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Biosynthesis of CGTase by immobilized alkalophilic bacilli and crystallization of beta-cyclodextrin: Effective techniques to investigate cell immobilization and the production of cyclodextrins

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ABSTRACT

Cyclodextrin glycosyltransferase (CGTase) catalyses the degradation of starch, producing α-, β-, and γ-cyclodextrins (CDs). Immobilized cells offer several advantages, such as their prolonged and repeated use, ease separation from the fermentation medium, and reduced risk of contamination. The biosynthesis of CGTase and β-CD was optimized by immobilization of *Bacillus firmus* strain 37 and *Bacillus sphaericus* strain 41 cells on a loofa sponge. Both microorganisms produced significant levels of CGTase for three consecutive cycles (94.2 U/mL on average), increased the relative formation of the β-CD, and inhibited the formation of other CDs. The crystallization of the CD produced by both bacterial strains, when corn starch was used as the substrate, resulted in a 64% recovery of β-CD. The purity of the β-CD was 89.5% when maltodextrin was used as the substrate for *B. firmus* strain 37. The Tilden–Hudson technique was used as a simple, inexpensive, and efficient method for monitoring the continuous production of CDs using immobilized cells. The innovative use of the Fourier Transform Infrared Spectroscopy technique by means of the Attenuated Total Reflectance method suggested that the interaction between the *B. sphaericus* strain 41 cells and the loofa sponge occurred by natural adsorption.

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1. Introduction

The microbial enzymes are generally unstable when isolated from their natural environment and also are easily denatured under working conditions. The direct immobilization of microbial cells that has a particular activity can minimize or even eliminate these problems. Immobilized viable cell systems have advantages over traditional batch fermentations, including improved biological stability, the ability to separate the cell mass from the bulk liquid for possible reuse, continuous operation at high dilution rates without culture washout, enhanced product yields, the capacity to recharge the system by inducing the growth of resting cells, and accelerated reaction rates because of increased cell density [1].

The methods commonly used for immobilizing cells are adsorption, covalent binding, entrapment in gels, and crosslinking using various types of support [2]. The immobilization method by passive adherence to surfaces is simple and has great potential for industrial application. The production costs have a modest increase if the used matrix is inexpensive, as the loofa sponge (*Luffa cylindrica*) [3]. This matrix is obtained from plants grown in many tropical and subtropical countries, it is biologically renewable, readily available and economically viable [4].

Cyclodextrin glycosyltransferase (CGTase), an enzyme found in bacteria, is generally extracellular produced and converts starch and related substrates into cyclodextrins (CDs) [5,6]. Previous studies have investigated the immobilization of microbial cells on a loofa sponge for the direct production of CDs; however, the production of CGTase has not been evaluated [7–9].

The CDs are formed by glucose units linked by α-(1,4) bonds. The most common CDs are α-CD, β-CD and γ-CD, with six, seven and eight glucose units, respectively [10]. The CDs have the shape of a truncated cone with interior cavity hydrophobic that allows the formation of inclusion complexes with hydrophobic guest substance, whereas the outer surface is hydrophilic, which makes the CDs soluble in water. This occurs due to the steric arrangement of glucose units [11]. The formation of inclusion complex can lead to changes in physical and chemical properties of the guest molecule [12]. Due to these features and properties, the CDs have extensive applications in different areas, such as pharmacy, food, cosmetics, textile industries, agriculture, environmental protection, analytical chemistry and biotechnology [12,13].

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The industrial production of CDs has been made by enzymatic reaction focused to produce a unique type of CD, using an organic complexing agent. There is also a solvent-free process, which produces a mixture of CDs that can be subsequently separated [11,14].

In water, the α-CD and γ-CD exhibit good solubility, while β-CD has the opposite behavior. Therefore, the separation of β-CD from reaction medium is not difficult due to its easy precipitation. Some techniques have been used to purify CDs, such as ion exchange chromatography and affinity chromatography, adsorption to synthetic polymers, molecular sieves and the use of membrane for filtration [12]. A well known technique for the industrial purification of materials is the crystallization method, which in essence, is the precipitation of solid crystals from a solution [15]. As will be explained latter, this was the method used in this work.

The process employed for the CDs production can be divided in four stages, as follows: (a) producing CGTase in the reaction medium from the cultivation of the microorganism, (b) obtaining the enzyme through their separation, concentration and purification; (c) application of the enzyme in the conversion of pre-hydrolyzed starch into cyclic and acyclic dextrin, and (d) separation, purification and crystallization of CDs [16]. In this study, the alkalophilic gram-positives microorganisms *Bacillus firmus* strain 37 and *Bacillus sphaericus* strain 41 were immobilized on a loofa sponge and were used as the direct source for the CGTase and CD production; therefore, two phases of the CD production process were eliminated: the cultivation of the microorganisms and the separation and purification of the enzyme from the medium. Using the crystallization process, the β-CD produced by the immobilized cells was purified without the addition of organic solvent.

The increase in the industrial application of CDs has promoted the use of inventive techniques for their production. In 1942, Tilden and Hudson [17] developed a fast and easy method to determine the activity of CGTase; however, the process involves many variables because it depends on the microscope and the human eye to determine the end point of reaction. There is a lack of reports in the literature evaluating the Tilden–Hudson technique for CD production. In addition, the use of Fourier Transform Infrared Spectroscopy (FTIR) technique by means of the Attenuated Total Reflectance (ATR) method is innovative for this study. It is well known that infrared techniques are powerful tools for physical chemical evaluation of the samples because they allow the detection of the presence and/or modifications of molecular functional groups optical bands that are related to the material microstructure [18]. Therefore, in this study, the Tilden–Hudson technique was used to monitor the β-CD production, and the FTIR-ATR method to investigate the interaction between the microbial cells and the loofa sponge fibers.

2. Materials and methods

2.1. General

The α-, β-, and γ-CDs and the phenolphthalein were purchased from Sigma (St. Louis, MO, USA). The soluble potato starch (article 101252) was purchased from Merck (Darmstadt, Germany). The maltodextrin (dextrin 10 from maize starch, article 31410) was obtained from Fluka (Buchs, Switzerland). The loofa sponges were purchased in the local market (Maringá, PR, Brazil). The other chemicals used were of analytical grade.

The bacterial suspensions were freeze-dried in a Christ Beta 1–16 freeze dryer. A Tecnal TE-424 orbital shaker was used to reactivate the cells for the immobilization procedures and for the enzyme and CD production. The crystallization stage was performed using a Tecnal TE-211 rotary evaporator to concentrate the β-CD solution and a Jouan GR2022 refrigerated centrifuge to collect the precipitate. For colorimetric quantification of the β-CD, a Tecnal SP 1105 spectrophotometer was used. The concentrations of the α-, β-, and γ-CDs were measured using a Varian ProStar 240 High Pressure Liquid Chromatographer (HPLC) equipped with an IR-CG 410 refractive index (RI) detector and an SGE aminopropylsilane column with particle size of 5 μm, a length of 25 cm and a 4.6-mm internal diameter. During the β-CD production cycles, an optical microscope (Leica DM 500) equipped with a digital camera (ICC 50) and image-capture system was used to visualize the CD–iodine complex crystals. To evaluate the interaction of the bacterial cells with the loofa sponge fibers, a FTIR-ATR Varian model 7000 unit was used. The spectra were recorded by averaging 128 scans at a resolution of 4 cm^{-1} in the spectral range between 4000 and 400 cm^{-1}.

2.2. Culture conditions and microorganism reactivation

In this research were used *B. firmus* strain 37, isolated from cassava culture, and *B. sphaericus* strain 41, isolated from soybean-soil culture. This choice was made because the CGTase enzymes produced by these microorganisms have been characterized and studied before [19,20].

The solid medium used for cultivating the microorganisms consisted of the following substances (w/v): 1.0% soluble starch; 0.5% polypeptone; 0.5% yeast extract; 0.1% K_2HPO_4; 0.02% $MgSO_4 \cdot 7H_2O$; 0.01% Congo red stain; 1.0% Na_2CO_3; and 1.5% agar [19,20]. Microbial cells were previously lyophilized and stored in freezer for subsequent use in the immobilization process. The reactivation of 30 mg of lyophilized cells occurred in 250-mL Erlenmeyer flasks containing 100 mL of liquid culture medium of same constitution of solid culture medium, but without the agar and stain. The reactivation process was performing in an orbital shaker (120 rpm) for 24 h at 37 °C.

2.3. Immobilization procedures

Immobilization of cells allows their continuous and repeated use, and represents the physical imprisonment of intact cells with the conservation of some desired catalytic activities [2].

The immobilization of the microorganisms by adsorption on the loofa sponge was performed in accordance with the method described by Pazzetto et al. [7]. From the dried fruit and after removing the seeds, the *L. cylindrica* loofa sponge was obtained and cut into discs of approximately 2–4 mm thick and with diameter of 23–25 mm. During 30 min, the disks were immersed in boiling water and, then, washed in water at room temperature. The discs were left for 24 h in distilled water, which was changed three times. After drying in an oven at 70 °C, the discs were sterilized in an autoclave.

The loofa-sponge discs (three in total) were added to the liquid culture medium containing the reactivated cells, and the culture was incubated at 37 °C in an orbital shaker at 120 rpm for 5 days. The discs were removed and were transferred to fresh medium for 5 days.

2.4. Enzyme and cyclodextrin production

For the production of the CGTase and the measurement of the CGTase activity, after immobilization, the matrices were washed in sterile saline and transferred to 250-mL Erlenmeyer flasks containing 100 mL of the liquid culture medium (the same liquid culture medium used for the reactivation and growth of the bacteria).

To investigate the possibility of using the immobilized cells to convert corn starch or maltodextrin into CDs without using crude or purified enzyme, after the immobilization, the matrices were washed in sterile saline and transferred into 250-mL Erlenmeyer flasks containing the reaction medium plus substrate (corn starch or maltodextrin).

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