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Effects of the immobilization of recombinant Escherichia coli on cyclodextrin glucanotransferase (CGTase) excretion and cell viability

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ABSTRACT

The excretion of recombinant enzymes is a preferred approach for protein expression because of the associated high level of expression, low level of proteolysis, ease of purification and more favorable folding environment. However, cell lysis is one of the major drawbacks in the excretion of enzymes when using *Escherichia coli* as a host. In this study, the effects of different polymer of hollow fiber membrane and culture conditions on the enzyme excretion, cell lysis and plasmid stability of immobilized *E. coli* were investigated. The cells immobilized on a hollow fiber membrane composed of a polyvinylidene fluoride (PVDF) polymer exhibited a 2–4-fold increase in CGTase excretion, over a 100% increase in plasmid stability and 28-60% reduction in cell lysis compared with free cells. Hence, the immobilization of *E. coli* using a hollow fiber membrane was demonstrated to increase enzyme excretion and cell stability.

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1. Introduction

Protein excretion is important, as it enables active and stable enzyme production, which is essential for successful biocatalysis. In recombinant protein expression, excreted proteins are preferable to intracellular proteins. Excretory production of recombinant proteins provides several advantages over intracellular protein production, including increased protein stability and solubility, correct formation of disulfide bonds and facilitation of downstream processing [1]. However, studies have shown that excretion of enzymes in *Escherichia coli* may cause cell lysis because of pressure build-up through overproduction of the expressed recombinant protein [2]. Thus, the production of recombinant proteins by excretion without (or with less) cell lysis caused by overexpression of the recombinant protein in the host cell is difficult. Various attempts have been made to reduce cell lysis resulting from the excretion of proteins, such adding glycine or Triton-X to the medium [3], using bacteriocin release protein (BRP) [4] and cultivating cells using favorable conditions [5].

Cell immobilization for the excretion of enzymes has various advantages over the conventional process using a free-cell system. These advantages include the repeated and expanded use of cells, preservation of plasmid-bearing cells, simplicity of performing a continuous process for extended periods of time, decreased risk of contamination, protection of the cells from environmental stresses and easier downstream processing [6–8]. Using cell immobilization techniques, increased protein excretion and reduced in cell lysis can be achieved. Various techniques for cell immobilization, such as adsorption on surfaces [9], covalent bonding to carriers [10], entrapment in a polymer gel [11] and self-aggregation [12], have been used to obtain cells with high levels of enzyme production and stability [13]. Therefore, the choice of immobilization technique and the mechanical properties of the matrix are significant factors affecting the enzyme activity and long-term stability of biocatalysts obtained from immobilized cells.

Calcium alginate, glass beads, polyacrylamide gel, alginate, silanized magnetite, agarose, polyurethane foam and carrageenan are the most commonly used matrices for the immobilization of cells. However, the use of alginate for gel entrapment is insufficient because the solidification of the gel requires the use of calcium chloride, which reduces the pH of the mixture and brings a negative effect on cell growth [10]. Furthermore, covalent binding to

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a matrix has the major disadvantages of high cost and low yield because of exposure of the cells to toxic reagents and rigorous reaction conditions.

The development of cell immobilization techniques using hollow fiber membranes has attracted considerable interest because of the high surface-to-volume ratio that can be obtained with this type of membrane [14]. Several studies have been conducted using hollow fiber membranes as a matrix for the production of β-lactamase [15], ammonia [14], ethanol [16,17] and ethylene [18].

In the present study, a hollow fiber membrane was employed as a matrix for cell immobilization to increase the excretion of CGTase, reduce the occurrence of cell lysis and preserve plasmid-bearing cells. *E. coli* were immobilized via adsorption and entrapment within the polymeric matrix based on the high porosity of the membrane. The major benefit of immobilization through adsorption is direct contact between nutrients and the matrix. Moreover, the limitation of diffusion imposed by cell immobilization through entrapment in a hollow fiber membrane is less severe because of the presence of liquid medium in the intra-capillary space of the membrane. In addition to being readily available and inexpensive, hollow fibers were selected as the matrix because of several other desirable properties, such as high mechanical strength, operational durability and lack of toxicity. Another advantage of hollow fiber membranes is the free exchange of nutrients and metabolic products, given the short diffusion distance between cells and the liquid medium [19].

Most studies for CGTase production thus far have focused on attachment to cotton [20]; entrapment in different gels, such as polyacrylamide, agar, alginate and polyvinyl alcohol-cryogel gels [9,11,21,22]; or covalent linking to a flat sheet membrane [10]. To the best of our knowledge, there have been no studies on the excretion of CGTase by *E. coli* that have been immobilized through a combination of adsorption and entrapment of the cells using a hollow fiber membrane. The results presented here suggest that immobilized cell is a promising method for CGTase excretion with less occurrences of cell lysis.

2. Material and methods

2.1. Bacterial strain and cell immobilization

The recombinant *Escherichia coli* strain carrying cyclodextrin glucanotransferase (CGTase) from *Bacillus* G1 used in this work was constructed previously by Jonet et al. [23]. *E. coli* strain JM109 and *E. coli* strain BL21 (DE3) were chosen to be the hosts for the cloning and expression of the constructed plasmid in this study, respectively. pET systems from Novagen were used as the vector backbones for cloning. Hollow fiber membranes (50 cm), products from Advanced Membrane Technology Research Center (AMTEC), Universiti Teknologi Malaysia were chopped to the required size (5 cm length) and then transferred to Luria Bertani broth (LB, 10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl) supplemented with 100 μg/ml ampicillin. The hollow fiber membranes were cultivated with cells from a glycerol stock at 37 °C and 200 rpm. After 18 h (optimization time for cell immobilization: data not shown), the membranes were thoroughly washed with sterile water to eliminate the non-immobilized cells. The immobilized cells were then transferred to 250-ml flasks containing 50 ml of expression medium.

2.2. Expression conditions

i The effects of the polymer membranes on cell immobilization were analyzed using six types of polymer. The polymers and the corresponding solvents used in this study were polyamide imide (PAI) with *N*-methyl-2-pyrrolidone (NMP), polyetherimide (PEI) with NMP, polysulfone (PS) with dimethylacetamide (DMC), polyvinylidene fluoride (PVDF) with NMP, PEI with NMP and carbon nano tubes (CNTs) and polyether sulfone (PES) with polyethylene glycol (PEG). The immobilized cells were expressed in LB medium with 0.01 mM inducer at 30 °C for 24 h. Table 1 shows the thickness and porosity of each polymer membrane.

ii The effect of the medium was studied using five different expression media: Luria Bertani, LB; Super Optimal Broth, SOB (20 g/L tryptone, 5 g/L yeast extract, 0.5 g/L NaCl, 0.186 g/L KCl, 2.4 g/L $MgCl_2$); Terrific Broth, TB (12 g/L tryptone, 24 g/L yeast extract, 4 ml glycerol, 2.31 g/L KH_2PO_4, 12.54 g/L K_2HPO_4); 2 × Yeast-Tryptone, 2 × YT (16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl); and Minimal Medium, M9 (0.6% Na_2HPO_4, 0.024% $MgSO_4$, 0.2% glucose, 0.1% NH_4Cl_2, 0.3% KH_2PO_4, 0.05% NaCl). For protein expression, the cells cultured in various media were immobilized on PVDF polymer with 0.01 mM inducer at 30 °C for 24 h.

iii The effect of the concentration of inducer, isopropyl β-D-1-thiogalactopyranoside (IPTG),was studied using concentrations of 0.005, 0.01, 0.05, 0.1, 0.5 and 1 mM. For protein expression, the cells were immobilized on PVDF polymer in SOB medium at 30 °C for 24 h.

iv The effect of the post induction temperature was studied by inducing protein expression at 20, 25, 30, 35 and 37 °C. The cells were immobilized on PVDF polymer in SOB medium with 0.01 mM inducer for 24 h.

Table 1
Thickness and porosity of hollow fiber membrane for each polymer.

Polymer	Thickness (μm)	Porosity (%)
PAI + NMP	120-123	78.0
PEI + NMP	153-166	74.0
PS + DMC	135 -154	71.0
PVDF + NMP	125-150	77.0
PEI + CNT + NMP	159-170	77.5
PES + PEG	152-161	76.0

A free-cell suspension (2% v/v) was used as a control in the experiment and was treated with the same growth and expression conditions as those used for the immobilized cells.

2.3. Analytical methods

CGTase activity was determined using the phenolphthalein assay [24]. Substrate buffer (1 ml) containing 40 mg/ml soluble starch in 0.1 M phosphate buffer, pH 6.0, was added to a 0.1 ml protein sample, followed by incubation at 60 °C for 10 min. The reaction was terminated by the addition of 3.5 ml of 30 mM sodium hydroxide. Then, 500 μl of 0.02% (w/v) phenolphthalein in 5 mM sodium carbonate was added to the reaction mixture, followed by incubation for 15 min at room temperature. The reduction in color intensity was measured at 550 nm. One unit of enzyme activity was defined as the amount of enzyme that forms 1 μmol of β-cyclodextrin from soluble starch per minute under the experimental conditions.

Cell viability was quantified by determining the amount of β-galactosidase in the extracellular medium using 0-nitrophenyl-β-D-galactopyranoside (ONPG). A total of 1 ml of substrate buffer containing 4 mg/ml of ONPG in 0.1 M phosphate buffer (pH 7.4) was added to 0.1 ml of sample, prior to incubation in a 37 °C water bath for 10 min. The reaction was stopped by adding 0.5 ml of 1 M sodium carbonate, and the absorbance was read at 420 nm. One unit of enzyme activity was defined as the amount of enzyme that forms 10^{-8} mol of ONP per minute under the experimental conditions.

Plasmid stability was determined based on the ratio of colony counts on a selective medium agar plate to that on a nonselective

medium agar plate. Immobilized cells were washed off of the hollow fibers through vortex mixing for 2 min in a test tube containing 10 ml of sterile distilled water. The cell samples were diluted to obtain colony counts within the range of 30-300. All plate counts were determined from the average of at least three replicates.

The dry cell weight of immobilized cells was determined according to Eq. (1).

$$X(\text{mg}) = (W_1 - W_0)x1000 \quad (1)$$

where X is the weight of dry cells, W_1 is the weight of dry cells and the dry hollow fiber membrane and W_0 is the weight of the dry hollow fiber membrane. The dry cell weight of the free cells was determined according to Eq. (2).

$$X(\text{mg}) = \frac{W_1 - W_0}{V_s}x50x1000 \quad (2)$$

where X is the weight of dry cells, W_1 is the weight of dry cells and the dry filter paper, W_0 is the weight of the dry filter paper and V_s is the sample volume.

3. Results and discussion

3.1. Effect of the polymer membrane on immobilized cells

The effects of the polymer membranes on the cell density of the immobilized cells were investigated. Fig. 1A shows the cell density based on the dry cell weight recorded for the different polymers. High cell densities, up to 15.30 mg, were achieved when PVDF polymer served as the immobilization matrix, whereas lower cell density values were measured when using a PAI polymer membrane (3.04 mg). The cell density on the PVDF polymer was directly correlated with the highest CGTase excretion (92.98 U/ml), as shown in Fig. 1B.

To achieve the most efficient immobilization of cells on a membrane, the physicochemical properties of both surfaces (the cells and membrane), such as their hydrophobicity and surface charges, have to be taken into consideration. Most bacterial cell surfaces exhibit a net negative charge under common physiological conditions [25,26]. PVDF consists of hydrogen atoms (positively charged) and fluoride atoms (negatively charged) and behaves as a hydrophobic polymer [27,28]. Therefore, the possible mechanisms responsible for the effective adsorption of *E. coli* cells onto a PVDF membrane include electrostatic interactions between the positively charged PVDF surfaces and the negatively charged cells and electrostatic interactions between positively charged domains on the cell surface and negatively charged surfaces of PVDF polymers. Regarding this latter mechanism, positively charged domains contribute to the formation of biofilms, which consist of unique combinations of polysaccharides, proteins, carbohydrates, DNA and lipids [29–31]. The combination of charges from the membrane and biofilm can cause a strong interaction between the membrane and cells. A third possible mechanism is that hydrophobic interactions at a very close distance may significantly contribute to cell adsorption onto a membrane [32].

The adsorption of *E. coli* to other polymers, such as PEI, only involves electrostatic interactions between the negatively charged cells and the positively charged surfaces of PEI. Atanasova et al. [10] showed that a flat sheet membrane must be treated with formaldehyde for 4 h to provide a positively charged surface that allows covalent linking between bacterial cells and the membrane. Interestingly, in the present study, the adsorption of *E. coli* onto the PVDF membrane occurred through a strong interaction between the cells and the membrane (two electrostatic interactions and a hydrophobic interaction), without requiring treatment with any chemicals. To our knowledge, there have been no previous studies on CGTase excretion in which cells are immobilized on a matrix with a combination of hydrophobic, negatively charged and positively charged surfaces.

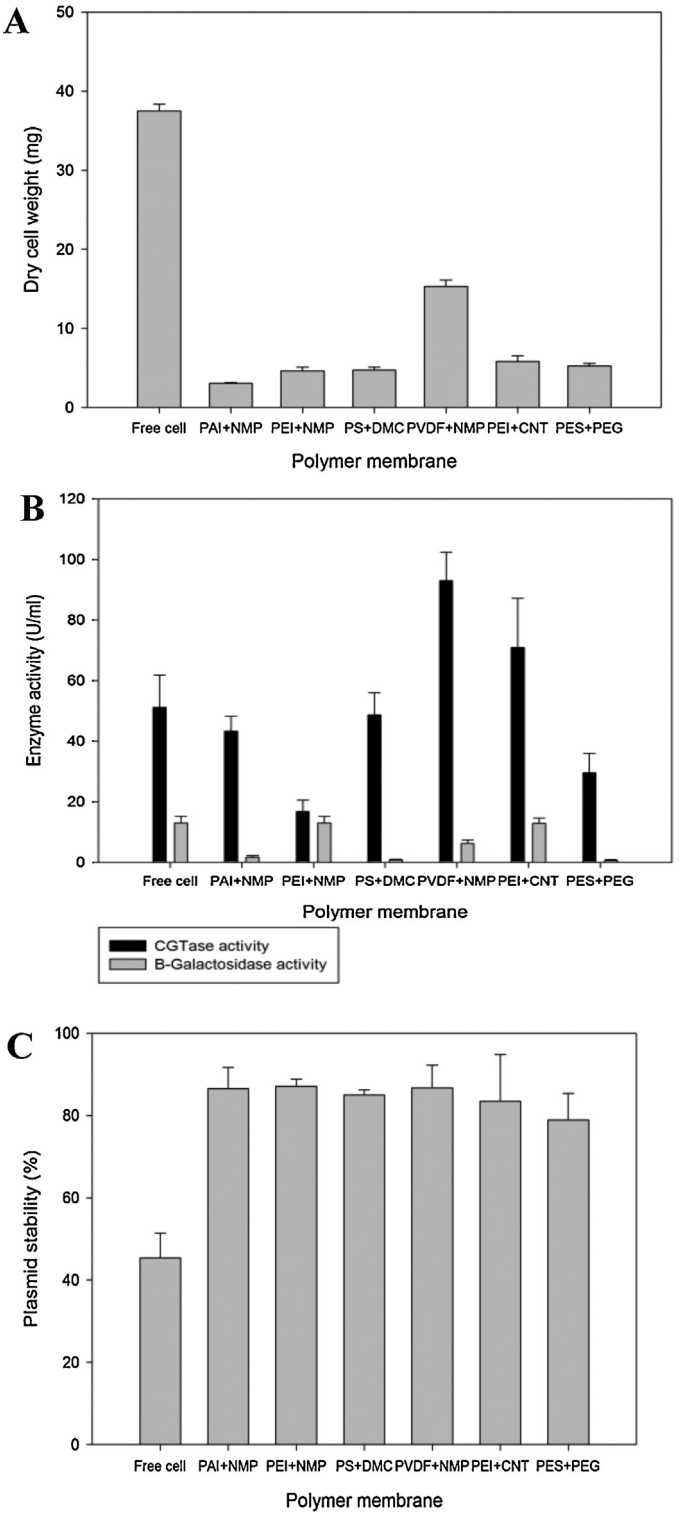

Fig. 1. Effect of the polymer membranes on immobilized and free cell. (A) Cell density (B) enzyme activity (C) plasmid stability. The medium used was LB. The constant value for each variable was 0.01 mM IPTG and 30 °C post induction temperature. Enzymes activity was measured after 24 h of post induction time.

Table 1 shows the porosity of each hollow fiber membrane. Higher porosity should immobilize more cells and produce higher CGTase excretion. However, in the present study, the efficiency of immobilization in terms of membrane porosity did not

significantly affect CGTase excretion. Porosities of 78% (PAI) and 77% (PVDF) showed significant differences in CGTase activity (43.25 U/ml and 92.98 U/ml, respectively). Therefore, we concluded that the porosity of the membrane did not significantly contribute to the efficiency of immobilization that eventually influences the CGTase excretion. Comparisons between immobilized and free cells regarding CGTase excretion, cell viability and plasmid stability were conducted under the following conditions: 0.01 mM IPTG and a 30 °C post induction temperature. The excretion of CGTase from immobilized cells was 92.98 U/ml, which was approximately 2 times higher than that from the free-cell culture (51.20 U/ml) when PVDF was used as an immobilization matrix, even though the biomass of free cells was higher (37.50 mg) than that of cells associated with PVDF (15.30 mg) as shown in Fig. 1A. Moreover, β-galactosidase activity, which represents the level of cell lysis, was 5.23 U/ml in PVDF-immobilized *E. coli*, which was approximately 60% lower than in free cells (13.01 U/ml), as depicted in Fig. 1B. An increase of 91% in plasmid stability was observed in *E. coli* immobilized using the PVDF polymer (86.70%) compared with free cells (45.41%), as illustrated in Fig. 1C. These phenomena occurred as a result of the high level of energy stored by immobilized cells, leading the cells to exhibit higher activity than free cells. The performance of attached cells may differ from that of free-living cells. Cell attachment has often been attributed to the formation of a biofilms anchoring the cells irreversibly after an initial reversible sorption [33]. These biofilms appear to assist cell–cell and cell–surface bridging interactions, anchoring cells to each other and to the surface of the matrix. Therefore, biofilms provide an ideal place for the exchange of plasmids, as the conjugation and segregation processes occur at a higher rate between cells in a biofilms than between free cells [34]. Therefore, in the present study, immobilized cells had higher plasmid stability than free cells, as shown in Fig. 1C. Moreover, a solid-water interface has often been shown to be more favorable for the growth and expression of microorganisms than the surrounding bulk liquid [35] because of the higher concentration of nutrients at the solid-water interface.

Sunitha et al. [36] showed that plasmid stability in recombinant *E. coli* used for the production of phytase in a free-cell system was reduced dramatically as phytase activity increased. Plasmid stability could only be maintained at 5% after 4 h of induction. Interestingly, in the present study, plasmid stability was maintained at up to 86.70% for 24 h after induction. In conclusion, cell immobilization results in higher CGTase excretion and plasmid stability while reducing cell lysis compared to a free-cell system.

3.2. Effect of the medium on immobilized and free cells

The effects of the five different media on CGTase excretion from immobilized *E. coli* are shown in Fig. 2A. The excretion of recombinant CGTase increased with the complexity and nutrient richness of the media. The maximum CGTase activity was observed in 2xYT (376.10 U/ml), followed by TB (355.98 U/ml), SOB (320.40 U/ml), LB (102.33 U/ml) and finally M9 (10.80 U/ml).

As shown in Fig. 2B, the maximum cell density of immobilized cells was observed in 2 × YT (29.40 mg), which was 1.32-fold higher than that in TB (22.23 mg) and 1.43-fold higher than that in SOB medium (20.53 mg). The cell density recorded in M9 medium was the lowest, with a dry cell weight of 11.44 mg. The nutrient richness of the 2 × YT and TB media enhanced the cell density on the hollow fiber membrane and hence promoted increased expression and excretion of recombinant CGTase. Conversely, the lower amounts of nitrogen and carbon sources in M9 medium decreased the cell density and CGTase expression, which led to a low level of excretion. Zhinan et al. [37] showed that 2 × YT was the best medium for human epidermal growth factor (hEGF) production compared with 14 other media. In studies conducted by Low et al. [38] and Kushoo et al. [39], TB medium was chosen for further evaluation because the richness of this medium significantly enhances protein expression. However, in the present study, SOB medium was selected for the subsequent expression analysis because of the associated high CGTase excretion and low cell lysis in addition to the preservation of plasmid stability. The use of SOB medium also decreased the production cost of fermentation compared with 2 × YT and TB because of the high price of yeast extract, which is a major component of 2 × YT and TB.

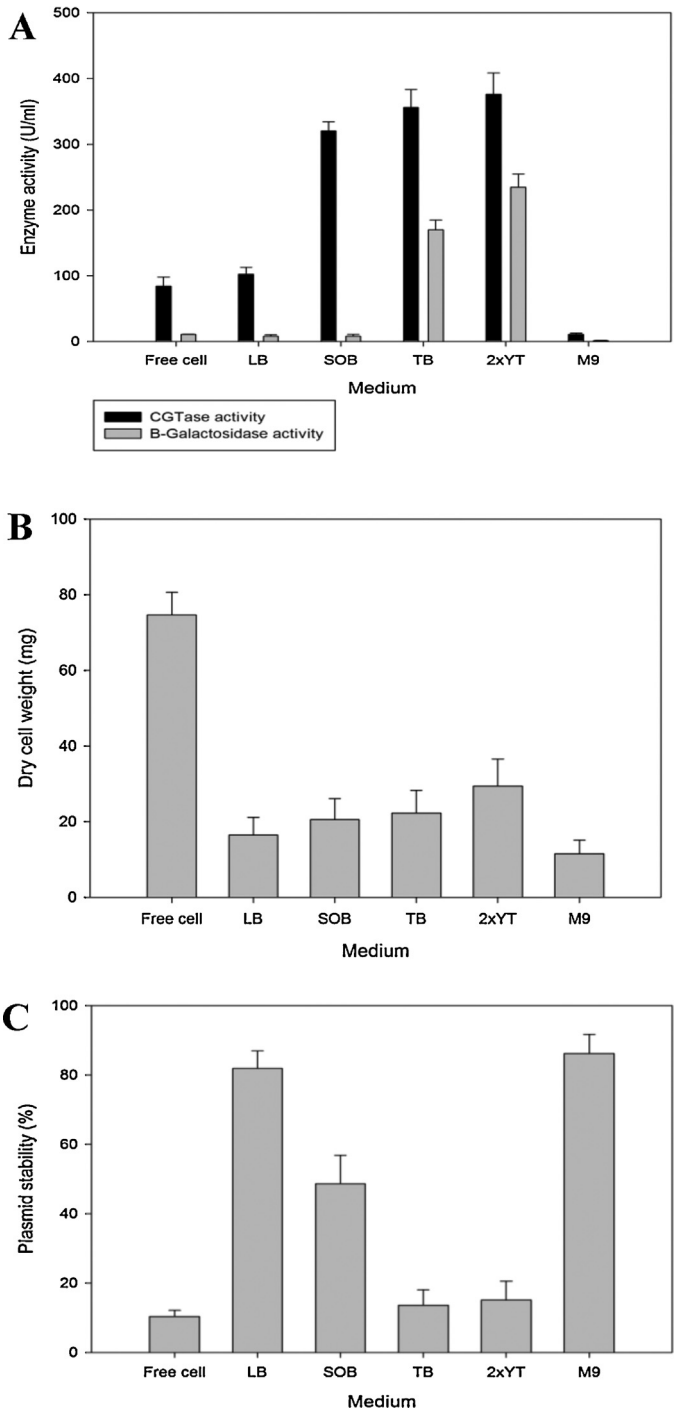

Fig. 2. Effect of medium on immobilized and free cell. (A) Enzymes activity (B) cell density (C) plasmid stability. The polymer membrane used was PVDF. The constant value for each variable was 0.01 mM IPTG and 30 °C post induction temperature. Enzymes activity was measured after 24 h of post induction time.

The CGTase excretion recorded in immobilized cells was 4-fold higher than that in free cells (84.13 U/ml) in SOB medium, despite the higher biomass of free cells (74.67 mg) compared to immobilized cells (20.53 mg). The high CGTase activity observed in the immobilized cells may have resulted from the ability of these cells to assimilate nutrients more readily than in free-cell culture [40], such as through obtaining nutrients adsorbed at the solid-liquid interface.

As indicated in Fig. 2A, the cell lysis observed in immobilized cells was quite high when 2 × YT and TB were used as expression media, leading to β-galactosidase activities of 234.47 U/ml and 169.67 U/ml, respectively, followed by LB (7.98 U/ml), SOB (7.88 U/ml) and M9 (1.17 U/ml), whereas in the free-cell culture, the β-galactosidase activity was 10.93 U/ml. The SOB and M9 media were associated with a lower occurrence of cell lysis compared with the other media tested, possibly because of the presence of Mg^{2+} in the surrounding medium that could have protected cell morphology while effectively suppressing cell lysis. Magnesium is believed to interact with the outer membrane (negatively charged) of cells and enhance the integrity and stability of the outer membranes [41], thereby controlling cell lysis. During *E. coli* growth and protein expression, disorganization of the cell structure can occur, including cell swelling, the development of a spherical form and the creation of large vacuoles, eventually ending in cell lysis [42]. The presence of Mg^{2+} helps to overcome these problems and maintain the structure of the cell. When Mg^{2+} interacts with the outer membranes of cells, proper organization of the outer membrane is maintained, including necessary alterations of the membrane structure for transport purposes. Therefore, the permeability and stability of the outer membrane increase. In the present study, Mg^{2+} is believed to enhance CGTase excretion to the extracellular space by preventing accumulation of CGTase in the periplasmic space, which eventually leads to cell lysis. This finding is supported by the results of Li et al. [43], who showed that divalent cations such as Ca^{2+} prevent cell lysis by maintaining sufficient membrane permeability. Birch and Walker [44] and Hu et al. [45] also found that magnesium appears to protect yeast cells during fermentation through a mechanism that results in decreased plasma membrane permeability.

Fig. 2C shows that 2 × YT and TB media were associated with low plasmid stability, 15.15% and 13.55%, respectively, whereas M9 and SOB were associated with higher plasmid stability, 86.15% and 48.61%, respectively. The presence of Mg^{2+} in SOB and M9 media influences cell attachment and subsequent biofilm formation [46]. Therefore, plasmid stability can be maintained at a high level when a greater amount of biofilm exists. However, the plasmid stability in LB medium was also high (81.84%) because of low CGTase excretion, which imposed a lower metabolic burden on the cell. In the free-cell system, the plasmid stability was only 10.35%, even though SOB was used as the expression medium. The increase in plasmid stability in immobilized cells resulting from the presence of a biofilm prevented desorption of daughter cells, therefore securing individual cells and their progeny in an appropriate surrounding.

3.3. *Effect of inducer concentrations on immobilized and free cells*

Immobilized cells were induced using different concentrations of inducer to determine the optimum excretion of CGTase. In the present study, *E. coli* was grown in SOB medium and induced with different concentrations of IPTG for 24 h. Maximum excretion of CGTase was observed when 0.01 mM IPTG was used, leading to activity of 339.72 U/ml, as shown in Fig. 3A. However, CGTase excretion was only 60.16 U/ml when the concentration of IPTG was 0.005 mM, which could occur because a small amount of IPTG is insufficient to trigger the transcription of the lac operon, thus affecting the expression and excretion of CGTase. CGTase

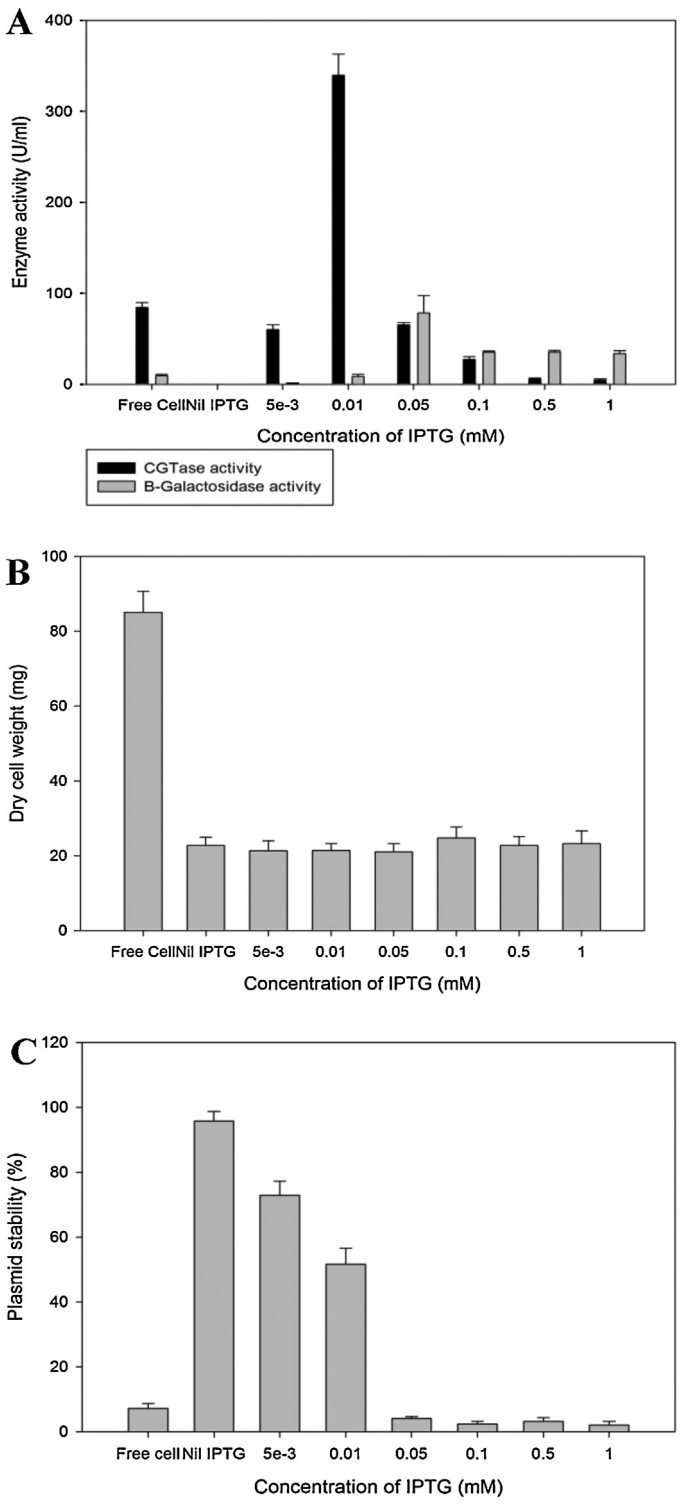

Fig. 3. Effect of inducer concentration on immobilized and free cell. (A) Enzymes activity (B) cell density (C) plasmid stability. The polymer membrane and medium used were PVDF and SOB, respectively. The constant value for variable was 30 °C post induction temperature Enzymes activity was measured after 24 h of post induction time.

excretion also decreased significantly when the IPTG concentration was increased from 0.05 to 1.00 mM. This decrease was the result of high gene induction leading to high CGTase expression and potentially promoting the formation of inclusion bodies. Inclusion bodies accumulated in the cytoplasmic space, impairing its translocation and overwhelming the excretion capability. The formation of inclu-

sion bodies eventually caused cell lysis. This finding was supported by Low et al. [38], who showed that CGTase excretion was higher when using a low concentration of IPTG. It has also been found that with a high synthesis rate, such as in the case of glycerophosphate oxidase, proteins overexpressed in *E. coli* become misfolded, resulting in partial loss of function [47].

As shown in Table 2, the expression of CGTase produced by immobilized recombinant *E. coli* exhibited expressed higher level of enzyme expression (339.72 U/ml) than other microorganisms or systems used for CGTase production. This finding indicates that immobilization of recombinant *E. coli* with hollow fiber membranes is a promising strategy to produce high levels of enzyme.

CGTase excretion by immobilized and free cells was compared using the optimal concentration of IPTG for immobilized cells (0.01 mM) for up to 24 h of post induction time. Immobilized cells showed increased CGTase excretion in comparison with the free-cell culture, as shown in Fig. 3A. An approximately 4-fold increase in enzyme excretion (339.72 U/ml) was observed in immobilized cells compared with the excretion obtained in free cells (84.60 U/ml), even though the biomass of immobilized cells (21.38 mg) was lower than that of free cells (85 mg). Generally, the maintenance and expression of heterologous protein production requires precursors for protein synthesis and energy, thereby generating a metabolic burden in cells. However, as shown in Fig. 3C, the plasmid stability in immobilized cells was 50% higher than that in free cells (7.15%) during the course of expression. This observation demonstrates that immobilized cells undergo different physiological changes compared with cells in a free-cell system. Maintenance of plasmid stability in immobilized cells was also reported by Chen et al. [48], who showed that cell immobilization decreased the probability of segregational plasmid loss and thereby increased the production of human epidermal growth factor (hEGF).

As shown in Fig. 3A, only a low level of β-galactosidase activity was detected in immobilized and free cells (8.70 U/ml and 9.28 U/ml, respectively). Because induction was conducted during the stationary phase of cell growth, the surface charge of the cells was more negative [49] than that during the exponential phase. Therefore, the presence of Mg^{2+} in the surrounding medium may enhance the ability of the cell surface to adsorb this ion (Mg^{2+}). Therefore, the integrity of the outer membrane was enhanced and the cells were protected from lysis.

3.4. Effect of post induction temperature on immobilized and free cells

Post induction temperature is an important factor in the expression of CGTase by recombinant *E. coli*. To determine the effect of post induction temperature on the excretion of CGTase using immobilized cells, five different temperatures (20, 25, 30, 35 and 37 °C) were investigated. The results (Fig. 4A) indicated that CGTase excretion in immobilized cells after 24 h of post induction time was improved significantly by increasing the post induction temperature from 20 to 30 °C. However, a further increase of post induction temperature reduced CGTase excretion. The highest CGTase excretion was achieved at 30 °C (318.80 U/ml), followed by 25 °C (245.94 U/ml), 20 °C (129.95 U/ml), 35 °C (41.79 U/ml) and, finally, 37 °C (39.49 U/ml). Lower temperatures may allow sufficient time for proper protein folding to occur. In addition, the metabolic stress generated by heterologous gene expression can be partially relieved by performing expression at lower temperatures [50].

Higher cell lysis was also observed when the post induction temperature was increased from 30 to 37 °C. As shown in Fig. 4A, the β-galactosidase release was higher at 35 °C (24.83 U/ml) and 37 °C (21.39 U/ml) than at 20 °C (1.05 U/ml), 25 °C (4.97 U/ml) and 30 °C (5.11 U/ml). The high temperatures induced metabolic stress in the cells and hence promoted cell lysis. Shi et al. [51] reported that cell lysis and proteolytic activity are reduced at low temperatures.

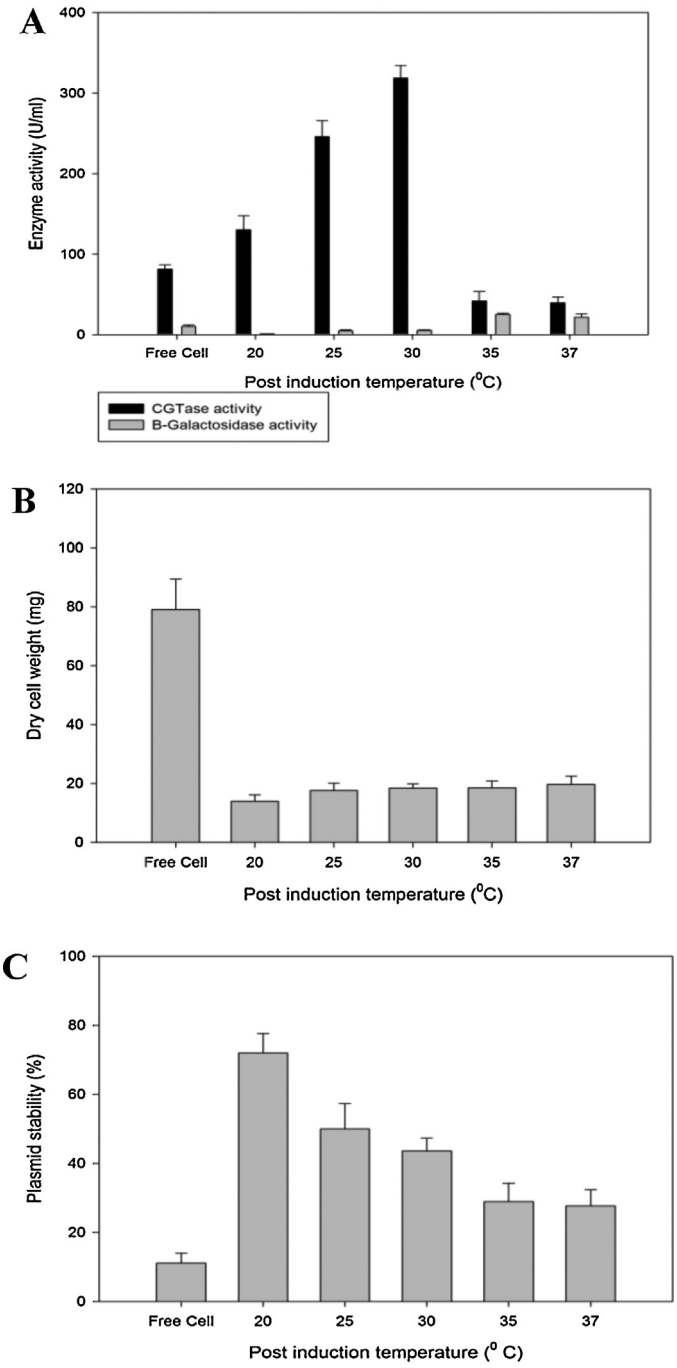

Fig. 4. Effect of post induction temperature on immobilized and free cell. (A) Enzymes activity (B) cell density (C) plasmid stability. The polymer membrane and medium used were PVDF and SOB, respectively. Enzymes activity was measured after 24 h of post induction time using 0.01 mM IPTG.

The results presented in Fig. 4B indicate that an increase in cell density will promote CGTase excretion. The cell density was only 13.90 mg at 20 °C (129.95 U/ml of CGTase activity), followed by 17.53 mg at 25 °C (245.94 U/ml of CGTase activity) and 18.38 mg at 30 °C (318.80 U/ml of CGTase activity). Thus, there is a parallel relationship between cell density and CGTase excretion. However, CGTase excretion decreased when the temperature was increased beyond 30 °C, even though the cell density was higher at 35 °C (18.44 mg) and 37 °C (19.59 mg). Although the growth of bacteria

Table 2
Comparison of CGTase expression from various microorganisms.

Microorganism	CGTase expression (U/ml)	Type of system	References
Recombinant *Escherichia coli*	339.72	Immobilization (hollow fiber membrane)	This study
Recombinant *Escherichia coli*	6.37	Immobilization (cotton)	[20]
Bacillus pseudalcaliphilus 20 RF	161.00	Immobilization (polysulfone membrane)	[10]
Klebsiella pneumoniae pneumonia AS-22	32.50	Free cell	[55]
Bacillus G-1	35.71	Free cell	[52]
Recombinant *Escherichia coli*	170.00	Free cell	[23]
Recombinant *Saccharomyces cerevisiae*	0.28	Free cell	[56]
Thermoanaerobacter sp. P4	0.17	Free cell	[57]
Thermococcus sp.	0.19	Free cell	[58]

was favored at these temperatures, inclusion body formation also tended to increase [52].

The effect of post induction temperature on plasmid stability in recombinant *E. coli* is shown in Fig. 4C. It is evident from this figure that the temperature significantly affected the stability of the recombinant plasmid. When the temperature was 20-30 °C, the plasmid was maintained stably (above 40%), whereas at higher temperatures (35 and 37 °C), the plasmid stability was significantly decreased. Plasmid stability decreases more rapidly because of the loss of flagella at higher cultivation temperatures [53], which reduces biofilm formation. The flagella are reportedly involved in microbial cell attachment for biofilm formation by overcoming the repulsive forces between the cells and matrix [54].

Comparisons between immobilized and free cells regarding CGTase excretion, cell viability and plasmid stability were conducted using the optimal temperature for immobilized cells (30 °C). The CGTase activity in the immobilized cells (318.80 U/ml) was found to be 4-fold higher than in free cells (81.51 U/ml) during 24 h of expression, even though the biomass of immobilized cells (18.69 mg) was lower than that of free cells (79 mg). Moreover, cell lysis in immobilized cells was only 5.11 U/ml, which was approximately 51% lower than that in free cells (10.34 U/ml). The plasmid stability recorded in immobilized cells was 43.96%, representing a 295% increase compared with free cells (11.14%), as shown in Fig. 4C. Therefore, it can be concluded that the immobilization method provides an ideal environment for microorganisms, especially for growth and expression.

4. Conclusion

This study indicated that the immobilization of recombinant *E. coli* using a hollow fiber membrane enhances CGTase excretion and cell stability compared with a free-cell system. The optimum amount of CGTase excreted by immobilized cell was 339.72 U/ml, approximately 6.6-fold higher compared to free cell culture (51.20 U/ml). It is clear from this work that a porous hollow fiber membrane is a suitable matrix for the immobilization of recombinant *E. coli*. The results also indicated that the maintenance of a plasmid-containing cell population in an immobilized cell system should be easier than that in a free-cell culture, thus resulting in more effective excretion of CGTase.

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