BIOMEDICAL ENGINEERING | RESEARCH ARTICLE

Cell immobilization of *Streptomyces griseobrunneus* by microcrystalline cellulose for production of cyclodextrin glucanotransferase enzyme

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Abstract: The cyclodextrin glucanotransferase are extracellular enzymes that can hydrolysis starch as a substrate to cyclodextrin. Encapsulation and immobilization technique of microbial cells within the polymeric network have widespread applications in the production of enzyme. Bacterial cells were isolated from soil by serial dilution method. The molecular identity was established using 16S rDNA sequence analysis, and the identified strain was immobilized in the synthesized composites. Physicochemical characteristics of the synthesized composites were determined by using field emission scanning electron microscopy, Malvern Zetasizer Nano ZS and FT-IR analyses. Enzyme activity was measured by phenolphthalein assay. *Streptomyces griseobrunneus* was identified according to the 16S rDNA sequence. FT-IR analyses indicated that there was an interaction between all of the elements. Scanning electron microscopy analysis showed a cross-section of composites without cells and cells attached to composites. Enzyme activity was 1109, 948 and 606 U/ml in alginate/chitosan/microcrystalline cellulose (MCC) sheets, agar/gelatin/MCC beads and free cells, respectively. It could be concluded that the polymeric immobilization...
was effective in improving enzyme production because of its excellent properties such as guarding the bacterial cells against inhibitors and toxins.

**Subjects:** Environment & Agriculture; Bioscience; Food Science & Technology

**Keywords:** Streptomyces; cyclodextrin glucanotransferase; bacterial immobilization; microbial enzyme; polymers

1. Introduction

According to the previous studies, many bacteria use starch as a carbon and energy source for growth. To utilize carbon and energy source, they produce a range of extracellular enzymes to convert the large molecules to utilisable small molecules. A large number of starch-hydrolyzing enzymes have been identified and characterized, including α-amylase, glucoamylase and cyclodextrin glucanotransferase (CGTase), among others (Qi & Zimmermann, 2005). Cyclodextrin glucanotransferase (CGTase EC.2.1.) is an extracellular enzyme that can catalyze several reactions such as coupling, cyclizing, disproportionation and hydrolysis functions that were used to produce cyclodextrin (CDs) from starch (Es et al., 2016). CGTase is a member of the α-amylase superfamily, which catalyzes the cleavage of the glycoside bond between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety. CGTase enzymes play a key role in the production of three types of cyclodextrin that are different in their degree of polymerization: α, β, and γ (Costa et al., 2015). CDs play a significant role in industrial processes in pharmaceutical, food and agricultural fields (Ibrahim et al., 2014). To further improve the pharmaceutical features of native CDs, chemically modified CDs have been synthesized (Darini et al., 2019). Microorganisms such as Bacillus sphaericus (Costa et al., 2012), B. stearothermophilus (Moriwaki et al., 2009) and Klebsiella sp. (Gawande & Patkar, 1999) are the only source for producing CGTase. Encapsulation and immobilization of microbial cells within the polymeric network have widespread applications in the production of enzyme and alcohol (Cheng et al., 2014; Nagashima et al., 2011). Bacteria immobilization improved the enzyme production which can further minimize the cost of downstreaming processing (Sharma et al., 2020). Another study showed that the recovered enzymatic activity was increased after immobilization of the bacterial cells (Vasconcelos et al., 2020). In addition, cell immobilization technology provides a promising solution, which not only allows the cell system to be used multiple times but also relieves feedback inhibition toward cells caused by metabolic products (Lu et al., 2020). Natural polymeric materials for cell immobilization are chitosan, agar, alginate, cellulose and gelatin, and the synthetic polymeric materials include polyethylene glycol (PEG), polyacrylamide, polyvinylpyrrolidone (PVP), etc. (Shrinivas et al., 2012). Natural macromolecules show the useful properties such as less toxicity and biocompatibility, which can be utilized for cell immobilization (Baysal et al., 2013; Datta et al., 2013). Gelatin is an amphoteric protein exhibiting high viscosity in water and synergistic effects with anionic polysaccharides (Doublier et al., 2000). Hydrogels have been lyophilized in order to afford a porous scaffold as well as enhance cell proliferation (Kirdponpattara & Phisalaphong, 2013). Calcium alginate–starch hybrid co-polymer was suitable for surface immobilization (Matto & Husain, 2009). Chitosan was used for cell immobilization due to high porosity, polycationic and water solubility at pH<6 (Khondee et al., 2015). A polymeric material is permeable to oxygen and nutrients to entrap microbial cells as well as increase protection against damaging agents such as hydrogen peroxide, acidic conditions, temperature changes and osmotic stress (Ding & Shah, 2009; John et al., 2011). Immobilization technique has been extensively preferred to genetic engineering on laboratory scale and industry, due to its low-cost simplicity and unsophisticated method (Zhang et al., 2009). The main aim of this study was to entrap S. griseobrunneus strain FSHH12 in alginate/chitosan/microcrystalline cellulose and agar/gelatin/microcrystalline cellulose composite to produce a higher and more efficient CGTase enzyme.
2. Materials and methods

2.1. Materials
Phenolpthalein, chitosan (CS), β-cyclodextrin (β-CD), tripolyphosphate (TPP), Cetyl trimethylammonium bromide (CTAB), alginate (ALG), microcrystalline cellulose (MCC), gelatin (GE), agar (AG), Rhodamin B (Rh B), Rhodamin 123 (Rh 123) and propidium iodide (PI) were purchased from Sigma (Sigma–Aldrich, USA). Nutrient broth (NB), nutrient agar (NA), starch and calcium chloride were obtained from Merck Chemicals (Darmstadt, Germany). All other materials used in this study were obtained from domestic providers in analytical grade.

2.2. Isolation and identification of the bacteria
Actinomycetes bacteria were isolated from soil samples collected from different areas in Kerman, Iran, according to the method described by Ameri et al. (2016). The enzyme activity was determined by phenolphthalein at 30°C according to Bergey’s Manual of Determinative Bacteriology (Bright & Bulgheresi, 2010). Then, 16S rDNA gene sequence was done for more emphasis. Finally, phylogenetic tree was performed by MEGA4 software according to the neighbor-joining method (Ohadi et al., 2017).

2.3. Agar/gelatin/microcrystalline cellulose sheets (AG/GE/MCC)
Briefly, agar powder was dissolved in deionized water and gelatin (1 g) was added into 100 ml of boiling distilled water (100°C) and stirred up to room temperature until a homogenous solution was obtained. The microcrystalline cellulose sheet (3 × 1 × 1 mm) was blended and mixed with the agar/ gelatin solutions in a weight ratio of 30:50:20 to prepare the cross-linked hydrogel. Then, the hydrogel was sterilized (15 min at 121°C) and 2 ml of bacteria suspension was added to the sterilized hydrogel. The homogeneous slurry mixture was poured into a tray plate (15 ml) and let it dry under sterilized conditions and freeze-dried. The dried sheet was cut into rectangular shapes of (2.0 × 2.0 × 0.2 cm) and seeded with 50 ml of the sterile NB medium in a 250-ml Erlenmeyer flask overnight (150 rpm and 30°C).

2.4. Alginate/chitosan/microcrystalline cellulose beads (ALG/CS/MCC)
Chitosan solution (w/v) in acetic acid 1% was maintained under vigorous stirring for 24 h, and then TPP (0.25 mg/ml) solution was added by dropping to the mixture under magnetic stirring at room temperature (Eslaminejad et al., 2016). 1% (w/v) microcrystal cellulose was solved in CTAB (2 mM). Chitosan nanoparticles and microcrystal cellulose solutions were mixed thoroughly with 15 ml of sterile sodium alginate solution 2% (w/v). Mixture was added drop wise to sterile CaCl₂ (50 ml, 0.1 M) under stirrer conditions to make beads. After stirring for 3 h at 4°C, the ALG/CS/MCC beads were filtered and then washed with deionized (DI) water to remove CaCl₂ and free cells. Then, 0.02 g (dry weight) of bacteria cells was suspended in 5 ml NB media containing 5 μl Rh 123 (5 μg/ml) and 5 μl PI (5 μg/ml) and then the bacterial suspension was seeded in NB supplemented with starch 1% and incubated for 72 h. Stained bacteria cells were examined by a fluorescence microscope (Nikon DQCFS, NIS-Element, Japan) at 540 nm and 448 nm.

2.5. Physicochemical characteristics of the synthesized composites
AG/GE/MCC sheets and ALG/CS/MCC beads were characterized by FT-IR (Bruker, Germany) as KBr discs (3500–50 CM-1). Morphological structures were investigated by Field emission scanning electron microscopy (FE-SEM) (Sigma VP, Carl Zeiss, Germany) and scanning electron microscopy (SEM) (VEGA/TESCAN-XMU) instrument. The mean particle size and zeta potential were measured by Malvern Zetasizer ZS series and Scattering Particle Size Analyzer (Malvern Co., UK).

2.6. Evaluation of the enzyme production and activity
Identified bacteria was seeded into 250 ml flask containing 50 ml NB culture medium and starch 1% and 30 mM NaCO₃ (pH 9) incubated for 72 h at 30°C and 200 rpm in a shaker incubator. Then, the culture media were centrifuged at 6500 rpm for 6 min to remove bacteria cell and the supernatant was used for enzyme activity assay.
Enzyme activity was measured by phenolphthalein assay (Gutenberg et al., 2013). A reaction mixture containing 0.5 ml of starch 1% (w/v) in 0.1 M phosphate buffer (pH 6.8) and 0.5 ml crude enzyme of the sample was shaken at 300 rpm and 30°C for 30 min. Control was included all except crude enzyme. Then, the suspension was centrifuged to separate the remaining insoluble starch (at 6500 rpm for 6 min). Enzymatic reaction was stopped by adding 30 mM NaOH solution and 0.5 ml of phenolphthalein 0.02% (w/v) in 5 mM Na2CO3 solution. The decrease in color intensity was estimated by the measurement of absorbance at 550 nm by spectrophotometry.

The standard curve of Rh B was plotted by the determination of fluorescence intensity (FI) at different concentrations (1–10 mM) at 540 nm (Spectrofluorimeter, Shimadzu, Japan). The absorption spectra of Rh B base aqueous solution in the absence and presence of β-CD and starch at different concentrations were obtained with an absorption maximum at 553 and 625 nm.

3. Results and discussion

3.1. Isolation and identification of the bacteria

The isolated bacteria strain was done according to serial dilution method; then, it was identified according to the biochemical characteristics and 16S rDNA gene sequencing methods and deposited to the GenBank as S. griseobrunneus strain FSHH12 under the accession number of KC626002. Figure 1 represents the BLAST and also phylogenetic tree analysis of S. griseobrunneus.

3.2. Physicochemical characteristics of the synthesized composites

Figure 2 shows the FT-IR pattern of the pure ALG (a), CS (b), AG (c), MCC (d), GE (e), AG/GE/MCC (f) and ALG/CS-np/MCC (h) sheets. Broad absorption bands at 2902–3334 cm\(^{-1}\), 2899–3391 cm\(^{-1}\), 2926 cm\(^{-1}\) and 2929 cm\(^{-1}\) show stretch O–H bond existing in the pure ALG, MCC, AG and GE, respectively. Additionally, the peaks at 1028 cm\(^{-1}\) (ALG), 1058 cm\(^{-1}\) (MCC), 1235 cm\(^{-1}\) (GE), 1018 cm\(^{-1}\) (AG) related to C–O band, which are removed, can be observed.

The characteristic peak at 3431.2 cm\(^{-1}\) is attributed to the NH\(_2\) group at CS. Furthermore, the peak absorbance C–N of the GE and CS at wavenumbers is recorded at 1030 cm\(^{-1}\), while the region of β-CD presented C–N band at 1157 cm\(^{-1}\) (Figure 3(a)). As Figure 3(b) shows, stretch band OH at 2931 cm\(^{-1}\), C–O band at 1274 cm\(^{-1}\) and N–H band at 3435 cm\(^{-1}\) correspond to Rh B, which are removed due to the interaction of the Rh B, β-CD (Ohadi et al., 2018).

Figure 4(a) presents the SEM of the cross section of freeze-dried sheets of ALG/CS/MCC without bacteria cells. The cross section of AG/GE/MCC beads (Figure 4(b)) was seeded with bacterial cells after 72 h (Figure 4(c)), and cells adhered to the inner surface (Figure 4(d)). It can be observed from the SEM micrographs that the growth, aggregation and attachment of bacteria cells in
microchambers existed in the polymer composites, preparing them to produce enzyme exiting from the chambers, while cells remain in their houses. Cross section of capsules showed bacteria cells into capsules similar to other reports (Cress et al., 2014). The morphological structures of chitosan nanoparticles by FE-SEM are shown in Figure 4(e). It was observed that the nanoparticles were spherical in shape with an average size of 38 nm and a zeta potential of +2.85.

Stained dead and alive bacteria by Rh 123 and PI were observed after 72 h incubation (Figure 5). These materials had a permeable effect on the outer membrane barrier.

3.3. Evaluation of the enzyme production and activity
Results showed that the amount of enzyme produced by cells immobilized in ALG/CS/MCC sheets and AG/GE/MCC beads were 1109 U/ml and 948 U/ml, respectively, while this was 606 U/ml on the free cells (Figure 6). Rh B method (Figure 6(a)) was seen to be at a high level at 72 h, and CGTase production was assessed by phenolphthalein method (Figure 6(b)). In Rh B method, supernatant

Figure 2. FT-IR spectra of (a) ALG, (b) CS, (c) AG, (d) MCC, (e) GE, (f) AG/GE/MCC composite, (h) ALG/CS-np/MCC beads.
obtained from cells entrapped into the ALG/CS/MCC beads, AG/GE/MCC sheets and the free cells showed 2494 U/ml, 1979 U/ml and 1496 U/ml enzyme, respectively. These findings are in contrast to other reports indicating that alginate-encapsulated bacterial cells produce much lower amounts of CGTase than free cells due to the limitation of mass transfer (Eș et al., 2016).

The same method has been used to evaluate CGTase by B. pseudocalphilus and B. amyloliquefaciens (Moriwaki et al., 2014). Figure 7 shows the production of the enzyme assayed according to the Rh B method in which FI of the enzyme was measured to be approximately 600 a.u (arbitrary unit).

CO₂ produced during the fermentation experiments seems to be capable of being released from the capsules (considerable to Durham tube test and also bubble produce in a tube). Therefore, capsules prepared from ALG/CS/MCC sheets and AG/GE/MCC beads which bear a lot of large lattices making them permeable to CO₂ may be more suitable for immobilization of microbial cells, compared to alginate alone (Cheng et al., 2014). A similar experiment was reported on immobilization of Clostridium acetobutylicum cells in calcium alginate–PVA–boric acid beads for butanol production (Reungsang et al., 2018). CGTase enzymatic activities investigated from B. firmus and B. sphaericus immobilized cells at 72 h were 110.0 U/ml and 97.0 U/ml, respectively (Moriwaki et al., 2014). Furthermore, B. cereus immobilized in chitosan showed higher CGTase activity than non-attached bacteria cells (Wang et al., 2018). CS in ALG beads was used to decrease the cell leakage by increasing the mechanical stability of the microspheres and reducing the porosity. Bacteria cell immobilization loofa was used for the production of the CGTase, which was decreased (Costa et al., 2015).

Immobilization of bacteria cells in beads with excellent mechanical properties, high cell densities, guarding the cells against inhibitors, toxins and commonly undesirable condition was reported (Kim et al., 2013). Also, production of the CGTase was increased by immobilized Bacillus sp. on the chitosan matrix (Abdel-Naby et al., 2011). Immobilization of yeast using a bacteria cellulose-alginate sponge for ethanol production was 45% more productive than Ca-ALG matrix (Ivanova et al., 2011). B. cereus immobilized in calcium alginate demonstrated a maximal CGTase activity of 47 U/ml, whereas, the maximum activity of CGTase by B. amyloliquefaciens cells entrapped in calcium alginate was 110 U/ml (Vassileva et al., 2003).
4. Conclusion
Considering the results, it can be concluded that fixing bacteria in the three-dimensional structures and void volumes of the ALG/CS/MCC beads and AG/GE/MCC sheets make them cages for bacteria to hide their products from outside of the cages.
Figure 5. Fluorescence of bacterial cells stained with PI (a) and Rh 123 (b).

Figure 6. Time-course of CGTase production measured by (a) method of Rh B, (b) method of phenolphthalein.

Figure 7. Reaction (FI) spectral (Rh B, CGTase) was determined by (a) control (2.5 mM concentration Rh B/β-CD), (b) free cells, (c) encapsulation of cell within AG/GE/MCC, (d) encapsulation of cell within ALG/CS/MCC.
Disclosure of potential conflicts of interest

There are no conflicts of interest.

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