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Enhancing β-Cyclodextrin Production by Immobilization of Recombinant *Escherichia coli* on Hollow Fiber Membrane: Screening the Reaction Parameters by Full Factorial Design

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ABSTRACT

Cyclodextrin (CD) is an organic compound that is nontoxic and has hydrophobic interior and hydrophilic peripheries, making it soluble in water. It is widely used in pharmaceuticals, food, and agrochemicals due to its unique characteristics. CD is produced via enzymatic conversion from starch, utilizing the cyclodextrin glucanotransferase (CGTase). The industrial production of β-CD requires a high yield of β-CGTase, but the output from *Bacillus* sp. is insufficient to meet this demand. Recombinant β-CGTase expressed in *E. coli* was utilized to address the low yield of CGTase from *Bacillus* sp. However, cell lysis and plasmid instability have been significant obstacles in the excretion and production of recombinant β-CGTase in *E. coli.* Cell immobilization has been suggested as a strategy to enhance β-CGTase excretion and β-CD production while preserving cell stability. The recombinant *E. coli* was immobilized using a hollow fiber membrane as a support. Many factors affect β-CD production and β-CGTase excretion in the immobilization system, such as temperature, pH, substrate concentration, reaction time and agitation rate. Thus, the objective of this study is to screen the significant reaction parameters that have a significant effect on β-CD production and β-CGTase excretion, using a full factorial design (FFD). A 2^5 FFD was applied, and the results were analyzed statistically. Substrate concentration and temperature were found to be the key factors of β-CD synthesis and β-CGTase excretion by immobilized cells. In conclusion, the findings of this study indicate that FFD is a promising approach to reduce the number of experiments and screen the significant parameters for the production of the desired product.

1. Introduction

Cyclodextrin (CD) is a non-toxic substance produced through an enzymatic conversion process from starch as a substrate utilizing CGTase enzyme. CD is considered to be a useful substance, giving

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various advantages to the food and pharmaceutical industries, among others [1,2]. CGTase is usually found in bacteria of the *Bacillus* genus, but the microorganisms can only excrete a low amount of CGTase, which makes it is very costly. To solve this issue, recombinant β-CGTase excretion was introduced in *Escherichia coli*. However, cell lysis frequently occurs when recombinant β-CGTase is excreted using *E. coli* as the host. Previously, researchers have excreted the enzyme for β-CD production using free cells, but these had low viability and were unstable, which reduced β-CD production [3]. Studies performed by Abdel-Naby *et al.,* [4] and Costa *et al.,* [5] using free cells also showed that the free cells could not be used repeatedly and produced less enzyme for β-CD production. Hence, the combination of mechanical containment behind a barrier and adsorption appears to be one of the most well-established and efficient cell immobilization techniques used to produce β-CD [6,7]. In this study, the direct production of β-CD via β-CGTase by the immobilized recombinant *E. coli* on the hollow fiber membrane with the presence of starch.

Many factors influence β-CD production efficiency, such as temperature, substrate concentration, pH, reaction time and agitation rate. Production efficiency may be increased by optimizing these factors, and optimization is often preceded by screening tests to find relevant parameters that have a significant impact on an experimental response [8]. However, the overall number of experimental runs frequently becomes unworkable when the number of parameters is high. The full factorial design (FFD) is beneficial for analyzing systems with a few factors and provides information regarding interactions between factors. It is therefore one of the approaches frequently used to screen factors and has the benefit of finding and isolating the significant parameters with the least amounts of tests, while still taking into account the effects of the interactions between the factors. FFDs have been applied in a variety of industries, such as in the determination of the conditions for umami chemicals to be extracted from fish by-products using ultrasound as an aid to enzyme action [9,10] and the simultaneous effects of the synthesis of Fe_3O_4 nanoparticles under sonication [11,12].

Many studies used the one-factor-at-a-time (OFAT) approach to produce β-cyclodextrin (β-CD) using β-CGTase with either free or immobilized cells [13,14]. As far as we are aware, no research had been undertaken using the FFD to determine the important factors influencing the β-CD production using β-CGTase excreted by the immobilized recombinant *E. coli* on hollow fiber membrane. This effective approach is more economical and environmentally beneficial. A two-level FFD was used in the current investigation to examine the impacts of a total of five reaction parameters (pH, temperature, substrate concentration, reaction time and agitation rate,) on production of β-CD and excretion of β-CGTase. The findings indicate that using the significant reaction parameters of immobilized recombinant *E. coli* on hollow fiber membrane by FFD is a promising approach to achieve high β-CD production with high excretion of β-CGTase.

2. Methodology

2.1 Chemicals and Materials

Polyvinylidene fluoride (PVDF) hollow fiber membrane was provided by the Separation and Membrane Cluster, Faculty of Chemical Engineering Technology and Process, Universiti Malaysia Pahang Al-Sultan Abdullah. All of the other chemicals and materials, such as yeast extract, β-CD, glycerol, tryptone, sodium chloride, phenolphthalein, ampicillin, potassium chloride, magnesium chloride, isopropyl-D-1-thiogalactopyranoside (IPTG), sodium hydroxide, sodium carbonate, dibasic sodium phosphate, citric acid, sodium phosphate monobasic, and soluble potato starch, used in this research were analytical grades and were acquired from a variety of sources, including Friendemann Schmidt, Sigma-Aldrich, and Merck (Parkwood, Australia).

2.2 Recombinant E. coli Strains and Plasmid

A strain of recombinant *E. coli* containing *Bacillus lehensis* G1 CGTase was previously created by Jonet *et al.,* [15]. The JM109 strain of *E. coli* was used as the cloning host, while BL21 (DE3) was selected as the expression host. The vector backbone for cloning was the Novagen pET-21a (+) system.

2.3 Immobilization Process of Recombinant E. coli

The immobilization of recombinant *E. coli* was performed by cutting the PVDF membranes into 5 cm lengths and transferring them into 50 mL Luria Bertani Broth (pH 7.0) containing 2.0 mL *E. coli* cell culture and 100 µg/mL ampicillin, to act as an antibiotic. The immobilization process was carried out at 37 °C with orbital shaking at 200 rpm. The PVDF membranes and immobilized cells were collected after 24 hr of immobilization, and sterilized distilled water was used to properly rinse out any non-immobilized cells.

2.4 β-CGTase Expression

An inducer, isopropyl β-D-1-thiogalactopyranoside (IPTG), was used to express CGTase after the recombinant *E. coli* had been immobilized. This study used the signal peptide gene (M5) from *Bacillus lehensis* G1 upstream of the CGTase gene to direct expression of the enzyme into the extracellular medium [15]. The immobilized cells with 100 µg/mL ampicillin were transferred to a 250 mL flask containing 50 mL of Super Optimal Broth, as an expression medium (20 g/L tryptone, 0.5 g/L NaCl, 5 g/L yeast extract, 2.4 g/L $MgCl_2$, 0.816 g/L KCl,) at pH 9 containing 0.01 mM IPTG [16]. The CGTase expression was carried out at 25 °C for 2 h with 200 rpm agitation rate.

2.5 Biotransformation of Starch to β-CD

The immobilized cells were added to 25.0 mL soluble starch in 0.1 M phosphate citrate buffer (pH 6.0). The enzymatic reaction was performed according to the FFD. Then, the starch hydrolysates were centrifuged at 10,000 rpm for 15 min at room temperature. The supernatant liquid was examined for production of β-CD and β-CGTase activity.

2.6 Reaction Parameter Screening by FFD

Reaction parameter screening was performed to identify the statistically significant factors that could affect the production of β-CD and β-CGTase excretion. FFD was used as the screening method, with the following parameters being considered: temperature, substrate concentration, agitation rate, pH and time. Table 1 shows the experimental value range for each factor, which was set in the Design Expert Software (State-Ease Inc., Statistics made easy, Minneapolis, MN, US. Version 12). A total of 35 sets of experiments were conducted in this study, with 3 center points, to determine the significance of parameters on β-CD production and β-CGTase excretion, as shown in Table 2.

Table 1
Independent variables and the levels of the screening design

Variables	Unit	Low Level	High Level
X_1: Substrate concentration	%	2	6
X_2: Temperature	°C	30	50
X_3: Agitation rate	rpm	150	250
X_4: Time	h	2	6
X_5: pH	-	5	7

Table 2
Experimental design of 2^5 full factorial design (FFD)

Run	Factor				
	X_1	X_2	X_3	X_4	X_5
1	6	30	250	2	5
2	6	50	150	2	7
3	6	50	150	2	5
4	6	30	150	2	5
5	2	30	150	6	5
6	4	40	200	4	6
7	4	40	200	4	6
8	6	50	250	2	5
9	6	30	250	2	7
10	2	30	150	2	5
11	2	30	250	2	5
12	6	50	250	2	7
13	2	50	250	2	7
14	2	30	250	2	7
15	2	30	150	2	7
16	2	50	250	6	7
17	4	40	200	4	6
18	6	50	250	6	7
19	6	30	150	6	5
20	2	50	150	6	5
21	2	30	250	6	5
22	6	50	150	6	7
23	2	50	250	6	5
24	6	30	250	6	5
25	2	30	250	6	7
26	6	50	150	6	5
27	2	50	150	2	7
28	6	50	250	6	5
29	6	30	150	2	7
30	2	50	150	6	7
31	2	50	250	2	5
32	6	30	250	6	7
33	2	30	150	6	7
34	2	50	150	2	5
35	6	30	150	6	7

2.7 Analytical Procedures

2.7.1 β-CD production by high performance liquid chromatography (HPLC)

The β-CD content was determined using a Zorbax Eclipse Plus C18, 4.6 x 150 mm HPLC column (Agilent Technologies, California, USA). The mobile phase was a mixture of acetonitrile, methanol,

and water (60:30:10) with the flow rate set at 1.0 mL/min and the column temperature set at 30 °C. A refractive index detector was used for the detection. A standard solution was prepared and calibration methods were used.

2.7.2 β-CGTase assay

The β-CGTase activity was determined using a phenolphthalein assay, according to the procedure designated by Rajput *et al.,* [17]. In brief, 1.0 mL of 40 mg/mL soluble starch in 0.1 M phosphate buffer (pH 6.0) was mixed with 0.1 mL enzyme, and the solution was then incubated at 60 °C for 10 min. The reaction was then 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. After 15 min of incubation at room temperature, the value of β-CD was measured spectrophotometrically at 550 nm. The β-CGTase activity is defined as the amount of enzyme that would produce 1 µmol of β-CD per min.

3. Results and Discussion

3.1 Screening of Reaction Parameters on β-CD Production and CGTase Excretion by FFD

A two-level factorial design was proposed in this research to highlight the important variables influencing β-CD production and β-CGTase excretion. By using a 2^5 FFD, the five variables substrate concentration, temperature, agitation rate, time, and pH were measured. Table 3 displays the experimental design and results. The maximum β-CD production (6.03 mg/mL) and β-CGTase excretion (37.47 U/mL) were attained by runs 27 and 13, respectively.

Table 3
Experimental design and result of FFD

Run	Actual Values					Responses	
	X_1: Substrate Concentration (%)	X_2: Temperature (°C)	X_3: Agitation Rate (rpm)	X_4: Reaction Time (hr)	X_5: pH	β-CD (mg/mL)	β-CGTase (U/mL)
1	6	30	250	2	5	3.96	19.33
2	6	50	150	2	7	4.39	24.49
3	6	50	150	2	5	4.17	23.52
4	6	30	150	2	5	4.55	18.57
5	2	30	150	6	5	4.03	23.12
6	4	40	200	4	6	4.58	25.93
7	4	40	200	4	6	4.58	25.93
8	6	50	250	2	5	5.00	28.41
9	6	30	250	2	7	4.81	21.79
10	2	30	150	2	5	4.00	22.50
11	2	30	250	2	5	4.94	28.05
12	6	50	250	2	7	4.63	28.49
13	2	50	250	2	7	5.34	37.47
14	2	30	250	2	7	5.06	28.78
15	2	30	150	2	7	4.17	26.44
16	2	50	250	6	7	4.93	28.01
17	4	40	200	4	6	4.29	24.25
18	6	50	250	6	7	4.23	23.56
19	6	30	150	6	5	3.25	18.12
20	2	50	150	6	5	5.38	30.68
21	2	30	250	6	5	4.55	25.79
22	6	50	150	6	7	3.70	27.03
23	2	50	250	6	5	4.76	27.03
24	6	30	250	6	5	3.82	21.48

25	2	30	250	6	7	4.48	28.27
26	6	50	150	6	5	3.84	22.58
27	2	50	150	2	7	6.03	34.48
28	6	50	250	6	5	4.08	23.33
29	6	30	150	2	7	3.87	21.04
30	2	50	150	6	7	4.69	34.99
31	2	50	250	2	5	6.01	33.56
32	6	30	250	6	7	3.71	20.95
33	2	30	150	6	7	4.18	24.80
34	2	50	150	2	5	4.43	25.05
35	6	30	150	6	7	3.47	20.35

The probability value (*p*-value) and percentage contribution of the reaction parameters for β-CD production and β-CGTase excretion are presented in Table 4. A low *p*-value (<.5) denotes a significant parameter to the model's development. The regression model was statistically significant, demonstrating that the model's prediction of the design was reasonable. Additionally, the *p*-value and percentage contribution also revealed that the major influences on β-CD production and β-CGTase excretion were the substrate concentration (X_1) and temperature (X_2).

Table 4
FFD *p*-value and percentage contribution for β-CD production and CGTase excretion

Terms	*p*-value		Percentage Contribution (%)	
	β-CD production	β-CGTase excretion	β-CD production	β-CGTase excretion
Model	.0006	<.0001	-	-
X_1	<.0001	<.0001	29.99	39.12
X_2	<.0001	<.0001	17.40	29.46
X_3	.0003	.0042	8.60	2.99
X_4	<.0001	.0154	15.47	2.03
X_5	.2489	<.0001	0.19	6.73

The coefficient of determination (R^2) was used to verify the model's accuracy [18]. The value of R^2 should be closer to 1 in order to have a good correlation between experimental and predicted values. The slight difference between the R^2 and adjusted R^2 values, which was 3.18% (β-CD production) and 3.20% (β-CGTase excretion), indicated that the non-significant terms have little chance of being involved in the model [19]. Table 5 also provides the adequate precision values, which gauge the signal to noise ratio. To have a good predictive power, the adequate precision needs to be higher than 4. For the β-CD production and β-CGTase excretion models in the current study, the adequate precision was calculated to be 24.105 and 21.800, respectively.

Table 5
Coefficient of determination of FFD for β-CD production and β-CGTase excretion

Coefficient of Determination	Values	
	β-CD production	β-CGTase excretion
R^2	0.9944	0.9317
Adjusted R^2	0.9628	0.9019
Predicted R^2	0.8120	0.8415
Adequate Precision	24.105	21.800

Eq. (1) and Eq. (2) are the regression equations that were obtained from the analysis of variance (ANOVA) for β-CD production and β-CGTase excretion, respectively; all terms were included, regardless of their significance.

$$\beta - CD\ production = 4.45 - 0.36X_1 + 0.27X_2 + 0.19X_3 - 0.26X_4 + 0.029X_5 - 0.11X_1X_2 - 5.00E - 0.003X_1X_3 - 0.072X_1X_4 - 0.020X_1X_5 - 0.046X_2X_3 - 0.016X_2X_4 - 0.012X_2X_5 - 0.066X_3X_4 - 0.024X_3X_5 - 0.049X_4X_5 + 0.088X_1X_2X_3 + 0.054X_1X_2X_4 - 0.014X_1X_2X_5 + 0.076X_1X_3X_4 + 0.081X_1X_3X_5 + 0.055X_1X_4X_5 - 0.032X_2X_3X_4 - 0.083X_2X_3X_5 - 0.032X_2X_4X_5 - 0.062X_3X_4X_5 + 0.46X_1X_2X_4X_5 + 0.19X_2X_3X_4X_5 - 0.12X_1X_3X_4X_5 \quad (1)$$

$$\beta - CGTase\ excretion = 25.69 - 3.00X_1 + 2.60X_2 + 0.83X_3 - 0.68X_4 + 1.24X_5 - 0.39X_2X_3 - 0.46X_2X_4 - 1.03X_3X_4 - 0.60X_3X_5 - 1.08X_2X_3X_4 \quad (2)$$

where X_1, X_2, X_3, X_4, and X_5 are the coded values of the reaction parameters of substrate concentration, temperature, agitation rate, time, and pH, respectively.

The immobilization of *E. coli* on the hollow fiber membrane for β-CD synthesis was found to be significantly affected by the interaction between substrate concentration and temperature, according to the experimental data analysis. The slope in the main effect in Figure 1 denotes the significant parameters for every factor. When compared to other parameters, the substrate concentration in Figure 1(a) has the steepest slope, indicating that it has the greatest impact on β-CD production. At a 2% substrate concentration, the β-CD production was 4.81 mg/mL, whereas it decreased to 4.09 mg/mL at a 6% substrate concentration. The high β-CD production at 2% substrate concentration might be due to the high β-CGTase excretion (28.69 U/mL), as shown in Figure 2(a). Meanwhile, the decrease in β-CD production at 6% substrate concentration might be the result of an oversaturation of substrate on the support's surface, which affects β-CD production. The effect of substrate concentration ranging from 2% to 20% was studied by Sakinah *et al.,* [20], and the results showed that β-CD production increased linearly at lower starch concentrations (2 to 8%). However, further increases in starch concentration above 8% did not contribute to substantial increases in the overall production of β-CD. This was caused by the substrate's high amylopectin concentration. Amylopectin interacts with the enzyme's active sites, resulting in high β-CD production. These results demonstrated that β-CD production is positively impacted by substrate concentration.

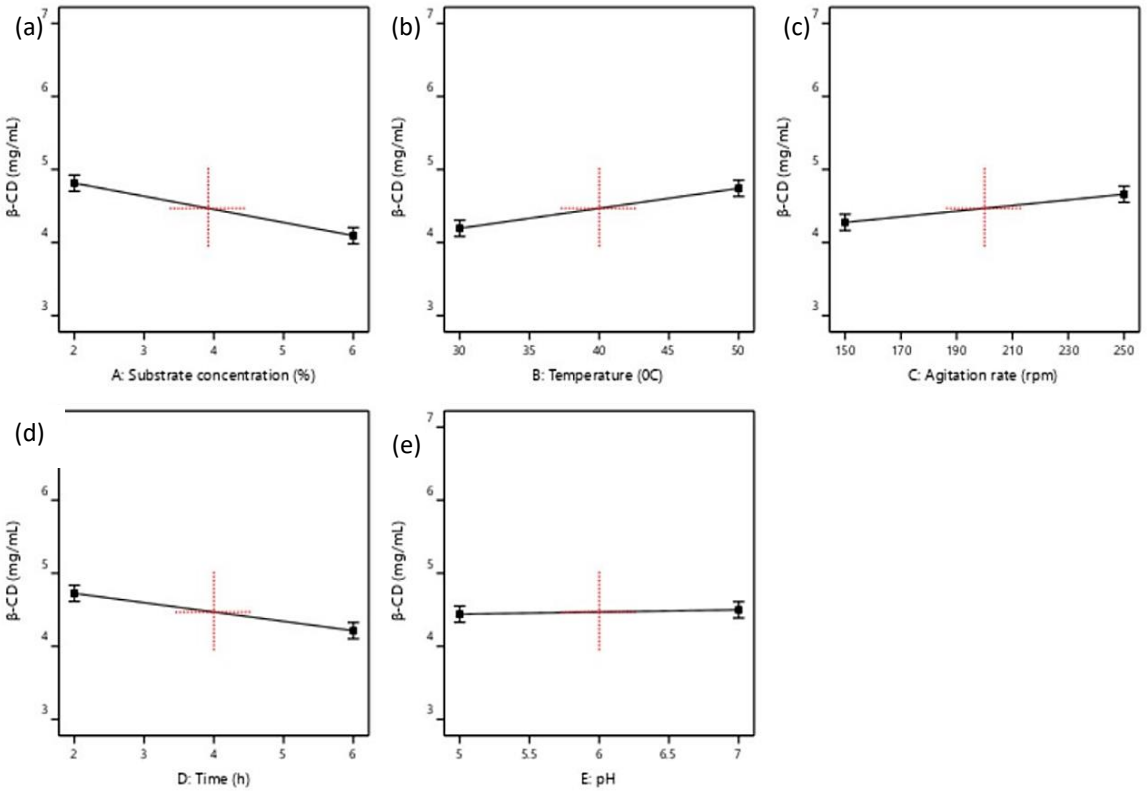

Fig. 1. Plot of the effect of (a) Substrate concentration (2 – 6%) (b) Temperature (30 – 50 °C) (c) Agitation rate (150 – 250 rpm) (d) time (2 – 6 h) (e) pH (pH 5 - 7) on the β-CD production from the immobilization of *E. coli* on hollow fiber membrane

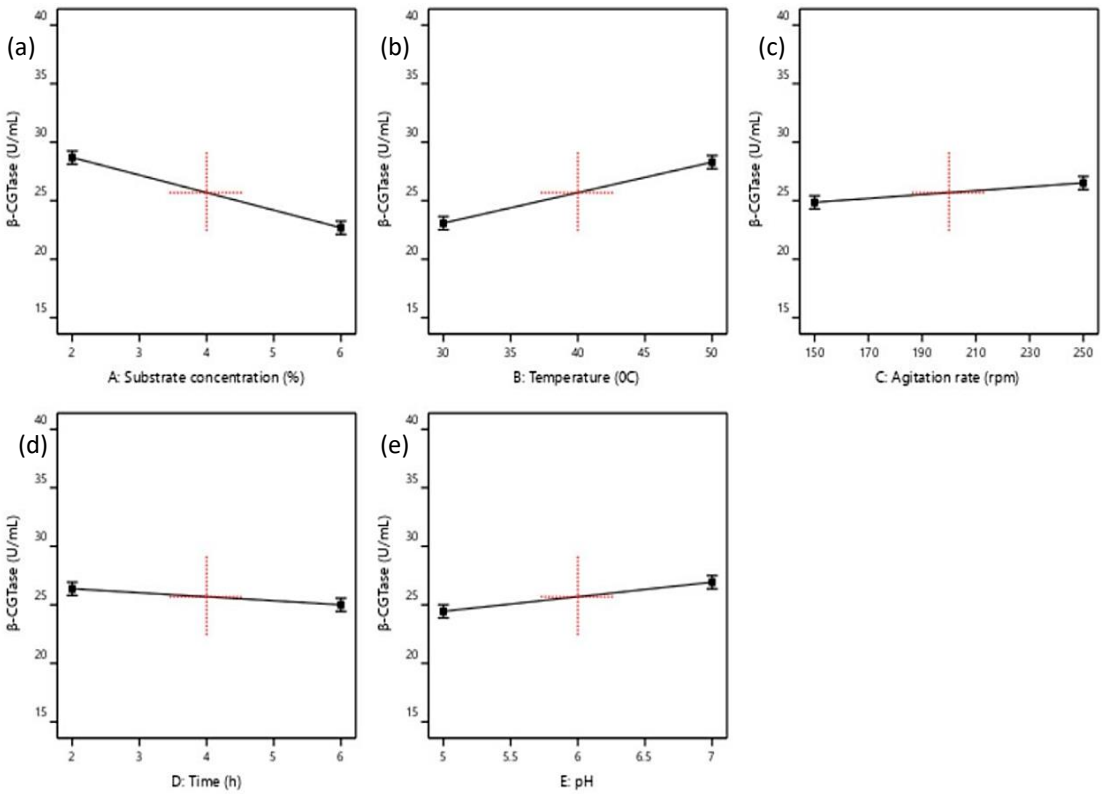

Fig. 2. Plot of the effect of (a) Substrate concentration (2 – 6%) (b) Temperature (30 – 50 °C) (c) Agitation rate (150 – 250 rpm) (d) time (2 – 6 hr) (e) pH (pH 5 - 7) on the β-CGTase excretion from the immobilization of *E. coli* on hollow fiber membrane

Figure 1(b) depicts the effect of the temperature on β-CD production. From 30 to 50 °C, β-CD production increased from 4.18 mg/mL to 4.73 mg/mL. This demonstrated that high β-CD production will occur at a slightly higher temperature. The kinetic energy also increased with temperature, causing more collisions between the enzyme and the substrate molecules. Hence, it can boost the interactions between enzyme and substrate molecules [21], resulting in high β-CD production. In addition, the amount of β-CGTase activity also contributed to the β-CD production. Figure 2(b) shows that the β-CGTase activity increased from 23.09 U/mL to 28.30 U/mL when the temperature increased from 30 to 50 °C. A study performed by Muria *et al.,* [22] discovered that the final concentration of CD in the mixture increased from 10.93 mM to 14.13 mM as the temperature was raised from 45 °C to 65 °C.

The β-CD production increased marginally from 4.26 mg/mL to 4.64 mg/mL as the agitation rate increased from 150 rpm to 250 rpm, as shown in Figure 1(c). The proper mixing of the reaction mixture will help the substrate and enzyme to have more contact, thus leading to high β-CD production [23]. The amount of β-CGTase excretion also affects β-CD production, as in Figure 2(c). The excretion of β-CGTase improved slightly, from 24.86 U/mL to 26.52 U/mL, as the agitation rate increased from 150 rpm to 250 rpm. This was due to the adequate air distribution, which accelerated cell growth and produced high levels of β-CD and β-CGTase excretion. A contradictory result was observed in a study carried out by Moriwaki *et al.,* [24], where the maximum β-CGTase activity and β-CD production (94.2 U/mL and 12.89 mg/mL, respectively) of immobilized alkalophilic *Bacilli* on loofa sponge was observed at an agitation rate of 120 rpm.

When the time was increased from 2 hr to 6 hr, β-CD production steadily reduced from 4.71 mg/mL to 4.19 mg/mL, as shown in Figure 1(d). This phenomenon occurred due to the accumulation of β- β-CD in the reaction mixture, which contributed to product inhibition when the time was extended. The slope in Figure 2(d) demonstrates that the excretion of β-CGTase was not significantly influenced by the reaction time. The CGTase excretion decreased from 26.37 U/mL at 2 hr to 25.01 U/mL at 6 hr; thus, it reduced β-CD production. Pazzetto *et al.,* [25] studied the β-CD production of *Bacillus firmus* strain no. 37 immobilized on a loofah sponge at different time intervals (5, 10, 20, and 30 days). The outcome revealed that the average β-CD production at 10–30 days was 18.14 mM. It is clear that the production of β-CD and the excretion of β-CGTase are unaffected by an increase in reaction time.

According to the flat slope in Figure 1(e) as the pH increased from pH 5 to pH 7, pH fluctuations had little impact on the ability of immobilized cells to produce β-CD. The β-CD production only increased from 4.42 mg/mL at pH 5 to 4.48 mg/mL at pH 7. Figure 2(e) shows that there was only a small increment of β-CGTase excretion, from 24.44 U/mL to 26.93 U/mL, when the pH increased from pH 5 to pH 7.

Figure 3 shows the FESEM of cross-sectional hollow fiber membrane and immobilization of *E. coli* on the surface of hollow fiber membrane. It is depicted more cells were immobilized on the surface of hollow fiber membrane through adsorption technique. High immobilization of *E. coli* on the hollow fiber membrane assisted in the high production of β-CD due to high stability of the *E. coli*.

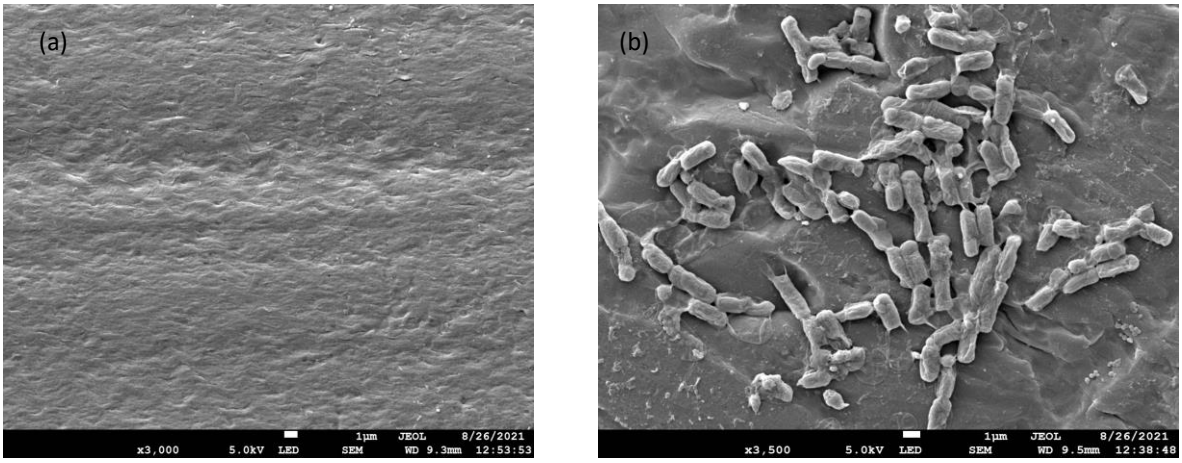

Fig. 3. FESEM showing (a) Cross-section of hollow fibre membrane (uninoculated) (b) Immobilized *E. coli* on the surface of hollow fiber membrane

4. Conclusions

The findings of this study show that the FFD is the appropriate tool to analyze the impact of multiple variables with a minimal number of tests. The results also showed that the substrate concentration and temperature act as the significant parameters in the production of high levels of β-CD by immobilized *E. coli* on hollow fiber membrane. Meanwhile, the agitation rate, reaction time, and pH only contribute slightly to β-CD production and β-CGTase excretion.

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