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Research Article
Reutilization of Microbial Cells for Production of Cyclodextrin Glycosyltransferase Enzyme

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Abstract

Background and Objective: Cell immobilization methods have been developed in order to improve enzymatic production. The aim of this work was to use immobilized bacteria on enzyme production to improve cell stability and to enhance the time of fermentation. **Materials and Methods:** The variables sodium alginate, number of cells, time of gelation, aeration and silica concentration in cells immobilization for CGTase production were evaluated. Statistical analysis of enzymatic response was performed using paired t-test (0.5%). **Results:** The better conditions for maximal production of CGTase (389.94 U mL^{-1}) with immobilization of *Bacillus circulans* ATCC 21783 were: 3% sodium alginate; 0.2 g L^{-1} initial cells; 100 rpm aeration and 3% silica. There was an increase of 83.05% in enzymatic production with cell entrapment in silica. The beads cell reutilization was possible during three cycles of fermentation during 216 h. **Conclusion:** The enzymatic production with the beads cells reutilization provided an increase in productivity and low costs of fermentation processes. The immobilization of cells in silica beads maintains cell stability and allows the reuse of the system during CGTase production.

Key words: Cyclodextrin glycosyltransferase, cell immobilization, microbial reutilization, reutilization, CGTase

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Cells immobilization may increase the enzymatic productivity in the fermentation with the increased cell stability. The immobilization of high cell concentrations facilitates the recovery of bacterial products and provides the reuse of microbial cells[1]. The internal structure of beads preserves the immobilized cells and enzymatic structure produced in this natural microenvironment. The metabolic activity is kept for a long time when compared to fermentation system for enzymes production[2].

The cyclodextrin glycosyltransferase (CGTase) is an enzyme produced predominantly by bacteria of the genus *Bacillus*, which catalyzes the reaction of cyclodextrins (CDs) from starch. The CDs are cyclic oligosaccharides formed by a variable number of glucose units linked by α-1,4 linkages. The α-CGTase, β-CGTase and γ-CGTase are the most common having 6, 7 and 8 glucose units, respectively.

The CDs has a hydrophobic internal surface and a hydrophilic external cavity. The truncated conical shape and the orientation of the hydrophilic groups confers physicochemical properties to the CDs. It is able to solubilize in aqueous medium and at the same time, encapsulate in their internal cavity of hydrophobic molecules[3]. The inclusion complexes solubilize and modify drugs, foods, cosmetics and others[4].

The microbial products are generally obtained by fermentation of free or immobilized cells. The immobilized cells are used for industries, such as catalysts in the fermentation process, which are advantageous when compared to conventional process. The physical confinement during *Bacillus circulans* ATCC 21783 cells immobilization may submit advantages in the production of the enzyme CGTase. The aim of the present study was to describe the behaviour of the enzymatic production from microbial immobilization, such as bacterial stability and enzymatic production.

MATERIALS AND METHODS

This study was carried out at the Laboratory of Industrial Microbiology in Bioscience Institute in Rio Claro, Brazil in, 2013.

Chemicals: All the reagents used were chemicals of commercial grade of Sigma-Aldrich, sodium alginate and calcium chloride in cell immobilization, Tris-HCl for buffer solution and phenolphthalein for enzymatic activity.

Microorganism: The strain *Bacillus circulans* ATCC 21783 was acquired from the American Type Culture Collection and is maintained in the Industrial Microbiology Laboratory of the Institute of Biosciences of Rio Claro (SP, Brazil).

Immobilization of cells

Imprisonment in sodium alginate: The cells of *Bacillus circulans* ATCC 21783 (0.1, 0.2, 0.3 and 0.4 g L^{-1}) were centrifuged with 10000 rpm for 10 min at 20°C. The cells were suspended in 20 mL of sterile distilled water and added to same volume of sodium alginate solution (1.5, 2, 3 and 6%). The mixture containing cells and sodium alginate was dripped in a solution of calcium chloride (0.2 M) for the gelation and to obtain the beads. It was kept for 0.5, 8, 18 and 24 h at 4°C in calcium chloride and washed with sterile distilled water to remove the excess of calcium ions and free cells. The beads were cultured in a rotary shaker (50, 100, 150 and 200 rpm) for the production of CGTase enzyme.

Imprisonment with use of silica were tested concentrations of silica (1, 1.5, 2 and 3%) mixed with sodium alginate (3%) and biomass and dripped in a calcium chloride solution (0.2 M). The beads were kept at 4°C and washed with sterile distilled water.

CGTase production: The *Bacillus circulans* ATCC 21783 was cultivated in 300 mL Erlenmeyer flasks containing 100 mL of nutrient medium modified[5] incubated in 150 rpm at 35°C. In the medium composition for the CGTase production, sorghum (1%) was used as carbon source. The immobilized cells were maintained in Erlenmeyers on the same conditions for the enzyme production. At the end of fermentation, samples were removed and centrifuged at 10000 rpm for 10 min. For the growth of the microorganisms and the enzyme production was utilized in all the experiments the sorghum grain as main source of carbon.

Enzymatic activity: Enzymatic Activity (EA) was performed following the method described by Makela *et al.*[6].

Statistical analysis: The values obtained from analysis of enzymatic activity were correlated with immobilization conditions and cell reuse using paired t-test (0.5%).

RESULTS

The present study described the cell immobilization of *Bacillus circulans* for the reutilization for production of CGTase. The variables of immobilization support presented interfered in the fermentation process. The mixture of silica with sodium alginate for the microorganism immobilization in order to obtain a better frame of support, allowed higher

stability with enzymatic production for several consecutive days (216 h). The efficacy of cells support was permitted the beads reutilization during three cycles of fermentation achieving increase of 83.05% of CGTase in relation the free cells with low efficiency in the enzymatic production.

Immobilization of cells from *Bacillus circulans*: Time of gelation 8 h was the best for the cellular stability during the enzyme production CGTase (39.01 U mL^{-1}) in 168 h of fermentation as shown in Fig. 1. The enzyme production process is relationated with the polymer stability of immobilization (192 h). The beads of 8 h shows better stability than 0.5 h and lower hardness than bead of 12 h, thereby providing better nutrients transport and release of the product through the matrix.

The change of the time of cellular immobilization of 0.5-8 h promotes better mass transfer of substrate in the bead and the output of the enzyme after had been produced by immobilized *Bacillus.*

Figure 2 showed the residence time of the beads in the $CaCl_2$ solution and it can change the morphology and porosity, which are associated with the nutrient exchange capacity and the movement of substance from microbial metabolism, such as CGTase.

In the Fig. 3a it is possible to observe that in the concentration of 3% of sodium alginate reached highest enzymatic production in 72 h of fermentation (66.08 U mL^{-1}). The influence of cell concentration inside of matrix during enzymatic production is observed in Fig. 3b. The highest enzymatic activity of 59.76 U mL^{-1} was reached after 144 h of fermentation and was obtained with a cell concentration of 0.20 g L^{-1}.

CGTase production: In this study the influence of the aeration in rotating agitator on the production of enzyme CGTase by immobilized cells was evaluated. This was observed in experiments with agitation of 150 and 200 rpm that had the production interrupted after 168 and 144 h of fermentation, respectively. The highest concentration of CGTase production was obtained with an aeration of 100 rpm during the fermentation process in 144 h (Fig. 4).

Beads reutilization with silica: Figure 5 showed that the enzyme production (EA) of non-immobilized cells. In Fig. 5b it is possible to observe that the silica used with sodium alginate for immobilization showed great efficiency for the production of CGTase. The silica concentration that determined the bead

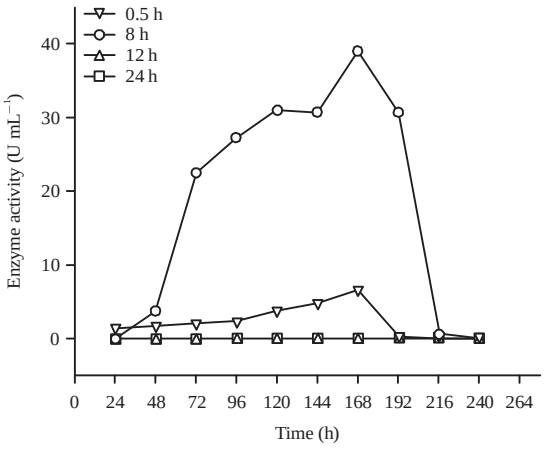

Fig. 1: EA for different gelation times with immobilized cells

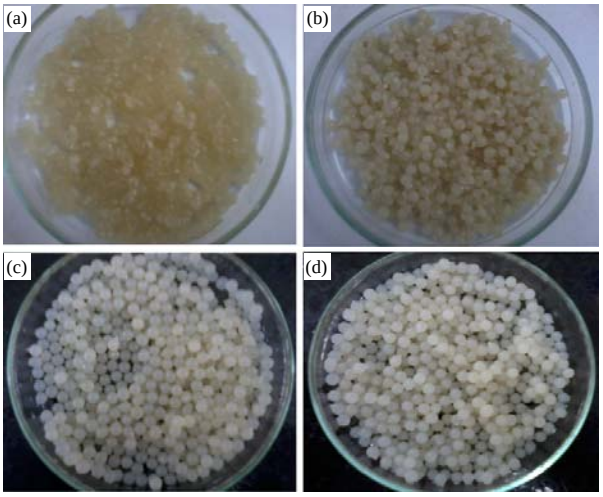

Fig. 2(a-d): Beads with immobilized *Bacillus circulans* using sodium alginate in different gelation times, (a) 0.5 h, (b) 8 h, (c) 18 h and (d) 24 h

stability for more time (216 h), reached a CGTase production of 389.94 U mL^{-1} at 120 h with 3% of silica, maintaining a higher production than 215 U mL^{-1} until 192 h. This is due to the use of higher silica concentration in the polymer matrix; increasing and facilitating the exchange of nutrients and gas, allowing an increase of 83.05% in relation to immobilization with sodium alginate only (66.08 U mL^{-1}, Fig. 5a). The beads with 3% of silica show the best results when the culture medium was replaced (Fig. 5c).

As the beads are being reutilized with the substitution of cultivation medium, the EA tends to diminish. In the first cycle, the higher EA found was of 389.94 U mL^{-1} in 120 h that was the highest enzyme activity obtained in all the process of beads reutilization. In the second cycle the EA decreased,

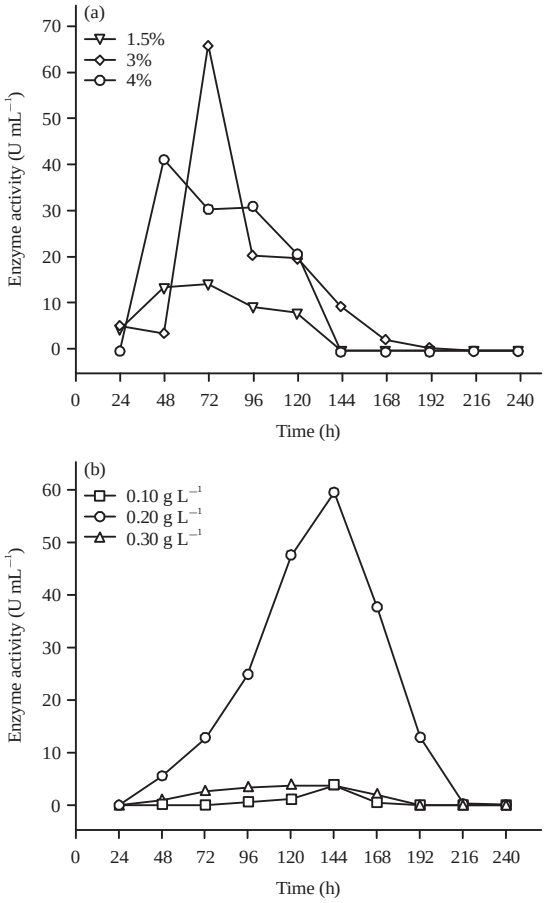

Fig. 3(a-b): Enzymatic activity in function of (a) Sodium alginate and EA and (b) Microbial concentration

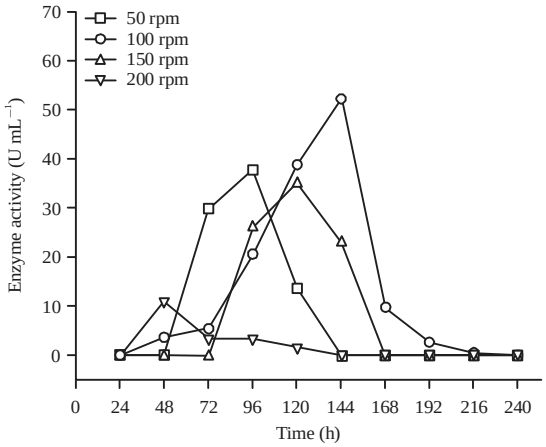

Fig. 4: Enzymatic activity of immobilized *Bacillus circulans* at aeration rates

reaching to the apex in 144 h and the enzymatic production of 17 U mL^{-1}, decreasing 52.61% in relation to first cycle. In the third cycle, there was a reduction in the EA, not surpassing 97.91 U mL^{-1} in 96 h.

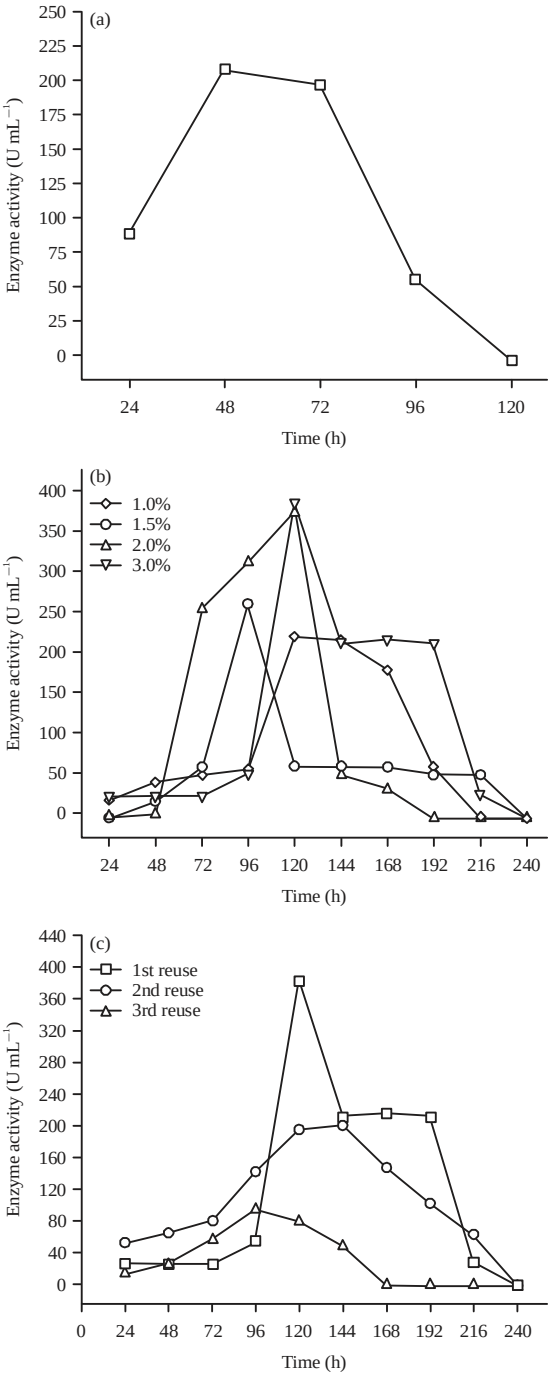

Fig. 5(a-c): Enzymatic activity of *Bacillus circulans* (a) Free cells, (b) Enzymatic activity of immobilized cells with silica concentrations and (c) Enzymatic activity of immobilized cells reutilized 1st, 2nd and 3rd reuse

DISCUSSION

In this study *Bacillus circulans* was immobilized in sodium alginate for cyclodextrin production. Wheat bran was

used as a carrier for the immobilization of *Lactobacillus strains*[7]. The *Rhodococcus* immobilization has been performed using chitosan microspheres[8]. Immobilization of *Rhizopus oryzae* was performed in fibrous matrix for acid latic production[9].

The better production of CGTase was 389.94 U mL^{-1} from immobilized cells. Immobilization of *Bacillus* strain in inorganic matrix for cyclodextrin production has been performed[10]. These microorganisms produced 94.2 U mL^{-1} of CGTase in 3 consecutive cycles[11]. An enzyme activity of 72.72% in 12 cycles was obtained using ethylenediamine and glutaraldehyde for cell immobilization[12]. Bacterial cell was immobilized in functionalized magnetic beads with an efficiency of 90%[13]. Daptomycin was produced by immobilized *Streptomyces roseosporus* onto several support matrices[14]. Probiotic yoghurts have been produced using immobilized cells of *Lactobacillus plantarum* 2035 on whey protein[15].

In this study was possible beads cell reutilization during three cycles of fermentation during 216 h.

Cost reduction in fermentation process using immobilized cells with silica confirms the efficacy of method with the increase of time of enzyme production using the same bacteria. A study performed by Zhao *et al.*[16] also improved the efficiency of lactic acid production for bacterial immobilization in 78.77% using silica and Ca-alginate gel[16]. In this work there was an increase of 83.05% in enzymatic production with cell in silica.

The ions Ca^{+2} in interior of the alginate support prevent the exit of cells and allow the growth and maintenance of immobilized cells[17]. The time of gelation of the sodium alginate is the most important aspect for realization of immobilization. It is important for the formation of the stable bead that occurs through of the diffusion reaction forming a stable structure to the production of the enzyme.

Bekers *et al.*[18] immobilized the cells of *Saccharomyces cerevisiae*, in spheres of stainless steel modified, for the production of ethanol. The authors reported that the immobilization increases the cells stability and the production of ethanol.

In this study CGTase was produced by immobilized Bacillus circulans. No effective technique was performed for production of enzymes in three cycles. In other studies the biosynthesis of CGTase was optimized by immobilization of *Bacillus firmus* and *Bacillus sphaericus* on a loofa sponge during three consecutive cycles[11]. The immobilized cells were utilized during five cycles of fermentation without loss of stability[18]. The authors report that the immobilized cells were capable of degrading all the hydrogen peroxide during 10 reutilizations without loss of efficiency[19].

The ideal concentration of cells to be immobilized depends on the type of cells and their metabolism. In cells with slow growth, there is the possibility of immobilizing a higher concentration of cells[20]. In a study conducted by Doleyres *et al.*[21] demonstrated that the highest concentration of the immobilized cells in the periphery of the bead occurs with greater diffusion of nutrients. This peripheral region can form colonies and occur output cells and their consequent rupture[20]. The growth of the cells imprisonment in the polymeric matrix occurs in the form of colonies in the small cavities being more intense in the periphery support. The force exerted by the growth and shearing resulting from the agitation causes the cell exit to pass through cavities where growth occurs[22].

Several studies were elaborate the use of organic and inorganic polymers as cell support that reacts minimizing the output of matrix cells[9]. As, strategy in improvement of sodium alginate microcapsules, were studied the silicification of the biopolymer to develop the bead stability while controls the diffusion through the membrane. Therefore, this strategy is used to optimize the properties of beads with strengthening of the microcapsules using sodium alginate[23].

However, the interactions between immobilized cell and the silica matrices may not be detrimental to biological activity. Studies are needed to evaluate the long-term toxicity of silica on microorganism response[24].

CONCLUSION

The results show that cell immobilization in calcium alginate is a promising method of bacterial stability in CGTase production. The conditions for maximal production of CGTase (389.94 U mL^{-1}) using immobilized cell were optimized. The use of silica increased 83.05% the enzymatic production from immobilized *Bacillus circulans*. The beads cell reutilization in fermentation process during three cycles of fermentation was performed during 216 h. The improve of CGTase production using beads cells reutilization may provide low costs of fermentation process.

SIGNIFICANCE STATEMENTS

Microbial enzymes present advantages as production in large scale and disadvantages as low yield due to microbial death. This study proposed the immobilization of *Bacillus circulans* for enzyme production.

This study discovers the possibility of reusing CGTase-producing bacteria in culture medium using sorghum as the

main source of carbon that can be beneficial for enzymatic production. This study will help the researcher to uncover the critical areas of reuse of microbial cells in fermentation processes, which many researchers were not able to explore. Thus a new theory on reuse of cell for the production of CGTase may be arrived at immobilized *Bacillus circulans* with sodium alginate, calcium chloride and silica.

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