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Effect of Tryptone Concentration on Cyclodextrin Glucanotransferase (CGTase) Excretion and Cell Lysis of Immobilized Recombinant *Escherichia coli*

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Abstract The recombinant enzyme excretion into the culture medium provides significant advantages over cytoplasmic expression. Nevertheless, the problems encountered during the excretion of recombinant enzyme are the plasmid instability and occurrence of cell lysis. Various attempts have been made to improve the recombinant enzyme excretion and plasmid stability with the low occurrence of cell lysis. The approaches include the modification of the nitrogen sources in the medium such as tryptone, the use of cell immobilization system and lowering the induction temperature. In the present study, the effects of different tryptone concentrations (1, 5, 10, 20 and 30 g/L) as nitrogen source in super optimal broth (SOB) medium on CGTase excretion and plasmid stability as well as cell lysis of the immobilized cell were studied. The recombinant *E. coli* was immobilized on polyvinylidene fluoride polymer (PVDF) hollow fiber membrane. The immobilized cells were expressed using 0.011 mM IPTG at 25°C, 200 rpm of agitation rate and pH 8.8 for 24 h of post induction time. The use of low tryptone concentration (5 g/l) produced high CGTase excretion (758.64 U/ml) and increased the plasmid stability (86% increment) with reduction of cell lysis (90% reduction) in comparison with the initial tryptone concentration (20 g/l). Hence, low concentration of tryptone could reduce the cost for CGTase production due to low amount of tryptone used in the fermentation process.

1. Introduction

Excretion of recombinant protein into the culture medium would be more advantageous than intracellular protein production because of the purification processes for target proteins would be



simplified and the improvement of the product quality, including solubility and stability. However, the recombinant protein excretion always relates to the occurrence of cell lysis [1]. Therefore, by using cell immobilization techniques, increased protein excretion and reduced in cell lysis can be achieved. The excretion of recombinant protein by the immobilized cells offers several advantages over free cells culture, including the reusability of the cells, enhanced plasmid-bearing cells stability and the enhancement of productivity [2-3]. Nevertheless, it is well known that the good performance of immobilized cells systems depends on the right selection of the immobilization matrix and the cultural conditions of the systems such as pH and temperature. The medium components also have a major influence on the production of recombinant protein especially in the immobilization system.

In the present study, a hollow fiber membrane was employed as a matrix for cell immobilization to increase the CGTase excretion and plasmid stability with reduce the occurrence of cell lysis. The polyvinylidene fluoride (PVDF) hollow fiber membrane was used as an immobilization matrix due to high CGTase excretion and plasmid stability with low occurrence of cell lysis compared to other polymer of hollow fiber membrane [4]. Cyclodextrin glucanotransferase, CGTase (EC 2.4.1.19) is an important industrial enzyme used for the production of cyclodextrin (CD) with the presence of starch [5]. CD have a cylindrical shape with a hydrophobic properties inside and a hydrophilic properties outside. Thus, they are able to form inclusion bodies with many hydrophobic molecules, changing their physical and chemical properties. The ability of these cyclic products to form inclusion complexes with many organic and inorganic compounds makes them widely studied for use in several sectors of the industry such as pharmaceuticals, food, cosmetics, agrochemical and textile [6-7].

Generally, high plasmid stability always correlates with high recombinant protein production. The plasmid instability has often been reported to cause significant losses of heterologous protein production by recombinant cells [4] due to the tendency of recombinant cells to lose their engineered characteristics during fermentation [5]. Silva et al. [8] reported that, the low concentration of tryptone could be used to overcome this limitation by favoring to attain the quality of plasmid, promote the plasmid yield and keeping the desirable plasmid structure. Tryptone is composed of amino acids mixture and used as the nitrogen source for the microbial cells. A study conducted by Wrobel and Wegrzyn [9], Ramerez and Bentley [10] and Young and Marchini [11] reported that the amino acid significantly affected the plasmid and product yield in the recombinant cell.

To the best of our knowledge, the effect of tryptone concentration on the excretion of CGTase by the immobilized recombinant *E. coli* has not been investigated. In the present study, the effect of tryptone concentration on the CGTase excretion, plasmid stability and cell lysis of the immobilized recombinant *E. coli* on PVDF hollow fiber membrane was studied.

2. Material and methods

2.1 Bacterial strain and cell immobilization

The recombinant *Escherichia coli* strain carrying cyclodextrin glucanotransferase (CGTase) from *Bacillus* G1 was constructed previously by Jonet et al. [12]. *E. coli* JM109 strain and *E. coli* BL21 (DE3) strain were chosen to be the hosts for the cloning and expression respectively, of the constructed plasmid used in this study. pET systems from Novagen were used as the vector backbones for cloning. The polyvinylidene fluoride (PVDF) hollow fiber membranes (50 cm) were chopped to the required size (5 cm length) and then transferred into Luria Bertani broth (LB, 10g/l tryptone, 5g/l yeast extract, 5g/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, the hollow fiber membranes were washed with the sterile water to eliminate the non-immobilized cells. The immobilized cells were then transferred to 250-ml flasks containing 50 ml of super optimal broth, SOB (20 g/l tryptone, 5 g/l yeast extract, 0.5 g/l NaCl, 0.186 g/l KCl and 2.4 g/l MgCl₂).

2.2 Effect of tryptone concentration on CGTase excretion and cell lysis of immobilized cell

The effect of tryptone concentration in SOB medium on CGTase excretion, plasmid stability and cell lysis of the immobilized cell was studied using concentrations of 1, 5, 10, 20 and 30 g/l. The initial concentration of tryptone (20 g/l) was used as a control. The immobilized cells were expressed on PVDF hollow fiber membrane using optimized cultural conditions, 0.011 mM IPTG, 25°C and pH 8.8 for 24 h of post induction time at 200 rpm of agitation rate [13].

2.3 Analytical methods

2.3.1 CGTase activity. CGTase activity was determined using the phenolphthalein assay [14]. One milliliter of substrate buffer containing 40 mg/ml soluble starch in 0.1 M phosphate buffer, pH 6.0 was added to 0.1 ml protein sample and incubated 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) of phenolphthalein in 5 mM sodium carbonate was added to the reaction mixture and incubated 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 min under the experimental condition.

2.3.2 β-Galactosidase activity (cell lysis). Cell lysis was quantified by determining the amount of β-galactosidase in the extracellular medium using 0- nitrophenyl-β-D-galactopyranosid (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 with 0.1 ml of sample before it was incubated in 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 moles of ONP per min under the experimental condition.

2.3.3 Plasmid stability. The plasmid stability was determined from the ratio of the colony counts on the selective medium agar plate and on the non-selective medium agar plate [15]. Immobilized cells were washed away from the hollow fiber membrane by vortexing for 2 min in a test tube containing 10 ml of sterile distilled water. Cell samples were diluted to determine colony counts within the range of 30-300. All plate counts were determined from the average of at least three replicates.

2.3.4 Cell density. The cell density of immobilized cell was determined as Equation (1).

$$X(\text{mg/ml}) = \frac{(W_1 - W_0)}{V} \times 1000 \quad (1)$$

where X is the cell concentration, W₁ is the weight of the dry cell and the dry hollow fiber membrane (g), W₀ is the weight of the dry hollow fiber membrane (g) and V is the total volume of the hollow fiber membrane (πr²l).

3. Results and discussions

Tryptone is composed of amino acids mixture and is commonly used as a nitrogen source for the microbial cell growth. The immobilized recombinant *E. coli* was expressed using SOB medium containing different concentrations of tryptone (1, 5, 10, 20 and 30 g/l). The original concentration of tryptone (20 g/l) in SOB medium was used as a control. The findings showed that lower tryptone concentration (5 g/l) drastically reduced the cell lysis occurrence and increased the plasmid stability without significantly changed the CGTase excretion level compared to the control (20 g/l). As shown in Figure 1, only a slight decrease (8%) in the CGTase excretion level (758.64 U/ml of CGTase activity) was observed when 5 g/l of tryptone was used compared to 20 g/l of tryptone (821.72 U/ml of CGTase activity).

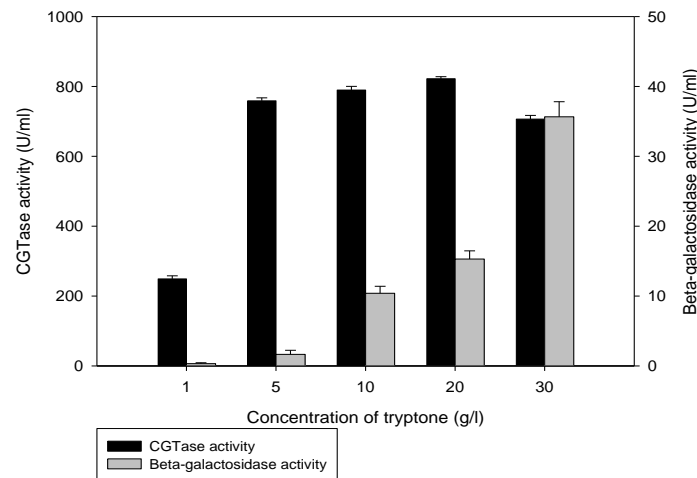


Figure 1. Effect of tryptone concentration on activity of enzymes by the immobilized recombinant *E. coli*. The cells were immobilized on PVDF hollow fiber membrane. The high CGTase excretion with low cell lysis was detected at 5 g/l of tryptone.

The high CGTase excretion was related with the high stability of plasmid due to the high amounts of plasmid-bearing cells, thus resulted in high CGTase expression. As shown in Figure 2, higher plasmid stability of the immobilized cell was observed when 5 g/l of tryptone was used which was 69.57% compared to 25.16% when 20 g/l of tryptone was used. The low nitrogen content in the fermentation medium promoted an extensive synthesis of microbial biofilms [16] thus contributed to better attachment of the cells to the membrane by anchoring the cells to each other and to the surface of the membrane [17]. This phenomenon assisted in improving the plasmids exchange between cells [18], hence increased the plasmid stability of the immobilized cell. As a result, the expression of recombinant enzyme was promoted. Moreover, the extensive synthesis of biofilms at low nitrogen content protected the cell from environmental stress [19] and decreased the possibility of cell lysis to occur. As shown in Figure 1, an 89% reduction of cell lysis was observed when low concentration of tryptone (5 g/l) was used with only 1.65 U/ml of β -galactosidase activity compared to 20 g/l of tryptone with 15.30 U/ml of β -galactosidase activity.

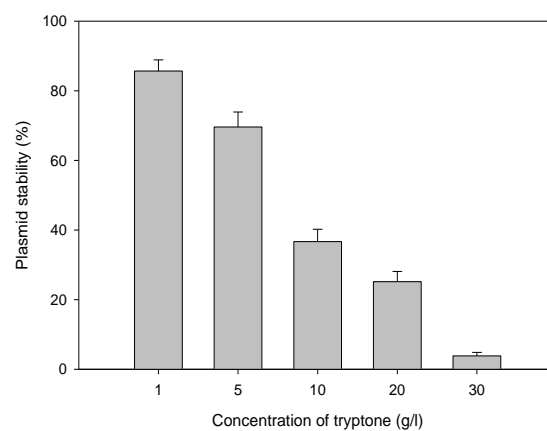


Figure 2. Effect of tryptone concentration on the plasmid stability of immobilized recombinant *E. coli*. The cells were immobilized on PVDF hollow fiber membrane. Highest plasmid stability was observed at 5 g/l of tryptone.

Higher concentration of tryptone affected the recombinant CGTase excretion. As shown in Figure 1, the CGTase excretion dropped from 821.72 U/ml to 706.57 U/ml when the tryptone concentration was increased from 20 g/l to 30 g/l. Tryptone serves as a nitrogen donor for the synthesis of nucleotides, leading to the formation of RNA, DNA and plasmid [20]. It is suggested that, cellular protein synthesis occurred at a proper rate when the concentration of tryptone was low. When the transcriptional and translational processes occurred at its optimum rate, it provided a sufficient time for the plasmid replication and protein synthesis [21]. In contrast, high rate of transcriptional and translational processes were reported to occur when high concentration of tryptone was used, leading to improper plasmid replication and protein synthesis. This phenomenon potentially promoted the formation of inclusion bodies and resulted in high occurrence of cell lysis due to the interference to the translocation pathway and eventually overwhelmed the excretion capability. As shown in Figure 1, the highest occurrence of cell lysis was detected in medium containing 30 g/l of tryptone with 35.64 U/ml of β -galactosidase activity. When high cell lysis occurred, the plasmid stability was significantly decreased to 3.85% (Figure 2) because of the reduction in the total plasmid-containing cells.

A study carried out by Vijayalaxmi et al. [22] showed that among the nitrogen sources tested, the peptone (86.8 U/ml activity) and feather hydrolyzate (108.4 U/ml activity) had a significant effect on the production of extracellular mannanase by immobilized *Bacillus halodurans* in sodium alginate beads. The nitrogen sources used in this study were ammonium sulfate, ammonium nitrate, ammonium chloride, casein, urea, tryptone, peptone, beef extract, yeast extract and feather hydrolysate. In addition, no mannanase was detected by the immobilized *Bacillus halodurans* when using tryptone as a nitrogen source.

Tryptone concentration also affected the cell density of the immobilized cell. As shown in Figure 3, the cell density of immobilized cell was reduced from 134.57 mg/ml to 104.73 mg/ml when the tryptone concentration was reduced from 20 g/l to 5 g/l. In contrast, the cell density of the immobilized cell was increased up to 169.70 mg/ml when 30 g/l of tryptone was used compared to 20 g/l of tryptone. The nitrogen source is incorporated into the cell mass in the form of proteins and nucleic acids, which constitutes 40% to 70% of cell dry weight [17]. Therefore, low concentration of tryptone (as nitrogen source) reduced the formation of proteins and nucleic acids in the cell, thus resulted in the decreased cell density of the immobilized recombinant *E. coli*. Therefore, tryptone concentration with 5 g/l was selected as the optimal concentration due to the high CGTase excretion and plasmid stability with low cell lysis.

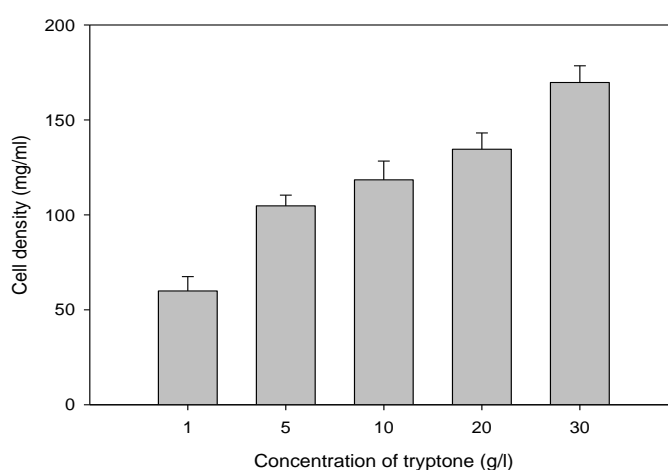


Figure 3. Effect of tryptone concentration on cell density of the immobilized recombinant *E. coli*. The cells were immobilized on PVDF hollow fiber membrane. The cell density of the immobilized cell was proportional to the tryptone concentration.

Gupta et al. [23] studied the effect of different nitrogen sources such casein, peptone, urea and gelatin on the production of α -amylase by using immobilized *Aspergillus niger*. The study found that the peptone produced the highest amount of α -amylase followed by urea, casein and gelatin. The study also stated that all the nitrogen sources with 0.03% concentration were used to assist the growth of immobilized cells and inducing the α -amylase production.

4. Conclusions

As a conclusion, 5 g/l was chosen as the optimal concentration of tryptone in SOB medium in the present study due to the high CGTase excretion (758.64 U/ml), high plasmid stability (69.57%) and low occurrence of cell lysis (1.65 U/ml of β -galactosidase activity) of the immobilized recombinant *E. coli*. In addition, the use of low tryptone concentration could also decrease the fermentation cost because of the high price of tryptone, which is a major component in SOB medium.

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