

# Enhanced biotransformation of starch to $\beta$ -cyclodextrin by using high viability of immobilized *Escherichia coli* on hollow fiber membrane as whole cell biocatalyst

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## ABSTRACT

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Application of biological processes to chemocatalysis through biotransformation of a substrate with assistance of whole cells increases efficiency of the entire process. Cell's ability to excrete enzymes into extracellular space is crucial in a process of whole cell biocatalysis. However, issues with cell lysis and stability are frequently encountered when using free cell biocatalysts, particularly *Escherichia coli*. In this study, cell immobilization was studied to overcome these bottlenecks. The effects of process parameters, such as agitation rate, concentration of substrate, temperature, pH, and reaction time, on the production of  $\beta$ -cyclodextrin ( $\beta$ -CD), the excretion of  $\beta$ -cyclodextrin glucanotransferase ( $\beta$ -CGTase), and cell lysis using immobilized recombinant *E. coli* on a hollow fiber membrane as whole cell biocatalyst were investigated. The optimum parameters were as follows: agitation rate, 200 rpm; starch, 4%; temperature, 40°C; reaction time, 4 h; and pH 6, with 5.25–6.14 mg/mL  $\beta$ -CD, 29.88–35.13 U/mL  $\beta$ -CGTase excretion, and 0.3–1.35 U/mL  $\beta$ -galactosidase activity. The immobilized cells exhibited a 11–14-fold increase in  $\beta$ -CD, 17–19-fold increase in  $\beta$ -CGTase excretion, and a 92% reduction of cell lysis compared with the free cells. Therefore, the high viability of the immobilized cell was considered valuable for the efficient biotransformation of starch to  $\beta$ -CD.

**Keywords:** cell immobilization; cell lysis; cyclodextrin; cyclodextrin glucanotransferase; cell biocatalyst

## 1. INTRODUCTION

Starch is an abundant polysaccharide that constitutes a large fraction of the total biomass generated in Malaysia. CGTase is a well-known enzyme that can positively react with starch (Khlestkin et al., 2018).

The production of cyclodextrin (CD) through intramolecular transglycosylation is one of the four reactions that CGTase with starch can catalyze.

Since CD has become increasingly widespread as a result of its various applications in industries, researchers have been hugely attracted to studying and

producing CD. Due to the high cost of enzymes on the market and the low production of enzyme from microorganisms, the commercialization of CD for industrial uses is incredibly challenging.

CGTase is naturally produced extracellularly in *Bacillus sp.* However, *Bacillus sp.* is rather difficult to use as an expression host compared with the conventional choice of *E. coli*. By using *E. coli* as an expression host, CGTase could be extracellularly overexpressed (Cui et al., 2017). Even though this elides the purification step to obtain CGTase, the drawback to this is the one-time-usage of the enzyme. This problem may be solved by immobilizing the enzyme. However, immobilized enzymes can be too unstable and unproductive for industrial applications. Enzymes produced extracellularly such as CGTase are generally preferred over intracellular proteins. Recombinant enzyme production outside of the cell can have advantages over production within the cell, since it can prevent intracellular proteolysis, make protein purification easier, and promote proper protein folding (Ahmad et al., 2018; Mudalip et al., 2018). However, the expression of recombinant proteins in *E. coli* presents additional challenges in terms of cell lysis and viability loss as a result of pressure developed in the periplasmic area brought on by high-level production of recombinant protein (Man et al., 2023; Newton et al., 2017). There have been many attempts to prevent or, at the very least, reduce this difficulty, such as by adding additives, using bacteriocin release protein, or focusing on the favorable conditions for cell cultivation (Fu et al., 2003; Jang et al., 1999). Moreover, whole cell immobilization (cell biocatalyst) may solve the problem of single-use enzymes as well as reduce cell lysis, leading to an efficient biotransformation process with high cell reusability.

Cell immobilization is the process of attaching a cell to or within a support via various techniques, such as entrapment, adsorption, encapsulation, and covalent bonding (Abdul Manaf et al., 2021). Cell immobilization may offer significant advantages to a cell by protecting it from shear forces during the enzyme production and can be reusable to keep producing the desired enzyme. Therefore, the need to obtain fresh enzyme from other sources can be eliminated.

Common cell immobilization techniques include entrapment, encapsulation, cross-linking, and adsorption. It has been proven that entrapment and encapsulation protect the cells and minimize the effects of mechanical shear, hydrophobic solvents, and gas bubbles during the process. However, the support structure could impose a mass transfer limitation on the cell that may throttle cell activity. Cross-linking could reduce cell leakage during the process of immobilization, but it is costly. Adsorption, in which the microbial cells are adhered to the outside of the support, is the simplest and oldest form of cell immobilization. As a result, the adsorption approach was chosen for this research since it is straightforward, affordable, and does not need further chemical pretreatment.

Many types of supports, such as hollow fiber membrane, alginate, silica, and chitosan, could be used for cell adsorption (Zdarta et al., 2018). In this study, the hollow fiber membrane was selected due to its high surface-to-volume ratio (more cells can be immobilized to

the support), availability, cost-effectiveness, and stability in all conditions (Jamil et al., 2020). It would not cause harm or release any toxic ingredients that may disrupt product synthesis. Moreover, it has high mechanical strength and good thermal property.

The process parameters for the biotransformation of starch, such as substrate concentration, agitation rate, pH, reaction time, and temperature, should be appropriately optimized to improve  $\beta$ -CD production. The parameters must be manipulated because they differ on the cell and support types and the cell immobilization method. Therefore, this study mainly aims to define the optimized process parameters of the immobilized recombinant *E. coli* for the biotransformation of starch to  $\beta$ -CD. The effects of process parameters on the CGTase excretion and cell lysis were also investigated. To date, there has been no study of the effects of process conditions of immobilized recombinant *E. coli* on hollow fiber membrane as the whole cell biocatalyst for the biotransformation of starch to  $\beta$ -CD. Therefore, this study aimed to investigate the direct synthesis of  $\beta$ -CD from starch using extracellular  $\beta$ -CGTase of immobilized cells on a porous hollow fiber membrane without the need for a cell disruption step.

## 2. MATERIALS AND METHODS

### 2.1 Materials

Hollow fiber membrane made from polyvinylidene fluoride (PVDF) was obtained from the Faculty of Chemical Engineering Technology and Process, Universiti Malaysia Pahang. The reagents and chemicals used were molecular and analytical grade. Standard  $\beta$ -CD was purchased from Sigma-Aldrich (St. Louis, USA). Ampicillin, citric acid, dibasic sodium phosphate, glycerol, glycine, isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG), magnesium chloride, phenolphthalein, potassium chloride, sodium carbonate, sodium chloride, sodium hydroxide, sodium phosphate monobasic, tryptone, and yeast extract were purchased from Merck (Darmstadt, Germany). Meanwhile, the substrate (soluble potato starch) was purchased from Friendemann Schmidt (Parkwood, Australia).

### 2.2 Process of immobilization for recombinant *E. coli*

The cloning and expression processes were conducted on the *E. coli* strains JM109 and BL21 (DE3), respectively. The cloning vector backbone used was the Novagen pET-21a (+) system. A 100 g/mL dose of ampicillin was given as an antibiotic. The hollow fiber membranes were chopped into 5 cm pieces and put into Luria Bertani broth (50.0 mL and pH 7.0) containing 2 mL of *E. coli* cell culture. The mixture was incubated for 24 h at 37°C with 200 rpm orbital shaking to complete the immobilization procedure. Immobilized cells were collected after incubation and given a thorough rinsing with sterile water.

### 2.3 $\beta$ -CGTase expression

This study used the signal peptide gene (M5) from *Bacillus lehensis* G1 upstream of the  $\beta$ -CGTase gene to directly express the  $\beta$ -CGTase into the extracellular

media (Jonet et al., 2012). A 250 mL flask containing 50 mL of super optimal broth (20 g/L tryptone, 5 g/L yeast extract, 0.5 g/L NaCl, 0.816 g/L KCl, 2.4 g/L MgCl<sub>2</sub>) containing 0.01 mM inducer (IPTG) at pH 9.0 was filled with the immobilized cells and ampicillin (100 g/mL) (Che Man et al., 2016). At 25°C with 200 rpm agitation, the CGTase expression process took place for 2 h.

## 2.4 Biotransformation of starch to $\beta$ -CD production

Initially, 25.0 mL of 4% substrate solution (soluble starch) in 0.1 M phosphate citrate buffer (pH 6.0) was added to the immobilized cells. The enzymatic reaction was conducted at 40°C for 4 h with 200 rpm shaking condition. At room temperature, starch hydrolysates were centrifuged at 10,000 rpm for 15 min.  $\beta$ -CD production,  $\beta$ -CGTase activity, and cell lysis were all examined (in triplicate) from the supernatant liquid.

## 2.5 Effect of process parameters of immobilized cells on biotransformation of starch, excretion of CGTase, and cell lysis

### 2.5.1 Effect of agitation rate

The medium was incubated while being agitated at different speeds of 50, 100, 150, 200, and 250 rpm to study the effects of agitation rate. In 0.1 M phosphate citrate buffer (pH 6.0), 25.0 mL of 4% substrate solution (soluble starch) was added to the immobilized cells. For 4 h, the enzymatic reaction was conducted at 40°C. Thereafter, the starch hydrolysates were centrifuged at 10,000 rpm for 15 min at room temperature.

### 2.5.2 Effect of substrate concentration

The effects of substrate concentration were determined by running the reaction with substrates of varying concentrations: 2%, 4%, 6%, 8%, and 10%. The immobilized cells were added together with 25.0 mL of substrate solution (soluble starch) in 0.1 M phosphate citrate buffer (pH 6.0) at 40°C under 200 rpm shaking condition for 4 h. The starch hydrolysates were then centrifuged at 10,000 rpm for 15 min at room temperature.

### 2.5.3 Effect of temperature

The effect of temperature was examined by incubating the medium at various temperatures of 20°C, 30°C, 40°C, 50°C, and 60°C. The immobilized cells were added together with 25.0 mL of 4% substrate concentration (soluble starch) in 0.1 M phosphate citrate buffer (pH 6.0) for 4 h under 200 rpm shaking condition. The starch hydrolysates were then centrifuged at 10,000 rpm for 15 min at room temperature.

### 2.5.4 Effect of reaction time

Researchers were able to examine the impacts of reaction time by incubating the SOB solution containing the  $\beta$ -CGTase enzyme for 2, 4, 8, 12, 16, 20, and 24 h. In 0.1 M phosphate citrate buffer (pH 6.0), 25.0 mL of 4% substrate solution (soluble starch) was added with the immobilized cells. At 40°C and 200 rpm, the enzymatic reaction took place. Thereafter, the starch hydrolysates were centrifuged at 10,000 rpm for 15 min at room temperature.

### 2.5.5 Effect of pH

Using several buffers at pH 5 and pH 6 (phosphate citrate buffer), pH 7 and pH 8 (Sorenson's phosphate buffer), and pH 9 and pH 10 (glycine-NaOH buffer) allowed researchers to examine the effects of pH. At 40°C with 200 rpm shaking for 4 h, the immobilized cells were combined with 25 mL of 4% substrate solution (soluble starch). Thereafter, the starch hydrolysates were centrifuged at 10,000 rpm for 15 min at room temperature.

## 2.6 Free cell preparation and biotransformation of starch, $\beta$ -CGTase excretion, and cell lysis

Free cells were prepared as a control in the experiment, operating under the same growth and expression conditions as the immobilized cell. The free cells from glycerol stock were transferred to 50 mL of Luria Bertani broth (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl) supplemented with 100  $\mu$ g/mL ampicillin in a 250 mL conical flask and incubated at 37°C and 200 rpm for 24 h. The free cells (2 mL) and ampicillin (100  $\mu$ g/mL) were then transferred to a 250 mL shake flask containing 50 mL of super optimal broth (20 g/L tryptone, 5 g/L yeast extract, 0.5 g/L NaCl, 0.816 g/L KCl, 2.4 g/L MgCl<sub>2</sub>) as the expression medium (pH 9.0) and 0.01 mM IPTG (Che Man et al., 2016). The  $\beta$ -CGTase expression was conducted at 25°C with 200 rpm for 2 h.

The free cells were added with 4% (w/v) of soluble starch (25 mL) for  $\beta$ -CD production. The enzymatic reaction was performed at optimized parameters according to the OFAT method. The samples were then centrifuged at 10,000 rpm for 15 min at room temperature (around 27°C). The supernatant liquid was analyzed (in triplicate) for  $\beta$ -CD production,  $\beta$ -CGTase activity, and cell lysis.

## 2.7 Analytical methods

### 2.7.1 $\beta$ -CD determination by high-performance liquid chromatography (HPLC)

The HPLC device, Zorbax Eclipse Plus C18, 4.6 x 150 mm (Agilent Technologies, California, USA), was used to detect  $\beta$ -CD. The flow rate was set at 1.0 mL/min, and the column temperature was set at 30°C. The mobile phase consisted of a 60:30:10 combination of acetonitrile, methanol, and water. The detection was conducted with a refractive index detector. The calibration procedures and the standard solution were prepared.

### 2.7.2 $\beta$ -CGTase assay

Rajput et al. (2016) reported that  $\beta$ -CGTase activity was assessed using a phenolphthalein assay. In short,  $\beta$ -CGTase enzyme (0.1 mL) was added to 1.0 mL of 40 mg/mL soluble potato starch in 0.1 M phosphate buffer (pH 6.0). The mixture was then incubated for 10 min at 60°C. The process was stopped by adding 3.5 mL of 30.0 mM NaOH and 0.5 mL of 0.02% (w/v) phenolphthalein in 5 mM sodium carbonate. The quantity of  $\beta$ -CD was spectrophotometrically determined at 550 nm after 15 min of incubation at room temperature. The  $\beta$ -CGTase activity is defined as the amount of enzyme that produces 1  $\mu$ mol of  $\beta$ -CD per min.



### 2.7.3 Cell lysis

B-galactosidase assay was used to examine cell lysis. The O-nitrophenyl-D-galactopyranoside (ONPG) reagent measures the quantity of  $\beta$ -galactosidase in the medium to quantify cell lysis (Pachelles et al., 2021). After adding 0.1 mL of the sample, 1.0 mL of the substrate buffer (4 mg/mL of ONPG in 0.1 M pH 7.4 phosphate buffer) was added. The mixture was then incubated in a water bath at 37°C for 10 min. The reaction was stopped by adding about 0.5 mL of 1 M sodium carbonate, and then the absorbance was measured at 420 nm. One unit of enzyme activity is the amount of enzyme that produces ONP (10–8 moles) per min.

### 2.7.4 Field emission scanning electron microscope (FESEM)

FESEM was used to take micrographs of the hollow fiber membrane (JOEL JSM7800F model). For fixing, the membranes were submerged overnight at 4°C in a glutaraldehyde solution (2.5%). They were thoroughly washed with distilled water and were then immersed for 10 min in ethanol solutions with concentrations of 50%, 70%, 80%, 95%, and 100% to dehydrate them. The membranes were then dried at the crucial stage. Before the FESEM micrographs of the fully dried membranes were taken, the membranes were coated with gold. At 5.0 kV, the membranes were magnified 6,000 times to be seen.

### 2.8 Statistical analysis

Each experiment and enzyme assay was performed in triplicate and values are expressed as the mean  $\pm$  standard deviation. The statistically significant differences among groups in each experiment were calculated by ordinary one-way analysis of variance (ANOVA) and Tukey's multiple comparison test. The statistical analysis was performed using GraphPad Prism version 8.0.2 (GraphPad Holdings, LLC, Boston, MA, USA). P values of <0.05 were considered statistically significant.

## 3. RESULTS AND DISCUSSION

### 3.1 Effect of agitation rate

Agitating a fluid mixture homogenizes it, which could be beneficial to the growth of recombinant cells and in this case  $\beta$ -CD production. Figure 1(A) shows a reduction in  $\beta$ -CD production, down to 3.02 mg/mL, at a low agitation rate of 50 rpm. Improper mixing of the reaction mixture could result in a low substrate dispersion and a low enzyme production. The low substrate dispersion in the reaction mixture led to less contact between the substrate and the enzyme that exaggerated the production of  $\beta$ -CD (Guo & Xu, 2006). Figure 1(B) shows that a low excretion of  $\beta$ -CGTase and a high occurrence of cell lysis happened at an agitation rate of 50 rpm.  $\beta$ -CGTase and  $\beta$ -galactosidase activities were 16.8 and 2.67 U/mL, respectively.

In order for cells to grow and produce the recombinant proteins, the concentration of dissolved oxygen is crucial. Mass transfer limitations cause the flux and to experience decreased cell lysis rates.

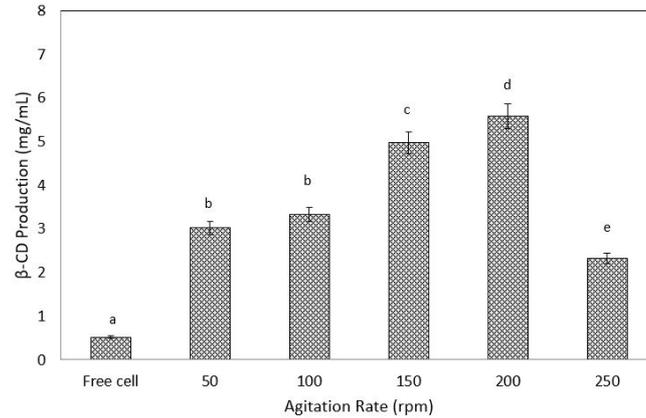
of nutrients and oxygen into the host cells to slow down at low agitation rates, which reduces the cellular activity and metabolism required for efficient protein synthesis. Due to these restrictions, the cells would be subject to a greater metabolic burden and would lyse (Chou, 2007).

At 200 rpm, the highest production of  $\beta$ -CD was observed (5.58 mg/mL), revealing that the mixing efficiency of the reaction mixture improved at optimum agitation rate (Abd Rahim et al., 2015). In Figure 1(B), the highest  $\beta$ -CGTase excretion was detected at 200 rpm with 31.85 U/mL of  $\beta$ -CGTase activity, which corresponds to a high  $\beta$ -CD. The immobilized cell lysis rate was lower at 200 rpm (0.72 U/mL) than at 50 rpm (2.67 U/mL). The lowest cell lysis rate of the immobilized cells was detected at 200 rpm, thus allowing for the continuous production of  $\beta$ -CD. A contradictory finding was detected in Moriwaki et al.'s (2014) research, where the maximum CGTase activity and  $\beta$ -CD production (94.2 U/mL and 12.89 mg/mL, respectively) of immobilized *Alkalophilic bacilli* on loofa sponge were observed at an agitation rate of 120 rpm after about 72 h of reaction. The production of  $\beta$ -CD was only 0.18 mg/mL.h, lower than in this study (1.4 mg/mL.h).

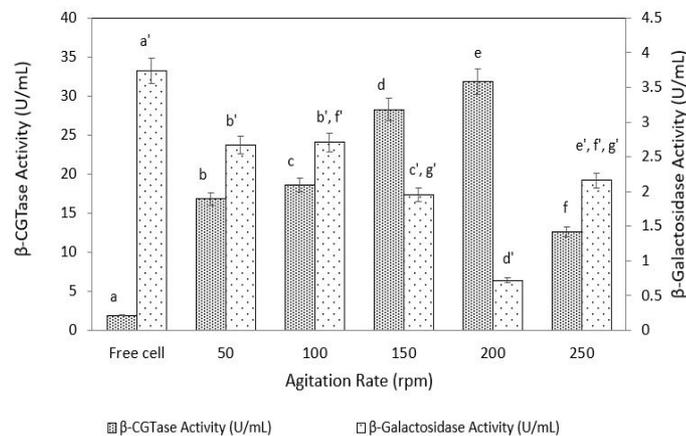
Vigorous agitation resulted in a lower  $\beta$ -CD production. At a stirring rate of 250 rpm,  $\beta$ -CD production decreased to 2.32 mg/mL. Vigorous agitation stressed the cells as they experienced severe shear forces that disrupted their biomolecular structures. This stress could lead to enzyme denaturation and even cell lysis, resulting in low product synthesis production (Ting et al., 2008). This was supported by the reduced CGTase excretion (12.64 U/mL) and a high cell lysis rate (2.16 U/mL) at 250 rpm. A different outcome was revealed in Pazzetto et al.'s (2012) study, where the  $\beta$ -CD production by immobilized *Bacillus firmus* strain 37 was high (23.5 mg/mL) at 100 rpm. As a result, it was suggested that agitating at a pace that was proper (200 rpm) was the best method for distributing air and media contents.

A comparison between immobilized and free cells in terms of their  $\beta$ -CD production was conducted at the supposed optimal agitation rate (200 rpm). Figure 1(A) also shows that the  $\beta$ -CD production by immobilized cells (5.58 mg/mL) was 11-fold higher than the free cells (0.52 mg/mL) after 4 h of reaction. Immobilized cells experienced lower mechanical stress, allowing them to dedicate more cellular energy toward product formation compared with free cells. Ganatsios et al. (2014) reported that in maltose fermentation, the activation energy using the immobilized *Saccharomyces cerevisiae* was lower than that using the free cells, a reduction of about 42%. The immobilization system is advantageous to the cells and enzymatic properties, such as the activity and stability of the cells (Nery & Kubota, 2016). In addition, the  $\beta$ -CGTase excretion (31.85 U/mL) was higher in the immobilized cells than in the free cells (1.75 U/mL). The free cells experienced considerably more cell lysis (3.72 U/mL) than the immobilized cells did. The sufficient mixing rate that could preserve cell morphology while efficiently suppressing cell lysis caused the immobilized cells to emit more  $\beta$ -CGTase.

(A)



(B)



**Figure 1.** Effect of agitation rate for immobilized cells and free cells; (A)  $\beta$ -CD production, (B) enzymatic activities for  $\beta$ -CGTase and  $\beta$ -galactosidase

Substrate concentration, 4%; temperature, 40°C; pH 6; and duration, 4 h. For the free cells, 200 rpm of agitation was applied. Mean values with the significant differences for each parameter are denoted by different letters if  $p < 0.05$  by ordinary one-way ANOVA and Tukey multiple comparison test.

### 3.2 Effect of substrate concentration

Figure 2(A) shows that the effect of variations in substrate concentration on  $\beta$ -CD production was studied by using different soluble starch concentrations (2%, 4%, 6%, 8%, and 10%). The maximum  $\beta$ -CD production achieved was 5.25 mg/mL at 4% substrate concentration due to high amylopectin content in the substrate used (potato starch). Amylopectin reacts with the enzyme active sites and results in a high  $\beta$ -CD production (Sakinah et al., 2014). Figure 2(B) shows that active sites were abundant in this system due to the very high excretion of  $\beta$ -CGTase (29.88 U/mL) that was detected at 4% substrate concentration. Muria et al.'s (2011) study showed a contradicting result, in which the sago starch substrate was fed into the culture at a concentration of 7.5%, resulting in a high production of  $\beta$ -CD out of  $\beta$ -CGTase from *Bacillus* sp. C26. Sago starch is an interesting alternative substrate for use with  $\beta$ -CGTase to produce  $\beta$ -CD, because it is cheap and has a

high production. Furthermore, sago starch could be charged into the reactor at a high concentration without turning the medium into a gluey paste like other starches would, due to the heat treatment that is part of the conventional sago preparation. Kitcha et al.'s (2008) study showed that some starches such as rice starch and sago starch may contain an inducer for  $\beta$ -CGTase production, with the effect of boosting the  $\beta$ -CD production of a microbial system fed with those biomass. In addition, at 4% of substrate concentration, the  $\beta$ -galactosidase activity decreased to 1.35 U/mL. Low  $\beta$ -galactosidase activity is associated with enduring  $\beta$ -CGTase activity, which naturally leads to high  $\beta$ -CD production.

A  $\beta$ -CD production was slightly different at substrate concentration of 2% in comparison with 4% substrate concentration even though the amount of  $\beta$ -CGTase and  $\beta$ -galactosidase activities were quite similar due to low amylopectin concentration in the substrate that slightly affected the  $\beta$ -CD production.

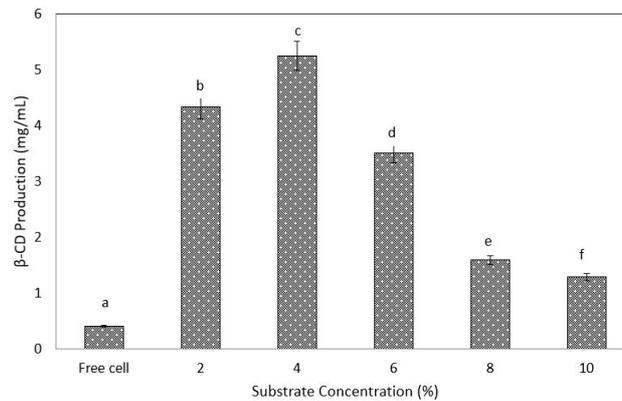
However, exceedingly high substrate concentrations only brought about a notable reduction in  $\beta$ -CD production. When the culture was cultivated in a medium with a 10% substrate concentration, the production of  $\beta$ -CD was only 1.28 mg/mL. Low  $\beta$ -CD production is directly caused by the reaction media



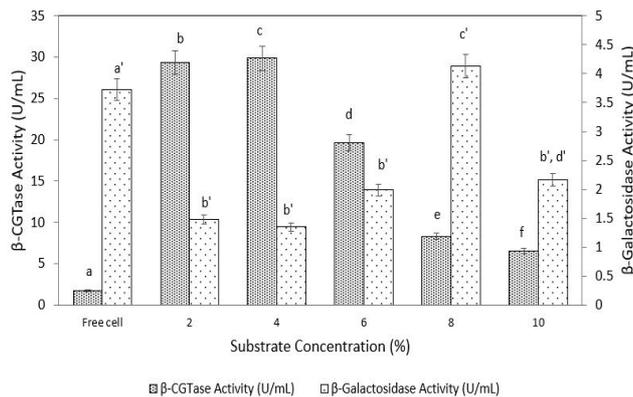
becoming a viscous paste due to high starch concentration, which is difficult to stir and prevents substrate from binding to the active sites of enzyme (Schöffler et al., 2013). Low  $\beta$ -CD production may also be due to a low excretion of  $\beta$ -CGTase. Figure 2(B) shows that the concentration of  $\beta$ -CGTase began to decrease from 19.65 U/mL to 6.57 U/mL as the substrate concentrations decreased from 6% to 10%. This is because enzyme active sites were becoming saturated at higher substrate concentrations, resulting in low  $\beta$ -CGTase excretion and  $\beta$ -CD production. Therefore, maximizing  $\beta$ -CD production requires determining the optimal substrate concentration in the reaction system. Cheirsilp et al.'s (2010) study reported a similar result when 7% starch concentration was used, where the  $\beta$ -CD production decreased due to the high viscosity of the solution, which reduced the ability of the enzyme to catalyze reaction. In addition, the cell lysis rate at 10% substrate concentration was quite high, reportedly being at 2.16 U/mL  $\beta$ -galactosidase activity. High occurrence of cell lysis meant that the reaction setup was not a viable industrial biocatalyst system.

The impact of substrate concentration on the  $\beta$ -CD production of immobilized and free cells was compared in Figure 2(B). The immobilized cells produced 5.25 mg/mL of  $\beta$ -CD, which was 13 times more than what free cells produced (0.40 mg/mL) using 4% substrate concentration. A better environment for microorganisms, particularly for growth and expression, can be provided via an immobilization system (Man et al., 2022). It may also reduce losses in biological activity in cells and enzymes. Pazzetto et al.'s (2012) study showed that the production of  $\beta$ -CD by immobilized *B. firmus* fed with 10% dextrin on loofa sponge was 19.26 mM. In contrast, the free cells under the same condition produced 5 mM of  $\beta$ -CD. Furthermore, the CGTase excretion (29.88 U/mL) of the immobilized cells at the optimum substrate concentration (4%) was higher than that of the free cells (1.75 U/mL) with low cell lysis (1.35 U/mL and 3.72 U/mL of  $\beta$ -galactosidase for the immobilized and free cells, respectively). Therefore, it can be concluded that the immobilized cells are more viable as a biocatalyst for industrial production of chemical products.

(A)



(B)



**Figure 2.** Effect of substrate concentration on immobilized and free cell; (A)  $\beta$ -CD production, (B) enzyme activities for  $\beta$ -CGTase and  $\beta$ -galactosidase

Temperature, 40°C; pH 6; duration, 4 h; and agitation rate, 200 rpm. The substrate concentration used was 4% for the free cells. Mean values with the significant differences for each parameter are denoted by different letters if  $p < 0.05$  by ordinary one-way ANOVA and Tukey multiple comparison test.

### 3.3 Effect of temperature

The effect of temperature on  $\beta$ -CD production was studied by running the reaction at five different temperatures: 30°C, 35°C, 40°C, 45°C, and 50°C. Figure 3(A) shows the trend of  $\beta$ -CD production against temperature. When the temperature increased from

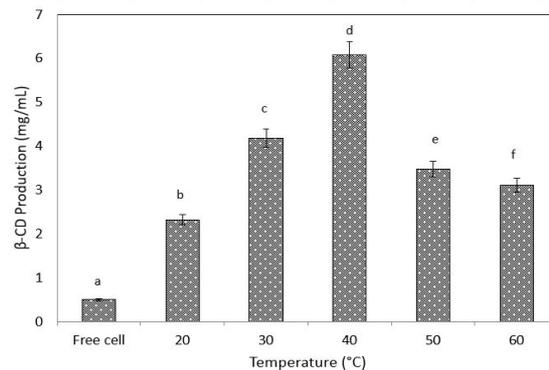
20°C to 40°C, the  $\beta$ -CD production increased.  $\beta$ -CD production was 2.33 mg/mL and 6.08 mg/mL at 20°C and 40°C, respectively. The kinetic energy also rose with temperature, leading to more collisions between the molecules of the substrate and the enzyme. Moreover, increased temperature can boost the interactions between enzyme and substrate molecules (Baskar et al., 2015). Figure 3(B) shows that the CGTase excretion (34.77 U/mL) was high at 40°C, contributing to a high  $\beta$ -CD production. At this temperature, it may allow proper protein folding to occur, thus contributing to the high  $\beta$ -CGTase expression. However, Man et al.'s (2022) study showed a contradicting result, in which the  $\beta$ -CGTase excretion was the highest at 30°C when using immobilized *E. coli* on hollow fiber membrane. No results on  $\beta$ -CD production were available in this study.

$\beta$ -CD production decreased as the temperature was raised above 40°C. At 60°C, the  $\beta$ -CD was only 3.11 mg/mL. This may be due to  $\beta$ -CGTase inactivation at high temperature, which prevented the substrate from binding to the enzyme's active sites and lead to low  $\beta$ -CD production (Kitcha et al., 2008). Figure 3(B) shows that the  $\beta$ -CGTase excretion was 17.31 U/mL at 60°C. In contrast, Muria et al. (2011), who performed a similar reaction but with a free-floating *Bacillus* sp. C26 cell strain that had been modified to be thermophilic, observed that the final concentration of  $\beta$ -CD in the mixture increased from 10.93 mM to 14.13 mM as the

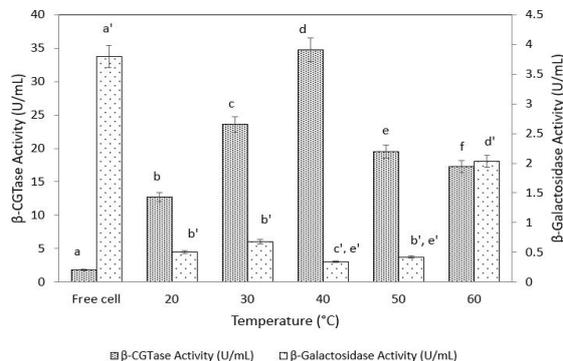
temperature was raised from 45°C to 65°C. High temperature was also reflected in the high cell lysis rate of 2.03 U/mL of  $\beta$ -galactosidase that was detected at 60°C. Therefore, the immobilized cell as whole cell biocatalyst was not suitable to be operated at the high temperature.

The effects of temperature of the immobilized cells on  $\beta$ -CD production, CGTase excretion, and cell lysis were compared to free cells at 40°C (the optimum temperature) as shown in Figure 3. Compared with free cells, which had a  $\beta$ -CD production of 0.50 mg/mL, the immobilized cells' production was 6.08 mg/mL, a 12-fold increase. A high  $\beta$ -CD production was facilitated by the immobilized cell's 20-fold increased  $\beta$ -CGTase activity compared with the free cells. As shown in Figure 4, due to the high cell concentration on the hollow fiber membrane's surface and lumen, the excretion of CGTase was higher in the immobilized cells than in the free cells. These conditions prevented the cells from being disrupted at high temperatures. This phenomenon can be demonstrated by the cell lysis rate recorded in this study, specifically the  $\beta$ -galactosidase of the immobilized cells, which was found to be 0.34 U/mL, which is around 91% less than that in free cells (3.72 U/mL). Compared with free cells that have been directly exposed to high temperatures, it is therefore claimed that cell immobilization approach can protect the cells from thermal denaturation (Ibrahim et al., 2011).

(A)



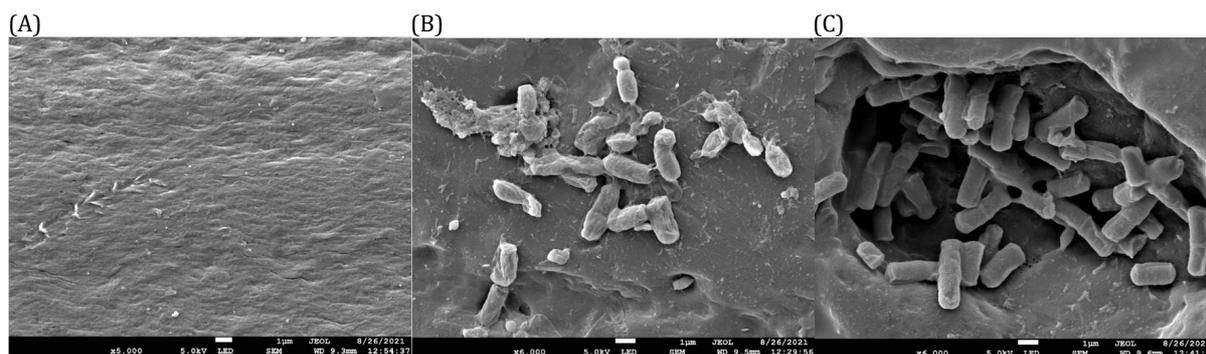
(B)



**Figure 3.** Effect of temperature on immobilized and free cell; (A)  $\beta$ -CD production, (B) enzyme activities for  $\beta$ -CGTase and  $\beta$ -galactosidase

Substrate concentration, 4%; pH 6; duration, 4 h; and agitation rate, 200 rpm. The temperature used was 40°C for the free cells. Mean values with the significant

differences for each parameter are denoted by different letters if  $p < 0.05$  by ordinary one-way ANOVA and Tukey multiple comparison test.



**Figure 4.** FESEM showing (A) cross-section structure of hollow fiber membrane (uninoculated), (B) recombinant *E. coli* immobilized on the surface of hollow fiber membrane at 40°C, (C) recombinant *E. coli* immobilized in the lumen of hollow fiber membrane at 40°C

Due to its high surface-to-volume ratio, the hollow fiber membrane is excellent as a support, as shown by the greater number of cells that were adhered to the membrane's surface and lumen in Figures 4(B) and (C). Moreover, the *E. coli* was only adsorbed on the surface of the hollow fiber membrane and in the lumen. This is because the average pore size distribution of PVDF ranged from 140 nm to 160 nm, which is smaller than the size of *E. coli* (2000 nm).

### 3.4 Effect of reaction time

Figure 5(A) shows that the impact of reaction time on  $\beta$ -CD synthesis was investigated by changing it from 2 h to 24 h. After 4 h of reaction, the maximal production of  $\beta$ -CD by immobilized cells, 6.00 mg/mL, was attained. The increase of  $\beta$ -CD overtime was possibly due to the extensive and frequent binding of the starch on the enzyme, thus increasing the enzymatic reaction in the reaction mixture (Salwanee et al., 2013). Therefore, high  $\beta$ -CD was produced. However, Pazzetto et al.'s (2012) study showed different results, whereas the  $\beta$ -CD production by immobilized *B. firmus* strain 37 on loofa sponge was high (20.73 mM) after 120 h of reaction. Interestingly, the productivity of  $\beta$ -CD (1.5 mg/mL.h) in this study was higher than that of the  $\beta$ -CD (0.17 mg/mL.h) in Pazzetto et al.'s (2012) study, suggesting that immobilized recombinant *E. coli* on a hollow fiber membrane was suitable to be used as whole cell biocatalyst, producing more  $\beta$ -CD in a shorter time.

Substrate concentration, 4%; pH 6; temperature, 40°C; and agitation rate, 200 rpm. The time used was 2 h for the free cells. Mean values with the significant differences for each parameter are denoted by different letters if  $p < 0.05$  by ordinary one-way ANOVA and Tukey multiple comparison test.

$\beta$ -CD production was reflected in the  $\beta$ -CGTase excretion. The highest  $\beta$ -CD production was directly correlated with the highest  $\beta$ -CGTase excretion, as was proven by the results of  $\beta$ -CGTase excretion shown in Figure 5(B). The excretion of  $\beta$ -CGTase after 4 h was high at 34.64 U/mL. Afzali et al.'s (2021) showed contradicting results whereby the  $\beta$ -CGTase activity of the immobilized *Streptomyces griseobrunneus* was the highest after 72 h (1,109 U/mL). Silva et al.'s (2021) study also showed contradicting results, where the  $\beta$ -CGTase activity (2.8 U/mL) from immobilized *B. firmus* strain 37 was higher after 48 h. It may be concluded that

the result obtained in this study was superior due to the  $\beta$ -CGTase having been excreted after only 4 h.

Figure 5(B) also shows that the rate of cell lysis increased over time. About 0.68 U/mL of  $\beta$ -galactosidase was released into the medium at 4 h, 2.21 U/mL at 8 h, 2.77 U/mL at 12 h, 2.98 U/mL at 16 h, 3.34 U/mL at 20 h, and 4.31 U/mL at 24 h. Expressing and excreting the enzyme took a significant toll on the cells that accumulated over time, manifesting in the steady increase in cell death (Bhatwa et al., 2021). The reaction time of 4 h was determined to be the optimal reaction time for  $\beta$ -CD in the present investigation using immobilized *E. coli* due to the high excretion of  $\beta$ -CGTase and low occurrence of cell lysis.

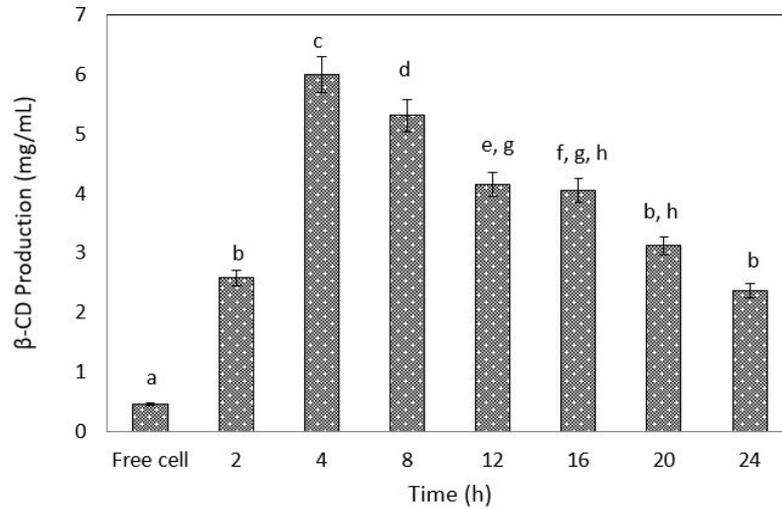
$\beta$ -CD production trended downward after 8 h to 5.31 mg/mL of  $\beta$ -CD and onward to 2.36 mg/mL  $\beta$ -CD production after 24 h of reaction as shown in Figure 5(A). As the reaction proceeded,  $\beta$ -CD accumulated in the reaction mixture that contributed to product inhibition. The  $\beta$ -CD accumulated on the active sites of the enzyme that prevented the substrate from binding on to it. Nevertheless, a study of immobilized *Bacillus sphaericus* on a loofah sponge performed by Delani et al. (2012) demonstrated that the maximum  $\beta$ -CD produced was 86.6 mM after 480 h. Figure 5(B) shows that the excretion of  $\beta$ -CGTase also started to decrease from 8 h (33.08 U/mL) to 24 h (12.92 U/mL). This is also the reason for the decreasing amount of  $\beta$ -CD production.

A comparison between the immobilized and free cells in terms of their  $\beta$ -CD production was conducted using the optimal reaction time (4 h). The immobilized cells resulted in 6.00 mg/mL of  $\beta$ -CD, 13 times more than the production of free cells (0.46 mg/mL) as shown in Figure 5(A). This might be because cells immobilized on the membrane maintain more CGTase activity than free cells do. Figure 5(B) shows that the excretion of  $\beta$ -CGTase by the immobilized cells was about 34.64 U/mL compared with free cells that only produce 1.75 U/mL of  $\beta$ -CGTase activity. Furthermore, there was only 0.68 U/mL of  $\beta$ -galactosidase activity when the immobilized cells were used compared with the free cells (3.72 U/mL). This phenomenon assisted in the continuous production of  $\beta$ -CD. A different result was shown by Yuvadatkun et al. (2018) on ethanol production using immobilized and free *Candida shehatae* on coconut bract, which were 17.2 g/L and 17 g/L and, respectively, at 24

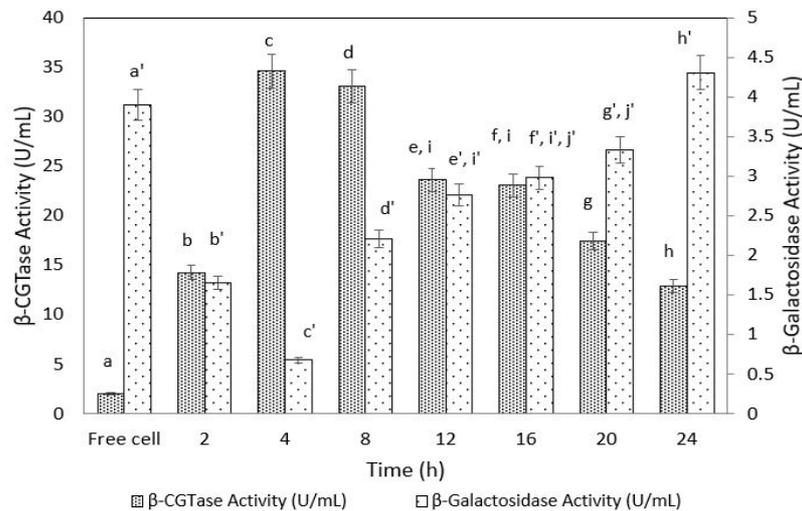
h of reaction. It can be concluded that the immobilized cells are more preferred than the free cells with the aim

of achieving high  $\beta$ -CD production with high  $\beta$ -CGTase activity and low occurrence of cell lysis.

(A)



(B)



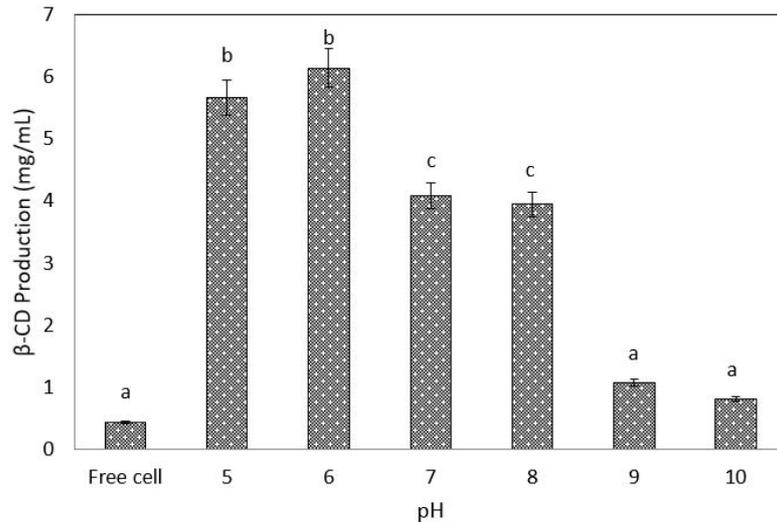
**Figure 5.** Effect of reaction time on immobilized and free cell; (A)  $\beta$ -CD production, (B) enzyme activities for  $\beta$ -CGTase and  $\beta$ -galactosidase

### 3.5 Effect of pH

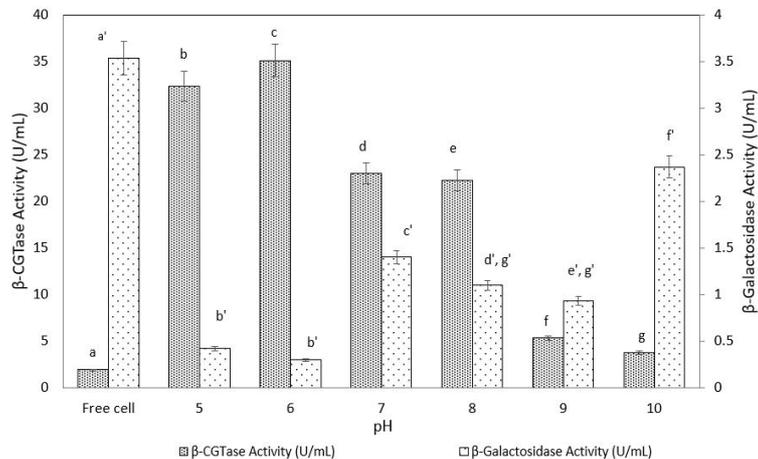
The production of  $\beta$ -CD by the immobilized cells was investigated by running the reaction at five different pH levels (pH 5–10). The highest  $\beta$ -CD production was noticed at pH 6 with 6.14 mg/mL of  $\beta$ -CD, as shown in Figure 6(A). The enzyme was more productive and the cells were more stable in an acidic medium. During the enzymatic reaction, one of the CGTase residues acts as basic residue [OH<sup>-</sup>], whereas other residues act as an acid [H<sup>+</sup>] catalyst (Abdul Manas et al., 2018). This calls for pKa cycling, an enzyme mechanism that regulates the ionization states of both residues involved in the process. It is crucial to control the pKa of the medium's

residues in order to enhance the enzymatic process that leads to the synthesis of the product (Ludwiczek et al., 2013). In Figure 6(B),  $\beta$ -CGTase excretion was found to be higher in acidic conditions (35.13 U/mL) at pH 6 and 32.36 U/mL at pH 5, corresponding to high  $\beta$ -CD production. A contradictory result was reported in a previous study performed by Martins et al. (2003), whereby the  $\beta$ -CD production by the alkaliphilic *Bacillus agaradhaerens* LS-3C entrapped in polyvinyl alcohol-cryogel beads was the highest (5 mg/mL) at pH 8. This is because the alkaline condition favors  $\beta$ -CD production compared with acidic condition (Schöffner et al., 2017).

(A)



(B)



**Figure 6.** Effect of pH on immobilized and free cell; (A)  $\beta$ -CD production, (B) enzyme activities for  $\beta$ -CGTase and  $\beta$ -galactosidase

Substrate concentration, 4%; duration, 4 h; temperature, 40°C; and agitation rate, 200 rpm. The pH used was pH 6 for the free cells. Mean values with the significant differences for each parameter are denoted by different letters for  $p < 0.05$  by ordinary one-way ANOVA and Tukey multiple comparison test.

$\beta$ -CD production decreased with increasing pH, from 4.08 mg/mL at pH 7 to 0.81 mg/mL at pH 10. The low excretion of  $\beta$ -CGTase in the medium plays an important role in the production of  $\beta$ -CD. As shown in Figure 6(B), from pH 7 to pH 10, the excretion of CGTase decreased from 23.01 U/mL of activity to 3.80 U/mL of activity. In an alkaline solution,  $\beta$ -CD production was low due to the excessive hydroxyl ions ( $\text{OH}^-$ ) in the reaction solution. These ions would not bind to the cell because both are negatively charged and they repel each other (van der Maarel et al., 2002).

The immobilized cells experienced high cell lysis rate in a highly alkaline environment, as evidenced by the 2.37 U/mL  $\beta$ -galactosidase activity at pH 10. Correspondingly, cell lysis rate was lower in an acidic medium, as evidenced by the  $\beta$ -galactosidase activity of only 0.42 U/mL at pH 5 and 0.3 U/mL at pH 6. Therefore,

the immobilized recombinant *E. coli* was best able to produce  $\beta$ -CD in acidic conditions (pH 5 and pH 6), as a result of the lower rate of cell lysis. Table 1 shows examples of the application of microbial biotransformation to produce common value-added product from common substrates in the literature.

A comparison study between the immobilized and free cells in terms of their  $\beta$ -CD production was conducted at the optimal pH (pH 6). Figure 6(A) shows that after 4 h of reaction, the  $\beta$ -CD production of the immobilized cells (6.14 mg/mL) was discovered to be 14 times higher than that of free cells (0.43 mg/mL). The drastic difference in the  $\beta$ -CD production between the immobilized and free cells may have been due to the support provided by the PVDF hollow fiber membrane to the immobilized cells. The immobilized cells could better maintain their shapes and experience less deformation in the turbulent medium environment, thus experiencing a lower rate of rupture than the free cells. This was supported by the low cell lysis rate of the immobilized cells at only 0.3 U/mL compared with the free cells (3.72 U/mL) as shown in Figure 6(B).

**Table 1.** Biotransformation of substrate to the desired product

Microorganism	Substrate	Support	Product	Reference
Recombinant <i>E. coli</i>	Soluble potato starch	Polyvinylidene fluoride hollow fiber membrane	1.54 mg/mL.h of $\beta$ -CD	This study
<i>Bacillus subtilis</i>	Corn Starch	Microencapsulated tributyrin	1.62 mg/mL.h of $\beta$ -CD	Li et al. (2020)
Recombinant <i>E. coli</i>	Xylulose	Multi-walled carbon nanotubes	0.26 mg/mL.h of xylitol	Abd Rahman et al. (2020)
Anaerobic bacteria	Glucose	PVDF membrane	4.11 mg/mL.h of biohydrogen	Engliman et al. (2020)
<i>Zymomonas mobilis</i> and <i>Scheffersomyces stipitidis</i>	Glucose	Polymeric membrane	0.87 mg/mL.h of ethanol	Nguyen et al. (2019)

Ibrahim et al.'s (2011) study reported a  $\beta$ -CD production of 8 mg/mL from free *Bacillus agaradhaerens* KSU-A11 with the additional of chemical (10 mM calcium chloride). The calcium chloride acted as a stabilizer for the enzyme, allowing it to achieve high  $\beta$ -CD production. In contrast, no additional stabilizer was required to enhance  $\beta$ -CD production by the immobilized cells in this study, since it is readily achievable with the high CGTase excretion rate.  $\beta$ -CGTase excretion by the immobilized cells was 35.13 U/mL compared with 1.75 U/mL by the free cells. From the results obtained, it could be concluded that the immobilized cells were more productive as cell biocatalyst compared with the free cells to achieve high  $\beta$ -CD production with high  $\beta$ -CGTase excretion and low cell lysis rate. The ideal environment that may shield the cell from external stress may be responsible for the immobilized cells' improved performance.

#### 4. CONCLUSION

The biotransformation of starch to  $\beta$ -CD may be improved using highly viable immobilized cells on porous hollow fiber membrane as a whole cell biocatalyst. The reaction parameters, such as agitation rate, substrate concentration, temperature, reaction time, and pH, must be carefully considered when trying to achieve a high production of  $\beta$ -CD. In this study, the optimized process parameters of the immobilized *E. coli* on the hollow fiber membrane for the biotransformation of starch to  $\beta$ -CD were as follows: 200 of agitation rate, 4% of substrate concentration, 40°C, pH 6, and 4-h duration. The excretion of  $\beta$ -CGTase and the occurrences of cell lysis also contributed in the production of  $\beta$ -CD. From the results, it can be concluded that the agitation rate should be moderate to minimize shear force while achieving a high enough mass transfer rate in the medium. In addition, the amount of starch used in the reaction mixture should ensure that the substrate can bind properly to the active site without facing difficulty in performing the reaction. The temperature of reaction process must not be so high as the enzyme will denatured and the cells lyse in high temperature. Furthermore, the substrate should have enough time to bind properly to the enzyme active site and having a medium with an acidic pH around the immobilized cells could enhance the potential production of  $\beta$ -CD.

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