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Heating cell immobilization of *Streptomyces griseus* and its variant for economical fructose production

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ABSTRACT

Aims: This study determined the optimum temperature for cell immobilization, the optimum time of fructose production by immobilized cell, and immobilized cell stability against repeated use in fructose production.
Methodology and results: Research on cell immobilization of *Streptomyces griseus* and the variant have been done. The *S. griseus* variant was resulted from UV mutation. The variant was able to produce fructose as hydrolysis product 3 times as much after 30 min. Heating was done at 50, 60, 70, 80 and 90 °C. The fructose production was performed at intervals of 4 h for 32 h. The results showed that the optimum cell immobillization temperature of *S. griseus* and its variant was 80 °C. The optimum time of fructose production by immobilizing cell of *S. griseus* was 28 h and its variant cells was 24 h. Immobilized cells of *S. griseus* can be reused for 6 × 28 h to produce fructose compared to variants cells was 5 × 24 h, respectively.
Conclusion, significance and impact of study: This study reported that immobilized cells of *S. griseus* can be reused and its variant were highly advantageous in the production of fructose. The amount of fructose production was increased as compared to the conventional method and the cost of production could be reduced as well.

Keywords: Cell immobilization, fructose, *Streptomyces griseus*, variant

INTRODUCTION

The high demands for sugars and the development of enzyme technology have increased the production of sweeteners, especially for glucose and fructose (Silva *et al*., 2010). Fructose is sweeter than glucose and safer for sugar metabolism. Moreover, D-fructose plays an important role as a diabetic sweetener because it is slowly reabsorbed by the stomach and does not influence the glucose level in the blood (Bhosale *et al*., 1996). Fructose can be produced from starch through the enzymatic hydrolysis and isomerization. The hydrolysis product of glucose then isomerized by glucose isomerase to produce HFS (High Fructose Syrup). HFS is used worldwide as a sweetener in canned goods, baked goods, processed foods, dairy products, and carbonated beverages. According to Vuilleumier (1993) HFS is preferred to be used in food industry because it does not pose the problem of crystallization as sucrose does**.** The glucose isomerase can be isolated from *Streptomyces*, *Bacillus*, *Actinoplanes* and *Arthrobacter* species.

The study using *Streptomyces griseus* was done by: (a) Puspaningsih *et al*. (1992), to simplify the fructose production stage of starch through the process of co-immobilization of glucoamylase enzyme with alginate matrix; (b) Purkan and Purwaningsih (1997), to optimize glucose isomerase production by the addition of xylose and glucose inducers; and (c) Widianti (1999), to increase the production of glucose isomerase by UV mutation. The mutation revealed that *S. griseus* variant were able to produce glucose isomerase 3 times as much after 30 min.

The conversion of glucose to fructose by using free Glucose Isomerase (GI) is not economical because the enzyme is intra-cellular and therefore the purification process is very difficult and expensive. This problem could be solved if the enzyme is immobilized. Current production of high-fructose syrups generally uses immobilized, rather than soluble enzymes according to Saxena (2016). The advantage of immobilized GI enzyme is its long life in the support material and the ability to be reused. Research by Hobbs (2009) showed that, it is possible to produce high-fructose syrups containing 42%,

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55% or 90% fructose by using immobilized enzyme technology.

The immobilization of whole microbial cells and their applications to bioprocessing have been of interest for nearly thirty years. Immobilization of whole cells for extra-cellular enzyme production offers several advantages, such as the ease to separate cell mass from the bulk liquid for possible reuse, facilitating continuous operation over a prolonged period, enhancing reactor productivity and ensuring higher efficiency of catalysis (Kar and Ray, 2008). Furthermore, the use of immobilized cells as industrial catalysts can be advantageous compared to batch fermentation process (Adinarayana *et al*., 2005).

Recently, the immobilized whole cell has been regarded as an alternative method of enzyme immobilization. Immobilization of enzymes is a tedious and time-consuming process. Moreover, the cost of isolation and purification of enzymes is high which is not necessary in whole cell immobilization (Yang *et al.*,1988; Elakkiya *et al*., 2016.). By heating the microbial cells on cell immobilization, it can act as a matrix that isolates enzymes for repeated isomerization reactions (Takasaki *et al*., 1969). Heating cell immobilization will be done in this study using *S. griseus* and its variant, to produce fructose.

The aims of this research were to determine the optimum temperature for cell immobilization by cell heating, the optimum time of fructose production by immobilized cell, and immobilized cell stability against repeated use in fructose production.

MATERIALS AND METHODS

Microorganism and culture

The strain of *Streptomyces griseus* and its variant was obtained from the Biochemistry Laboratory, Department of Chemistry, Faculty of Science and Technology Universitas Airlangga. All chemicals were analytical grade purchased from Merck (Germany), Becton Dickinson (France) and Sigma Aldrich (USA).

Solid medium consists of 1% D-glucose, 0.02% $MgSO_4 \cdot 7H_2O$, 0.5% yeast extract and 1.7% bacto agar. The medium was sterilized by autoclave at 121 °C for 15 min. The culture plates were incubated for 5-7 days at 30 °C. For GI production, the spores were transferred to 100 mL of liquid medium consisting of 0.5% yeast extract, 0.3% peptone, 0.3% casein, 0.02% $MgSO_4 \cdot 7H_2O$, and 1% xylose. After incubation under constant shaking (175 rpm) for 42 h at 30 °C, the pellet cell was collected by centrifugation at 3000 rpm for 15 min and washed with 0.85% NaCl.

Cell immobilization of *S. griseus* and its variant

The pellet cells of *S. griseus* and its variant were suspended into phosphate buffer pH 7. Each 5 mL of cell suspension in a covered reaction tube was heated at 80 °C for 24 h in a water bath. The pellet cell was collected by centrifugation at 3000 rpm for 5 min. The pellets were referred as immobilized cell which was tested for enzyme activity. The activity was determined by incubating the immobilized cell and 5 mL of the substrate (0.1 M D-glucose and 0.01 M $MgSO_4 \cdot 7H_2O$ in phosphate buffer pH 7) in a closed reaction tube with stirring at 65 °C for 5 min and determined its concentration by cysteine-carbazole method (Bhatia and Prabhu, 1980).

Determination of optimum temperature of immobilized *S. griseus* cell and its variant

Immobilized cell of *S. griseus* and its variant were suspended into phosphate buffer pH 7. Each 5 mL of cell suspension in a covered reaction tube was heated at different temperature (50, 60, 70, 80, dan 90 °C) for 24 h in a water bath. Immobilized cell separated by centrifugation at 3000 rpm for 20 min and was ready for activity detection. The activity was measured by incubating the immobilized cell and 5 mL of the substrate (0.1 M D-glucose and 0.01 M $MgSO_4 \cdot 7H_2O$ in phosphate buffer pH 7) in a closed reaction tube with stirring at 65 °C for 5 min and determined its concentration by cysteine-carbazole method (Bhatia and Prabhu, 1980).

Determination of optimum time for fructose production by immobilized cell

Optimum time for fructose production was measured by incubating immobilized cell (product of optimum temperature of immobilized cell) and 10 mL of substrate in a covered Erlenmeyer with stirring at 65 °C. The fructose production was taken for every 4 h and determined its concentration by the cysteine-carbazole method (Bhatia and Prabhu, 1980).

Stability of immobilized cell

The stability of repeated usage of immobilized cell was determined by incubating immobilized cells (products of optimum immobilization temperature) and 5 mL of the substrate in a covered Erlenmeyer flask with stirring at 65 °C. The optimum time for fructose production was fixed to 32 h. Furthermore, immobilized cell was washed with phosphate buffer pH 7 and can be reused for next application until GI activity in immobilized cells was low. This can be seen from the concentration of fructose by the method of cysteine-carbazole (Bhatia and Prabhu, 1980).

Determination of fructose concentration

The fructose concentration was determined by the modified method of cysteine-carbazole (Dische and Borenfreud, 1951). This method is specific for the fructose concentration of 10-60 µg/mL. The reaction was done by mixing of fructose with 1 mL of 2% L-cysteine HCl follow by addition of 5 mL 75% H_2SO_4 and stirred. The reaction is carried out at a temperature of 4 °C. Then 0.15 mL of 0.12% carbazole was added into solution and stirred. The reaction mixture was heated at 40 °C for 30 min, moved

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to 4 °C, then kept the reaction at room temperature for 4 min. The colour formation was measured by absorbance at wavelength range 560-565 nm. The standard fructose curve was made with varying concentrations of 5-30 µg/mL.

RESULTS AND DISCUSSION

Production of GI enzymes in *S. griseus* cells and its variant

The GI enzyme is intra-cellular and inductive enzyme (Bhatia and Prabhu, 1980). To produce a maximum GI enzyme, an appropriate inducer is required. Purkan *et al*. (1997) reported that the xylose inducer increased GI production compared to the glucose and glucose-xylose mixed inducers. Xylose also act as carbon source, while nitrogen sources are obtained from peptone, casein hydrolysate and yeast extract. Phosphate is used to synthesize nucleic acids and as a source of energy. Magnesium and sodium are used for cell wall synthesis, nucleic acid and maintaining membrane structure (Crueger and Crueger, 1989). Mg^{2+} also serves as an activator (Godfrey and Reichelt, 1983).

Puspaningsih *et al*. (1992) determined the growth curve of *S. griseus* using dry weight measurement. The result showed that the maximum dry weight cell growth of *S. griseus* was 42 h, while *S. griseus* variant can be harvested after 10 h respectively (Widianti, 1999). During cell harvest, the GI activity of *S. griseus* was the highest. The growth difference between *S. griseus* and its variant occurs due to the errors on translation process, thus causing important proteins/enzymes needed for cell metabolism during the growth phase are not functional (Puspaningsih, 1995).

Determination of optimum temperature of immobilized *S. griseus* cell and its variant by heating

The breakdown of cells without destruction of the enzyme makes the GI more expensive (Bailey and Ollis, 1986). Therefore, in this study we used microbial cells, for immobilization process that will be simpler and economical (Jack and Zajic, 1977).

Determination of optimum immobilization temperature is important for generating high GI activity in immobilized cells. The amount of activity can be known from the concentration of fructose as the product. Cell immobilization has some advantages when compared with free cell culture. The reaction speed can be accelerated, a high dilution rate can be used in continuous fermentation without cell washing, and it is less susceptible to the effect of inhibitory compounds and nutrient depletion (Marques *et al*., 2006). The main advantage of immobilization Is the low sensitivity of cells to temperature and pH when compared to free enzymes (Lee *et al*., 1984). The process of immobilization was done by heating. Takasaki *et al*. (1969) reported that the type of microbial cell immobilization by heating without chemical treatment. When *Streptomyces sp* cells having high GI activity was heated at 60-85 °C for 2-20 min, the GI will be retained within the cell. It was stated that the enzyme trapped in the cell would not come out of the cell, although the cells were heated for a long time and the conditions were suitable for the extraction of enzymes from cells with autolysis (Chibata, 1978). Heating causes inactivation of other enzyme intrusions that contribute to autolysis and retains GI enzymes, thus immobile cells may be used repeatedly (Bhatia and Prabhu, 1980).

In this study, immobilized cell temperature of *S. griseus* varied between 50-90 °C. The result of optimum immobilization temperature can be seen in Table 1, while the optimum immobilization temperature of *S. griseus* cell and its variant were 80 °C.

Determination of optimum time of fructose production by immobilized cell

The enzymatic production of fructose can be obtained by isomerization of glucose by isomerase glucose enzyme (GI) (Bailey and Ollis, 1986). The determination of the optimum time of fructose production is very important in producing high fructose levels. In this condition, the GI in immobilized cells will work on the optimum activity in converting glucose to fructose.

The immobilized cell was contacted with the substrate at 65 °C with stirring. The concentration of fructose production at an interval of 4 h was determined. The optimum time for fructose production by immobilized cell is shown in Table 2. It showed that the optimum time of fructose production of *S. griseus* immobilized cell was 28 h and its variant was 24 h. GI catalyze the reversible reaction of glucose to fructose, and reverse fructose to glucose until reach the certain equilibrium point (Wiseman, 1985).

Table 1: Data of immobilization temperature variation by heating.

Temperature (°C)	Fructose concentration (µg/mL)	
	S. griseus	Variant
50	37.7164 ± 0.0265	63.9398 ± 0.0346
60	43.3582 ± 0.0200	94.9716 ± 0.0400
70	43.6716 ± 0.0346	96.5479 ± 0.0265
80	51.7313 ± 0.0265	101.6708 ± 0.0220
90	43.1940 ± 0.0344	95.3657 ± 0.0426

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Table 2: Data of optimum time of fructose production by immobilized cell.

Fructose production time (h)	Fructose concentration (µg/mL)	
	S. griseus	Variant
4	57.9851 ± 0.0344	121.7686 ± 0.0368
8	81.0746 ± 0.0256	139.1078 ± 0.0448
12	100.4478 ± 0.0440	152.9064 ± 0.0374
16	122.4179 ± 0.0326	167.0870 ± 0.0486
20	135.8806 ± 0.0460	176.9388 ± 0.0382
24	151.4328 ± 0.0512	200.1892 ± 0.0444
28	172.9851 ± 0.0442	184.0322 ± 0.0354
32	94.4029 ± 0.0346	-

Table 3: Data of repeatedly used of immobillized cell.

Number of uses	Fructose concentration (µg/mL)	
	S. griseus	Variant
1	179.4925 ± 0.0224	124.3301 ± 0.0342
2	167.6119 ± 0.0376	122.1627 ± 0.0466
3	142.4179 ± 0.0324	121.1775 ± 0.0368
4	135.9701 ± 0.0422	116.2516 ± 0.0426
5	131.0746 ± 0.0478	93.7894 ± 0.0488
6	87.4925 ± 0.0386	90.0457 ± 0.0342

Stability of immobilized cells

The immobilized cell stability test for repeated use was conducted to find out how long the immobile cell can be used for the isomerization reaction of glucose to fructose. The results of stability testing of immobilized cells against repeated use in fructose production is shown in Table 3. The immobilized cells of *S. griseus* cell can be used repeatedly for fructose production for 6 × 28 h, and its variant for 5 × 24 h.

From the Table 3, we observed that enzyme activity decreased as the immobilized cells were used continuously. This could be due to changes in the conformation of the enzyme's active center caused by too frequent contact with the substrate. In the first application, the conformation of the active center of the enzyme is allegedly slightly altered and the permanent damage occurs when too often contacted with the substrate. This situation causes the decline in the activity (Tyasrini, 1994). The results of this study indicated that the reused immobilized cells are very advantageous in the production of fructose, because in addition to reduce costs, the fructose produced is also high.

CONCLUSION

The optimum cell immobillization temperature of *S. griseus* and its variant was 80 °C. The optimum time of fructose production by immobilizing cell of *S. griseus* was 28 h and its variant cells was 24 h. Immobilized cells of *S. griseus* can be reused for 6 × 28 h to produce fructose compared to variants cells was 5 × 24 h, respectively. Cell immobilization of *S. griseus* and its variant by heating is potentially used for fructose production.

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