacillus licheniformi*s was used, which produced CGTase extracellularly. During the experiments the batch, fed-batch and semi-continuous fermentation techniques were compared in terms of enzymatic production. All cultivation processes were carried out in a desktop lab scale fermenter.

Keywords: Cyclodextrin glycosyltransferase, fermentation, cyclodextrins, *Bacillus licheniformis*

1. Introduction

Cyclodextrin glycosyltransferase (CGTase, EC 2.4.1.19) is a starch-degrading enzyme, which is a member of the α-amylase family. The formal name of CGTase is [1,4-α-D-glucan 4-α-D-(1,4-α-glucano)-transferase(cyclizing). Kuriki et al. [1] reported that CGTase has the same four highly conserved regions as the α-amylases. CGTase catalyses four kinds of transglycosylation reactions (Fig. 1): cyclization, coupling, disproportionation and hydrolysis. These reactions are all transglycosylations, in which cyclization is intramolecular, coupling and disproportionation are intermolecular, and hydrolysis is the conversion of sugar to H_2O [2]. The formation of cyclodextrin (CD) by the enzyme CGTase is an intermolecular transglycosylation reaction [3].

Many microorganisms are capable of producing CGTase, e.g., *Bacillus macerans* [4, 5], *Bacillus amyloliquefaciens* [6], *Bacillus clarkii* [7], *Bacillus megaterium* [8], *Bacillus subtilis* [9], *Bacillus licheniformis* [10, 11], *Bacillus firmus* [12,13], *Bacillus circulans* [14,15], *Bacillus ohbensis* [16, 17], *Geobacillus stearothermophilus* [18], *Thermoanaerobacter* sp. [19], *Klebsiella pneumoniae*, and *Klebsiella oxytoca* [20].

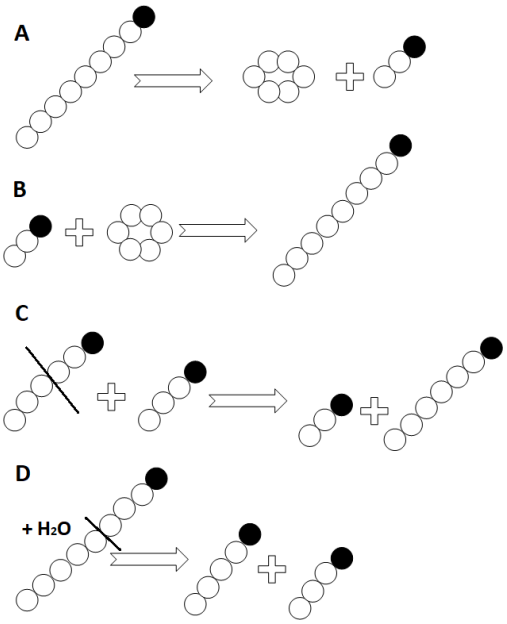

Figure 1: CGTase-catalysed reactions: A: cyclization, B: coupling, C: disproportionation, D: hydrolysis

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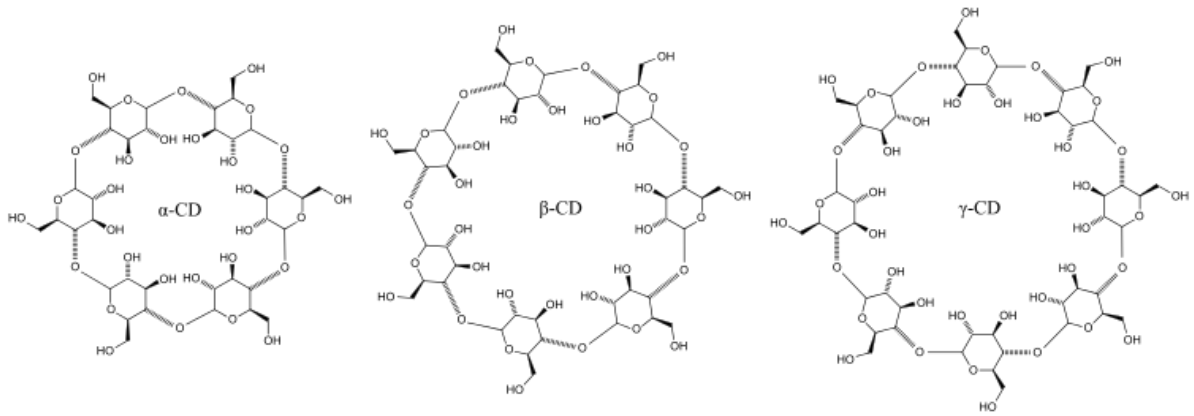

Figure 2: General structure of cyclodextrins (n: number of glucopyranose units, $n = 6$ α-CD, $n = 7$ β-CD, $n = 8$ γ-CD) (Figure adapted from: https://commons.wikimedia.org/wiki/File:Cyclodextrin.svg CC BY-SA 3.0) (08.07.2019))

The molecular weight of CGTases may vary from 60 to 110 kDa, typically its proteins have a mass of 75 kDa [21]. The most important demand of metal ions for them is Ca^{2+}, which protects the protein against heat denaturation. Most CGTases are strongly inhibited by Zn^{2+}, Cu^{2+} and Fe^{2+} [22].

Cyclodextrins, produced from starch or its derivatives via enzymatic conversion, proceed through an intramolecular transglycosylation reaction using CGTases and to a lesser extent α-amylases [3]. They are cyclic oligosaccharides composed of α-1,4-glycosidic-linked glucosyl residues [23]. Three different types of cyclodextrins exist and are characterised according to the number of glucosyl residues in the molecule: α-, β- and γ-cyclodextrins consist of 6, 7 and 8 glucose units, respectively (Fig. 2). Cyclodextrins are cyclic molecules with a hydrophilic exterior and a hydrophobic cavity that enables them to form specific inclusion complexes with small hydrophobic molecules [24]. Cyclodextrins are chiral non-reducing oligosaccharides. Glucose is the decomposition product of all cyclodextrins in acidic solutions.

The rate of hydrolysis follows the order of $\gamma > \beta > \alpha$. Under acidic conditions, cyclodextrins are more slowly hydrolyzed than maltooligosaccharides. The glycosidic bonds in the cyclodextrins can be hydrolyzed by α-amylase, but β-amylase is unable to perform this hydrolysis. The rate of enzymatic hydrolysis is the fastest for γ-CD, followed by β- then α-CD. All CDs are very stable and soluble in alkaline solutions at high pH. CDs are more resistant to acid or alkaline degradation than starch. CDs do not even degradate at temperatures as high as that of caramelization ($> 200\,^{\circ}C$, sterilization) under both dry or aqueous conditions of between pH 2 and 12. They are also stable up to $250\,^{\circ}C$ under an inert atmosphere of, for example, nitrogen [20, 25, 26].

The widespread use of cyclodextrins is due to their specific structure. Since each guest molecule is uniquely surrounded by the CD (or one of its derivatives), it is microencapsulated from a molecular microscopic point of view. This can result in beneficial changes to the chemical and physical properties of guest molecules, e.g., light- or oxygen-sensitive materials can be stabilized; very volatile substances fixed; the chemical reactivity of molecules modified; the solubility of materials improved; changes between phases achieved from liquid substances into powders; degradation of microorganisms avoided; bad smells and tastes masked; and pigments or colors of materials coated. As a result of these characteristics, CDs (and their derivatives) can be used in analytical chemistry, agriculture, the pharmaceutical as well as food industries and other masking areas. CGTase can be used for the transglycosylation of stevioside to rebaudioside through which the edulcorant quality can also be improved by increasing the substitution of steviol glycoside with the help of cornstarch hydrolyzate and CGTase [27–30].

2. Materials and Methods

2.1 Cultivation of the bacteria

The applied bacterial strain was *Bacillus licheniformis* B.01470 (DSM 13) purchased from the National Collection of Agricultural and Industrial Microorganisms in Hungary. *Bacillus licheniformis* is a Gram-positive, rod-shaped, endospore forming, facultatively anaerobic bacteria. Nutrient agar was used to maintain the bacterium in Petri dishes [31]. In our research, three types of fermentation techniques for the production of CGTase were compared: batch, fed-batch, and semi-continuous fermentation techniques. All cultivation processes were carried out in a benchtop lab scale fermenter (Fig. 3) (Biostat Q, B. Braun Biotech International, Germany). In the fed-batch fermentation, after 24 hours 15 v/v % of the medium was fed into the bioreactor. During the semi-continuous fermentation at the end of each cycle, 80 % of the broth was replaced by fresh media.

For the experiments in the bioreactor, Horikoshi II medium was used for the cultivation of bacteria containing 1.0 % soluble starch, 0.5 % peptone, 0.5 % yeast extract, 0.1 % K_2HPO_4, 0.02 % $MgSO_4 \cdot 7\,H_2O$, and 1.0 % Na_2CO_3 (all concentrations are given in w/v in distilled water) [32].

Figure 3: The bioreactor used in the experiments

2.2 The modelling of microbial growth

In order to monitor the growth of bacterial cells, samples were taken during fermentations and the optical density (OD) measured at 600 nm.

Microbial growth is described by

$$\mu_x = \frac{1}{x}\frac{\mathrm{d}x}{\mathrm{d}t}, \tag{1}$$

where μ_x is the specific growth rate of the microbe. It was evaluated through fitting the generalized logistic function

$$Z = \frac{Z_{\max}}{1 + \exp(a + bt + ct^2 + dt^3)} \tag{2}$$

to the measured cell dry weight (CDW) values (calculated from at OD600). To fit the curve, SigmaPlot Version 12.0 software was applied. If the coefficient of determination (R^2) was not high enough, the last two members of the generalized logistic function were omitted resulting in

$$Z = \frac{Z_{\max}}{1 + \exp(a + bt)}, \tag{3}$$

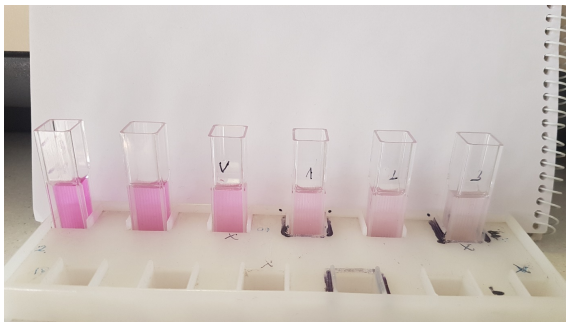

Figure 4: Colorimetric analysis of CGTase activity

which also corresponds to the modified Monod model. The derivative of the fitted function is

$$\frac{\mathrm{d}Z}{\mathrm{d}t} = -Z\left(1 - \frac{Z}{Z_{\max}}\right)\frac{\mathrm{d}H}{\mathrm{d}t} \tag{4}$$

where

$$\frac{\mathrm{d}H}{\mathrm{d}t} = b + 2ct + 3dt^2 \tag{5}$$

is the derivative of the internal function. The auxiliary variable Z in Eq. 2, 3, and 4 was x (biomass in g/L), S (substrate in g/L), and P_i (product in g/L), respectively, while $Z_{\max}$ was $x_{\max}$, S_0 and $P_{i,\max}$, respectively. If the fit was successful, then, by using determined constants of the model, the velocities and specific growth rates could be calculated by derivation from Eq. 4.

2.3 Measurement of CGTase activity

During the fermentations, samples were regularly extracted into Eppendorf tubes, which were centrifuged at 12,000 rpm for 6 minutes, then the cell-free supernatants were used to determine the enzyme activity.

The measurement of extracellular CGTase activity was adapted from the method of Kaneko et al. [33] with slight modifications (with a reduced concentration of phenolphtalein). The colorimetric reaction (Fig. 4) was measured by a spectrophotometer at 550 nm.

The experiments were conducted in 15 ml centrifuge tubes in a water bath at 40 °C. First, 4.5 ml 50 mM Tris-HCl buffer (pH = 9) was added containing a 1 % (w/v) water-soluble starch suspension, then 0.5 ml of cell-free supernatant containing the extracellular CGTase enzyme was introduced and homogenized thoroughly with a vortex mixer.

Then four 0.5 ml samples were taken from each tube which were boiled for 5 minutes to inactivate the enzyme. The boiled samples were transferred into 2 ml cuvettes that contained a staining solution (1.2 ml 0.06 mM phenolphthalein in 0.5 M Na_2CO_3 solution). Four absorbances at 550 nm of a given assay were plotted against time and the gradient (mmol/min) converted into enzyme activity with the help of a molar extinction coefficient ($32,263\ \mathrm{M^{-1}cm^{-1}}$) resulting in the CGTase activity in unit/ml supernatant.

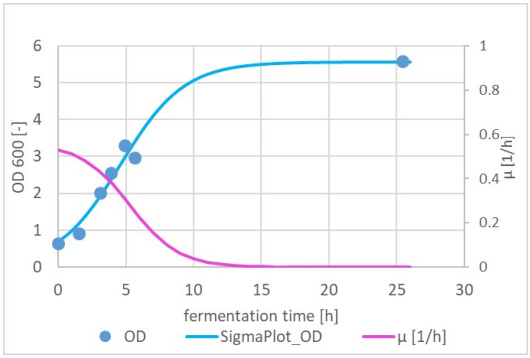

Figure 5: Microbial growth during the batch fermentation

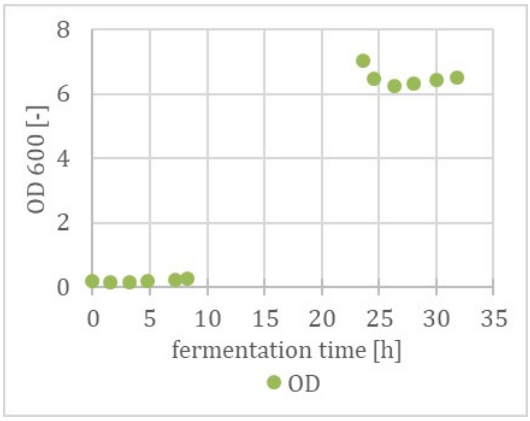

Figure 6: Microbial growth during the fed-batch fermentation

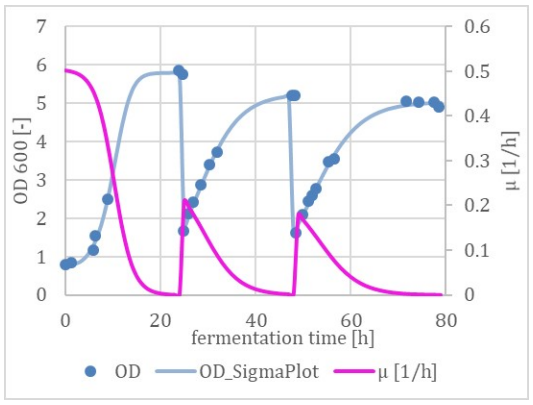

Figure 7: Optical density and specific growth rate changes during the semi-continuous fermentation

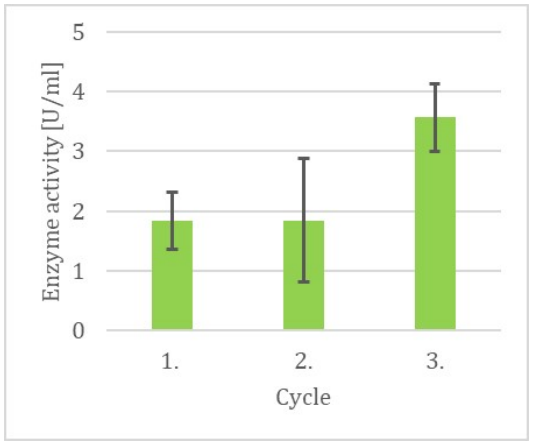

Figure 8: CGTase activities at the end of each cycle

3. Results and Discussion

3.1 Batch fermentation

Fig. 5 shows the microbial growth during the batch fermentation and also represents the changes in the specific growth rate. The maximum value of the specific growth rate was 0.53 1/h. At the end of the fermentation, the final activity of CGTase was 0.3 U/ml and the productivity was 11.8 mU/(ml h).

3.2 Fed-batch fermentation

Fig. 6 represents microbial growth during the fed-batch fermentation. Unfortunately, due to poorly scheduled sampling, it was not possible to adjust the generalized logistic equation, therefore, it was impossible to calculate the specific growth rate. A fresh medium of 15 % was injected after 24 hours. The final enzyme activity was 0.5 U/ml and the enzyme productivity was 12.3 mU/(ml h).

3.3 Semi-continuous fermentation

The semi-continuous fermentation is shown in Fig. 7, which consisted of 3 cycles. The highest value of the maximum growth rate was during the first cycle (0.5 1/h). As the fermentation progressed, the maximum specific growth rate decreased.

Fig. 8 shows that the enzyme activity of CGTase increased as the fermentation progressed.

3.4 Comparison of the different fermentation techniques

Table 1 summarizes the enzyme activities and productivities achieved by each fermentation technique. The maximum specific growth rates reached in the batch and semi-continuous fermentations were approximately the same, which is characteristic of when the microorganism can multiply.

The enzyme activities at the end of the fermentations

Table 1: Comparison between the results of the different fermentation techniques

Type	μ_{max} [1/h]	Final enzyme activity [U/ml]	Productivity [mU/(ml h)]
Batch	0.53	0.3	11.8
Fed-batch	n.d.	0.5	12.3
Semi-continuous	0.50	2.4	29.95 ± 0.3

rose as the complexity of the fermentation technique increased. While the fed-batch fermentation elongated the declining phase of the microbial growth cycle, in the semi-continuous fermentation technique an attempt was made to operate in the exponential growth phase. It is assumed that this difference caused the higher activity and productivity in the case of the semi-continuous fermentation.

4. Conclusion

In our experiments, the effect of the fermentation technique on the activity of the produced enzyme CGTase was investigated. There was no significant difference between the activities of the produced CGTase and productivities of the systems when the batch and fed-batch fermentations were compared. In contrast, bacteria produced a much more active enzyme during the semi-continuous fermentation, moreover, the productivity of this system was also significantly higher than that of the other two fermentation techniques.

From the results, it can be assumed that the microbes produce the enzyme during the exponential growth phase, since no significant difference was observed between the batch and fed-batch fermentations. Meanwhile, a repeated exponential growth phase resulted in a much higher activity and productivity. This suggests that CGTase production follows growth associated-type product formation.

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