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Optimization of Process Parameters of Immobilized *Escherichia Coli* for Cyclodextrin Production

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ABSTRACT

The growing interest toward cylclodectrin (CD) application in industries is due to its unique structure that able to form inclusion complexes with substances. However, the problems arise during the CD synthesis using the commercial enzyme (cyclodextrin glucanotransferase) are low production rate and the process is expensive. Hence, cell immobilization system was used. The objective of this study is to optimize the process parameters using Response Surface Methodology to increase the CD production of the immobilized cell. High CD production was achieved after the optimization process. The reusability of immobilized cell also can be reuse up to six cycles using the optimized parameters. The findings propose that optimization process increased the CD production and made it more valuable for industrial application.

1. Introduction

The peculiar structure of cyclodextrin (CD), which allows for the formation of inclusion agents, has drawn attention to the use of CD in industries [1]. Cyclodextrin glucanotransferase (CGTase) degrades starch molecules via an enzymatic process to produce CD. CGTase is normally produced in *Bacillus* sp., but the main problem that usually arise is that the production of CGTase by *Bacillus* sp. is low. Hence, it is inconvenient to be applied in industries. The solution to this problem is the genetically engineered *Escherichia coli* (*E. coli*) that able to excrete CGTase to the medium. *E. coli* is widely employed as a host cell due to its unique ability to manufacture and purify the needed enzyme by using a simple approach [2]. The main concern in the CD production process using the free enzyme are usually the high cost of the downstream process and the low CD production rate. As a result, cell

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immobilization is a possible alternative method for mitigating the negative effects of employing the free enzyme by making the manufacturing process more efficient and cost-effective.

The whole-cell immobilization method is advantageous since it improves the cell stability and recyclability [3]. Adsorption, covalent binding, entrapment, and crosslinking are the methods of immobilization of cells. Adsorption technique is the most basic, affordable, and widely utilized technique of cell immobilization [4,5]. As a result, the adsorption method was employed in this study since it is simple, efficient, and environmentally friendly for the industrial applications. The selection of an appropriate support [6,7] is also an important step in assuring the success of immobilization and maximizing the microbial cell efficiency. Hollow fibre membrane was chosen as the support in this study due to its vast surface area, low cost, absence of toxicity, and excellent in mechanical strength [8,9].

Several factors, including pH, agitation rate and substrate concentration have a substantial impact on the CD synthesis. Previously, the classic approach of altering one factor at a time (OFAT) was employed to determine the optimal response parameters [10] of an immobilized cell. However, it is requires a significant amount of time and is incapable of identifying the relationships between the factors involved [11]. Thus, response surface methodology (RSM) may investigate the impacts of numerous variables with interactions while reducing the number of tests [12].

Hence, the aim of this study is to optimize the process parameters on production of CD by the immobilization of recombinant *E. coli* on hollow fibre membrane using the Response Surface Methodology (RSM). To date, there have been no studies on optimization of the CD production by the immobilized recombinant *E. coli* on hollow fibre membrane. Previous study focused on the CD production by the immobilized cells on a loofa sponge, chitosan and agar-gel beads [1,13,14]. Moreover, there are a few studies on the CD production by the immobilized cells using OFAT approach [15,16]. Thus, this approach suggested that the high CD production will be produced by using the optimized process parameters of the immobilized cells and the reusability of this biocatalyst is suitable to be used in various industrial applications.

2. Methodology

2.1 Material and Chemicals

All chemicals and materials used such as magnesium chloride, potassium chloride, isopropyl -D-1-thiogalactopyranoside (IPTG), sodium carbonate, sodium hydroxide, dibasic sodium phosphate CD, glycerol, tryptone, yeast extract, sodium chloride, ampicillin, phenolphthalein, citric acid, sodium phosphate monobasic and soluble potato starch were analytical grades, purchased from the multiple of companies, including Sigma-Aldrich (Germany) and Merck (Parkwood, Australia). Faculty of Chemical Engineering Technology and Process, Universiti Malaysia Pahang provided the polyvinylidene fluoride (PVDF) hollow fibre membrane.

2.2 Bacterial Strains and Cell Immobilization Process

Genetic Laboratory from Universiti Teknologi Malaysia, Skudai Johor provided the recombinant *E. coli* carrying CGTase from *Bacillus* G1 used in this work. For cloning, the host used was *E. coli* JM109 while the expression host used was *E. coli* BL21 (DE3). pET-21a (+) system from Novagen was used for vector backbone. For immobilization process, 50 ml of Luria Bertani broth (5 g/L yeast extract, 10 g/L tryptone, 5 g/L NaCl) together with 100 mg/mL of ampicillin as an antibiotic were mixed with 50 cm (chopped to 5 cm) of hollow fibre membranes in 250 mL of conical flask. Then, the cells from the glycerol stock were added to the flask and the immobilization process was conducted at pH 7, 37 °C

and 200 rpm. After 24 h, the immobilized cells were collected and the membranes were then cleaned with buffer.

2.3 CD Production

After immobilizing the recombinant *E. coli*, isopropyl -D-1-thiogalactopyranoside (IPTG) was utilised as an inducer to produce the CGTase. The signal peptide gene (M5) from *Bacillus lehensis* G1 was used to guide CGTase expression into the extracellular media. The immobilized cells and ampicillin (100 g/mL) were transferred to a 250 mL flask containing 50 mL of SOB (20 g/L tryptone, 5 g/L yeast extract, 0.5 g/L NaCl, 0.816 g/L KCl, 2.4 g/L MgCl2) at pH 9 containing 0.01 mM IPTG. About 25 mL of substrate solution (soluble starch) in 0.1 M phosphate citrate buffer was added to the immobilized cells (pH 6.0). The enzymatic reaction was carried out on the parameters recommended by the RSM. The starch hydrolysates were then centrifuged at room temperature for 15 min at 10000 rpm. The liquid supernatant was tested for CD production.

2.4 Optimization of the Process Parameters on CD Production by the Immobilized E. coli on Hollow Fiber Membrane using Response Surface Methodology (RSM)

To maximise the CD production of recombinant *E. coli* immobilised on the hollow fibre membrane, response surface approach was used. The optimization was created using a central composite design (CCD), with a total of 20 tests using six-star points and three replicates at the centre points. The tested parameters were pH, substrate concentration, and agitation rate, with CD production collected as the response.

Table 1
Actual values of the design variables for the optimization process

Factor	Low-level star point (-1.682)	Low-level factorial (-1)	Centre point (0)	High-level factorial (+1)	High-level star point (1.682)
x_1 : pH	7.32	8	9	10	10.682
x_2: Substrate concentration (%)	4.32	5	6	7	7.68
x_3 : Agitation Rate (rpm)	115.91	150	200	250	284.09

2.5 Reusability of the Immobilized Cell

The reusability of the immobilized CGTase was conducted in 6 successive batches (6 h per batch). The immobilized cell was transferred into 250 mL flask containing 50 mL of SOB and 10 mL of 6% (w/v) soluble starch in 0.05 M phosphate buffer (pH 6.0). The samples were incubated at 40 °C with a shaking speed of 200 rpm for 15 min. At the end of each cycle, the immobilized cell was collected from the reaction mixture and washed thoroughly with 0.05 M phosphate buffer (pH 6.0) to remove any substrate or products. Then, the immobilized cell was introduced into a new SOB medium and new substrate solution to start a new cycle. Then, the starch hydrolysates were centrifuged at 6000 x g for 15 min at room temperature. The supernatant liquid was examined for CD production.

2.6 HPLC Analysis

High Performance Liquid Chromatography (HPLC) with quaternary pump was used to determine the -CD (Agilent 1260 Infinity Quaternary LC, California, USA). Zorbax Eclipse Plus C18 columns, 150 mm x 4.6 mm, were utilised (Agilent Technologies, California, USA). Prior to analysis, the mobile phase, which was composed of HPLC grade acetonitrile and ultrapure water in a 60:40 ratio, was filtered using a 0.2 m nylon membrane filter. The flow rate of the mobile phase was fixed at 1.0 ml/min, and the column temperature was kept constant at 30 °C. A refractive index detector (RID) was used for detection, and Agilent ChemStation 4.0 was used for analysis.

3. Results and Discussion

3.1 Optimization of Process Parameters of CD Production by Immobilized E. coli on Hollow Fiber Membrane using Response Surface Methodology (RSM)

Response surface methodology (RSM) was applied to determine the optimum process parameters for CD production by immobilized *E. coli*. Table 2 shows the process parameter of pH (X_1), substrate concentration (X_2) and agitation rate (X_3) with the CD production collected as the response. The CD production collected was ranged from a minimum value of 6.36 mg/mL to a maximum value of 11.48 mg/mL based on findings as shown in Table 2.

Table 2
Experimental design and results of the central composite design

Run	Factor 1 X_1: pH	Factor 2 X_2: Substrate concentration (mg/mL)	Factor 3 X_3: Agitation rate (rpm)	Response 1 CD production (mg/mL)
1	8.00	7.00	250.00	7.22
2	10.00	5.00	150.00	8.45
3	8.00	7.00	150.00	8.10
4	9.00	4.32	200.00	10.43
5	10.00	7.00	250.00	7.20
6	9.00	6.00	200.00	11.41
7	9.00	6.00	115.91	8.72
8	10.00	7.00	150.00	7.20
9	9.00	6.00	200.00	11.41
10	9.00	6.00	284.09	6.80
11	9.00	6.00	200.00	11.41
12	9.00	6.00	200.00	11.48
13	7.32	6.00	200.00	7.66
14	9.00	6.00	200.00	11.48
15	10.00	5.00	250.00	7.40
16	9.00	7.68	200.00	8.70
17	9.00	6.00	200.00	11.41
18	8.00	5.00	150.00	9.98
19	10.68	6.00	200.00	6.36
20	8.00	5.00	250.00	7.79

Table 3 summarises the analysis of variance (ANOVA), which includes the list of sources, sum of squares, and P-value. According to ANOVA analysis, the model with a P-value less than 0.05 was statistically significant, implying that it was appropriate for this experiment. Meanwhile, the P-value of 0.0859 for this model's 'lack of fit' indicates that the model was fit with the response data gathered and was desirable for the following experiment. Based on the calculated *P*-value in Table 3, effects

of pH (x_1), substrate concentration (x_2), agitation rate (x_3) and interaction effects of x_1x_2, x_1x_3, x_2x_3, ${x_1}^2$, ${x_2}^2$ and ${x_3}^2$ are the significant factors that influenced the CD production with the *p*-value < 0.0001.

Table 3
ANOVA for response surface quadratic model

Sources	*p*-value
Model	<0.0001*
x_1-pH	<0.0001*
x_2-substrate concentration	<0.0001*
x_3-agitation rate	<0.0001*
x_1x_2	<0.0001*
x_1x_3	<0.0001*
x_2x_3	<0.0001*
${x_1}^2$	<0.0001*
${x_2}^2$	<0.0001*
${x_3}^2$	<0.0001*
Lack of fit	0.0859

*Significant factor: p-value < 0.05

The contour curve of CD generation as a function of pH, agitation rate, and substrate concentration is shown in Figure 1. The CD synthesis rose from 8.85 mg/mL to 11.48.8 mg/mL when the pH increased from 8 to 9. At pH 9, the maximum CD production (11.48.8 mg/mL) was obtained. This might be owing to the cells' tight adhesion to the hollow fibre membrane. The interactions between cations (K^+, Mg^{2+}, and Na^+) and anions (OH^- and negative charge of the cell's surface membrane) contributed to cell attachment and CD synthesis. A study conducted by Man *et al.,* [17] on the immobilization of recombinant *E. coli* onto hollow fibre membrane to express CGTase showed that the optimum pH condition was at pH 9 with 8144 U/mg of CGTase. The strong attachment of cells to the support contributed to the high CGTase expression.

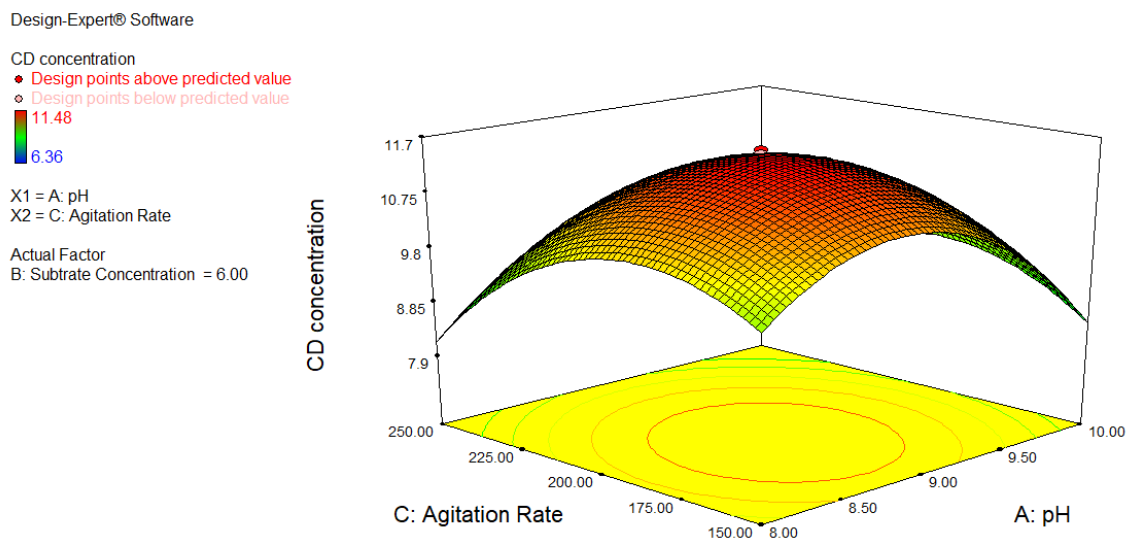

Fig. 1. Response surface plot for CD production: Agitation rate vs pH at constant substrate concentration (6%). The CD production by immobilized cells was measured after 6 hr

As the pH further increased to pH 10, the CD production was decreased to 8.85 mg/mL. An environment that is too alkaline was not suitable for the growth of recombinant *E. coli*. Thus, this phenomenon affected the CD production. Moreover, the high amount of hydroxyl ions (OH^-) at pH 10 created repulsive force between the OH^- and the negative charge of the surface of the *E. coli*, causing a weak attachment of the *E. coli* onto the hollow fibre membrane that contributed to low CD production.

Figure 2 shows the effect of substrate concentration on CD production. As the substrate concentration increased to 6%, the CD production also increased. The highest CD concentration was at 6% with 11.48 mg/mL of CD. At 7% of substrate concentration, the CD production decreased to 8.7 mg/mL. The oversaturation of the substrate might occur that caused more substrate-substrate interaction instead of substrate-enzyme interaction which resulting in the decrease of CD production. A contradict result was shown by Kumar *et al.,* [18] on hydrogen production by the immobilized *Enterobacter cloacae* IIT-BT 08 using lignocellulosic materials as solid support whereas the highest production of hydrogen was 62 mmol/l.hr at 1.0% of glucose concentration. However, when high glucose concentration (>1%) was used, it created the substrate inhibition, thus disrupted the enzymatic reaction between the substrate and enzyme.

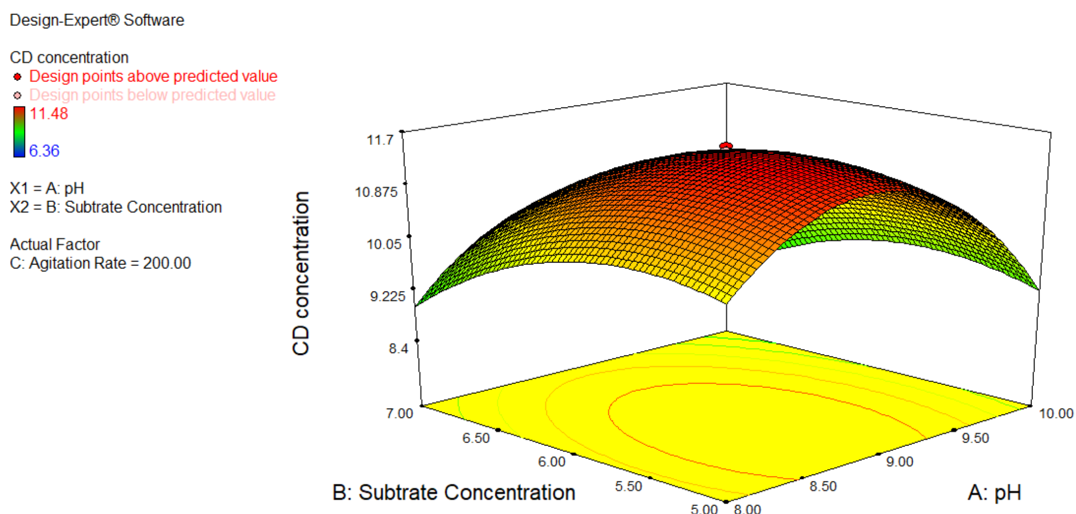

Fig. 2. Response surface plot for CD production: substrate concentration vs pH at constant agitation rate (200 rpm). The CD production by immobilized cells was measured after 6 hr

The contour curve of CD synthesis as a function of agitation rate and substrate concentration with constant pH is shown in Figure 3. The CD manufacturing rose when the agitation rate increased from 150 to 200 rpm. At 200 rpm, the greatest CD output was 10.95 mg/mL. The interaction between the CGTase and the substrate increased as the agitation rate rose. As a result, CD output surged. A research conducted by Costa *et al.,* [19] obtained a contradictory result on CGTase production from *Bacillus circulans* strain DF 9R immobilised on loofa or synthetic sponge at 120 rpm, with the greatest CGTase output being 0.91 and 0.95 U/mL, respectively.

The CD concentration reduced to 8.9 mg/mL when the agitation rate increased to 250 rpm. This might be owing to the high strong force that produced cell separation, resulting in a decrease in CD production. Martins *et al.,* [20] found that immobilised cells of the alkaliphilic *Bacillus agaradhaerens* encapsulated in polyvinyl alcohol-cryogel beads to make CD produced the most (3 mg/mL) at 190 rpm. So, the agitation rate must be taken into account in order to sustain cell growth and cell immobilisation and thus enhance the output of desired product.

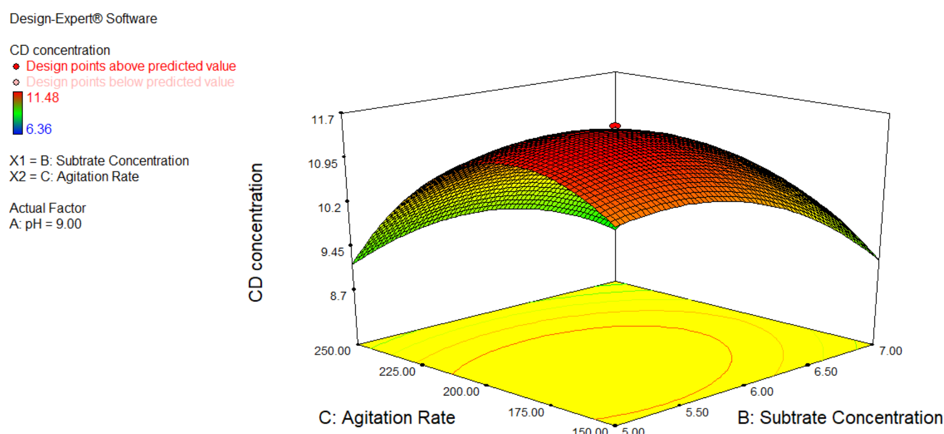

Fig. 3. Response surface plot for CD production: Agitation rate vs substrate concentration at constant pH (pH 9). The CD production by immobilized cells was measured after 6 hr

The production of CD from immobilized cell were analysed and the optimum conditions for each factor were predicted as shown in Table 4. Under the optimized process parameters (pH 9, 6% and 200 rpm), the maximum CD production predicted was 12.5 mg/mL. To validate the accuracy of the model, the experiment with the proposed process parameters was performed. The experimental result showed that the optimal value of CD production was 11.6 mg/mL which was suggested that both experimental and predicted results of the CD production were in a good agreement. The experimental result of the process parameters before and after the optimization procedure were compared and tabulated in Table 4.

Table 4
Summary of the optimized process parameters on CD production

Process Parameters:	Before optimization	After optimization
pH	6	9
Substrate concentration (%)	7	6
Agitation rate (rpm)	200	200
Response:		
CD production (mg/mL) by immobilized cell (predicted):	-	12.5
CD production (mg/mL) by immobilized cell (actual)	6.8	11.6
CD production of free cell (mg/mL)	3.3	6.01

The CD production by the free cell was greatly improved under the optimized process parameter, the maximum CD production gained by the free cell was 6.01 mg/mL. However, maximum CD production gained by the immobilized cell (11.6 mg/mL) was still higher than the free cell. This is might due the enhanced biocatalyst stability provided by the cell immobilization that caused the CD production to increase [21]. A study conducted by Vassileva *et al.,* [22] on CGTase production by immobilized cells of *Bacillus circulans* ATCC 21783 on agar gel showed that the immobilized cells produce CGTase 2-folds higher compared the free cell which was 200 U/mL.

3.3 Reusability of the Immobilized Cell on CD Production

Cell immobilization is often preferred by the industry because of its advantage in term of cost reduction by reusability of biocatalyst. In this study, the reusability of the immobilized cells to produce CD was studied through six successive cycles. Based on Figure 4, the production of CD by the

immobilized cells on the second cycle was reduced to 5 mg/mL as compared to the first cycle which was 11.2 mg/mL. It is might be due to the detachment of the cell from the support and affected the CD production. For the free cell, the CD production for the first cycle was only 6 mg/mL and then shifted to 3 mg/mL for the second cycle. This phenomenon occurred might be due to the cell lysis that affected the CGTase excretion [23]. Thus, the CD production was decreased. After sixth successive cycle, the CD production was 2.2 mg/mL for the immobilized cell and 1.03 mg/mL for the free cell. This showed that the immobilized cells provide suitable approach to be used for the CD production. Another study conducted by Stasiak-Różańska *et al.,* [24] on the reusability of immobilized *Gluconobacter oxydans* ATCC 621 encapsulated by sodium alginate for glycerol oxidation depicted that the immobilized cells does not lose the bioactivity even after several successive batch. The oxidation of glycerol catalysed by immobilized cell can maintain 39% of its initial activity even after seven cycles of re-application. The reason for the loss of activity was might be due to the mechanical forces on biocatalysts during centrifuging and filtration procedure after each cycle. Thus, cell immobilization is proven to increase the productivity by applying reusability of the cell.

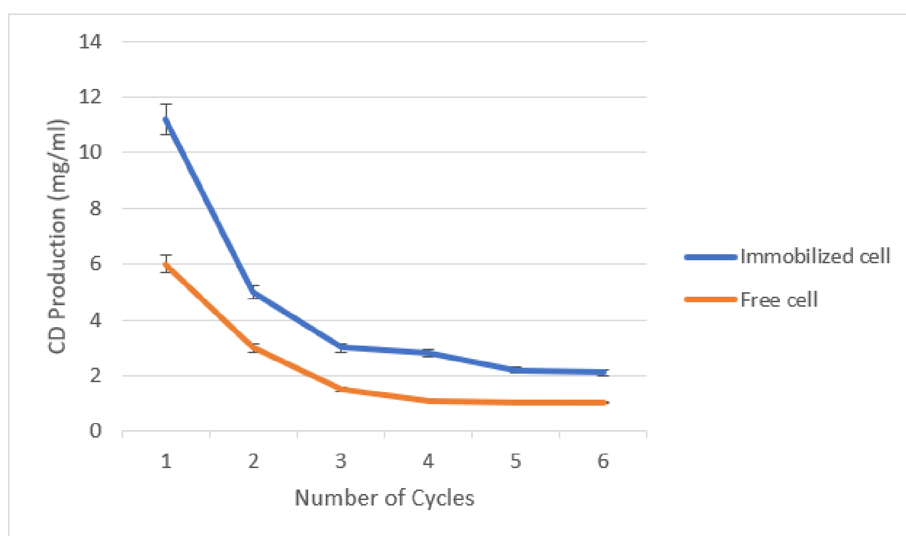

Fig. 4. Reusability of the immobilized and free cell measured for every cycle until the sixth cycles for CD production. The process parameters for immobilized and free cells were as follows: 35 °C, 200 rpm of agitation rate, 6 h of contact time by using LB broth at pH 6

Based on the Table 5, the cumulative CD production by the immobilized cell was 26.3 mg/mL which was approximately 2-folds higher than the free cell (13.63 mg/mL). These results showed that the immobilized cell on hollow fibre membrane greatly improved the production of CD by allowing the reusability of the cells. Research conducted by Mazzer *et al.,* [25] on the reusability of immobilized *Bacillus firmus* strain 37 by using inorganic matrices and alginate gel demonstrated that the production of the CD was improved by retaining 50% activity even in the 3rd reuse cycle. This is because the cell immobilization provides better operational cell stability during the production process.

Table 5
Cumulative CD production throughout six cycles

System	CD Production (mg/mL)
Immobilized cell	26.3
Free cell	13.68

4. Conclusions

The optimal process parameters create a favourable environment to increase the CD production. Under the optimized process parameters, the maximum CD production (11.6 mg/mL) by immobilized cells was achieved. The immobilized cells allow the reusability of biocatalyst up to sixth cycles with 3-folds higher in CD production compared to the free cells. These findings showed that the immobilization of recombinant *E. coli* on the hollow fibre membrane can be employed in industry with direct synthesis of CD without cell disruption.

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