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Research Note

Ethanol Production from Glycerol by the Yeast *Pachysolen tannophilus* Immobilized on Celite during Repeated-Batch Flask Culture

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Abstract We investigated a novel process for production of ethanol from glycerol using the yeast *Pachysolen tannophilus*. After optimization of the fermentation medium, repeated-batch flask culture was performed over a period of 378 hr using yeast cells immobilized on Celite. Our results indicated that the use of Celite for immobilization of *P. tannophilus* was a practical approach for ethanol production from glycerol, and should be suitable for industrial ethanol production.

Keywords Celite, Ethanol production, Glycerol, Immobilization, *Pachysolen tannophilus*

Glycerol has become an abundant byproduct of the transesterification reaction of the natural oils and fats used as feedstock for biodiesel production [1, 2]. There have been numerous studies on the conversion of glycerol into valuable products [3, 4], including the conversion of biodiesel-derived glycerol to ethanol for the complete conversion of the oil and fat feedstock to biofuels [5-7]. Economic analysis revealed that the cost of ethanol production using glycerol as the substrate was lower than that for production using corn starch [8].

Bacteria and yeast can convert glycerol to ethanol; however, the metabolic pathways involved are very different [6-8]. The yeast *Pachysolen tannophilus* converts glycerol to ethanol under controlled aeration conditions [6, 7], and involves the activity of the mitochondrial electron transport chain [9, 10], thus a limited air supply is required.

In this study, we investigated a novel process for ethanol production from glycerol using the yeast *P. tannophilus* with the goal of developing a more practical process for ethanol production from glycerol. First, the medium for ethanol production from glycerol was optimized on the basis of the cost-effectiveness of the medium ingredients, such as the use of industrial-grade yeast extract and corn steep liquor (CSL). Secondly, we immobilized the yeast cells on Celite (a low cost commercial diatomaceous earth immobilization substrate) by adsorption, and used the immobilized yeast to perform repeated-batch culture for ethanol production from glycerol. Celite has been used previously as an immobilization carrier for yeast cells and enzymes [11, 12]. However, the immobilization of *P. tannophilus* on Celite has not previously been reported and there have been no reports on the immobilization of *P. tannophilus* for ethanol production from glycerol. Finally, we evaluated the effectiveness of Celite as an immobilization carrier during repeated-batch culture, as compared to cell recycle using centrifugation.

Glycerol and Celite 545 were purchased from Daejung Chemicals and Materials Co. (Siheung, Korea) and Yakuri Pure Chemicals Co. (Kyoto, Japan), respectively. Yeast extract and peptone were obtained from Becton Dickinson (Franklin Park, NJ, USA). Industrial-grade yeast extract was purchased from Choheung Corp. (Ansan, Korea). Thin-layer chromatography (TLC) plates were purchased

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from Macherey-Nagel GmbH & Co. KG (Düren, Germany). CSL was kindly supplied by Samyang Genex Corp. (Seoul, Korea). All other chemicals used were reagent-grade.

The yeast strain used in this study was *P. tannophilus* ATCC 32691. For the seed culture, the yeast was cultivated in a 100 mL Erlenmeyer flask in YPD (10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose) or YPG medium (10 g/L yeast extract, 20 g/L peptone, and 20 g/L glycerol), in an incubator shaker maintained at 30°C and 150 rpm.

For optimization of ethanol production from glycerol, a series of YCG1 media was prepared containing glycerol concentrations ranging from 5 to 60 g/L, 5.0 g/L industrial-grade yeast extract, 20.0 g/L CSL, 3.0 g/L $(NH_4)_2SO_4$, 2.4 g/L KH_2PO_4, and 1.2 g/L $MgSO_4 \cdot 7H_2O$. In addition, a series of YCG2 media was prepared containing an initial glycerol concentration of 40 g/L in the same media as described for the YCG1 media series. However, for the YCG2 media series, when the glycerol concentration was increased beyond 30 g/L, up to a maximum concentration of 100 g/L, the other ingredients were also increased proportionally. A previous study [13] demonstrated that a balanced batch culture medium could be designed on the basis of this principle.

The initial batch shake flask cultures were performed with YCG2 medium containing 40 g/L glycerol in 100 mL Erlenmeyer flasks at 30°C with shaking at 150 rpm. After the residual glycerol concentration was less than 10 g/L, the medium was replaced with new YCG2 medium containing 40 g/L glycerol using two methods. In the first method, the entire culture was centrifuged to separate the yeast cells and the pelleted yeast cells were transferred to a new flask containing an equal volume of fresh YCG2 medium (40 g/L glycerol). In the second method, when the residual glycerol concentration was less than 10 g/L, Celite was added into the culture broth at a concentration of 2.0 g/L and the culture was allowed to settle for 30 sec, producing a Celite layer containing the yeast on the bottom of the flask. This was followed by removal of the culture broth by pipetting. Afterward, fresh YCG2 medium containing 40 g/L glycerol was added into the flask containing the yeast cells immobilized on Celite. These two medium replacement methods were repeated three times. Scanning electron microscopy (FEI Quanta 400; FEI Co., Hillsboro, OR, USA) was performed on the Celite-immobilized yeast after washing with deionized water and drying for 24 hr at 60°C to fix the yeast cells [14, 15].

The concentration of yeast in the culture media was assessed by measuring the optical density at 600 nm (OD_{600}) using a spectrophotometer (Spectronic; Thermo Scientific, Rockford, IL, USA). The residual glycerol concentration was measured using quantitative TLC [16]. Ethanol concentrations were measured by gas chromatography, as described previously [17]. The specific growth rates of the flask cultures were estimated over a period of zero to 24 hr of culture, and the ethanol yields were estimated by dividing the amount of produced ethanol by the amount of consumed glycerol.

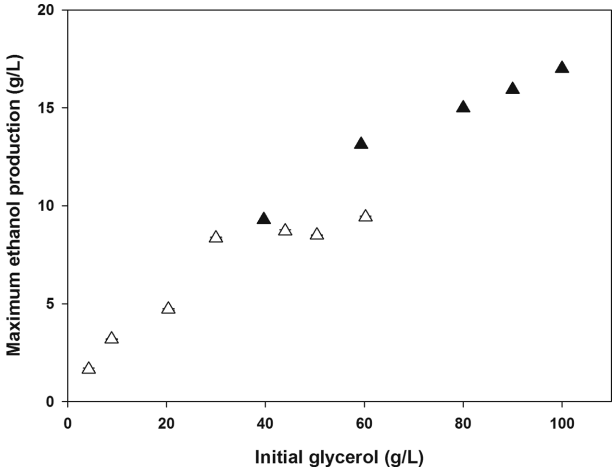

Fig. 1. Maximum ethanol productions from glycerol in the flask culture of *Pachysolen tannophilus*. White symbols (△) indicate the maximum ethanol production using the YCG1 media series. Black symbols (▲) indicate the maximum ethanol production using the YCG2 media series.

To optimize the culture medium, shake flask cultures of *P. tannophilus* were performed using the YCG1 media series and ethanol production was monitored as an indicator for medium optimization. The maximum ethanol production was used to for culture medium optimization because the OD_{600} measurement of cell growth was considered to be somewhat compromised by the dark color and insoluble debris present in the CSL and industrial-grade yeast extract. As shown in Fig. 1, the maximum ethanol production increased gradually with increases in the glycerol concentration up to a glycerol level of 30 g/L. However, the maximum ethanol production reached a plateau after the glycerol concentration in the YCG1 medium was increased beyond 30 g/L, and this was considered to be the result of nutrient deficiency. Therefore, 30 g/L glycerol was used as the basis to determine the optimal concentration of the other ingredients in the YCG2 medium series.

To optimize the ingredient balance of the culture medium, the flask cultures were performed using the YCG2 media series, in which the initial glycerol concentrations were varied from 40 to 100 g/L and the other ingredients were increased in proportion to the increase in the glycerol concentration relative to the concentrations used for the YCG1 series using a glycerol concentration of 30 g/L. As shown in Fig. 1, the maximum ethanol production increased gradually throughout the YCG2 media series indicating a proportional increase in the other ingredients relative to glycerol produced a more balanced medium up to a concentration of at least 100 g/L glycerol.

Although measurement of yeast cell growth based on the OD_{600} was considered somewhat inaccurate, the specific growth rates of the flask cultures were roughly estimated in order to observe the effect of initial glycerol concentration

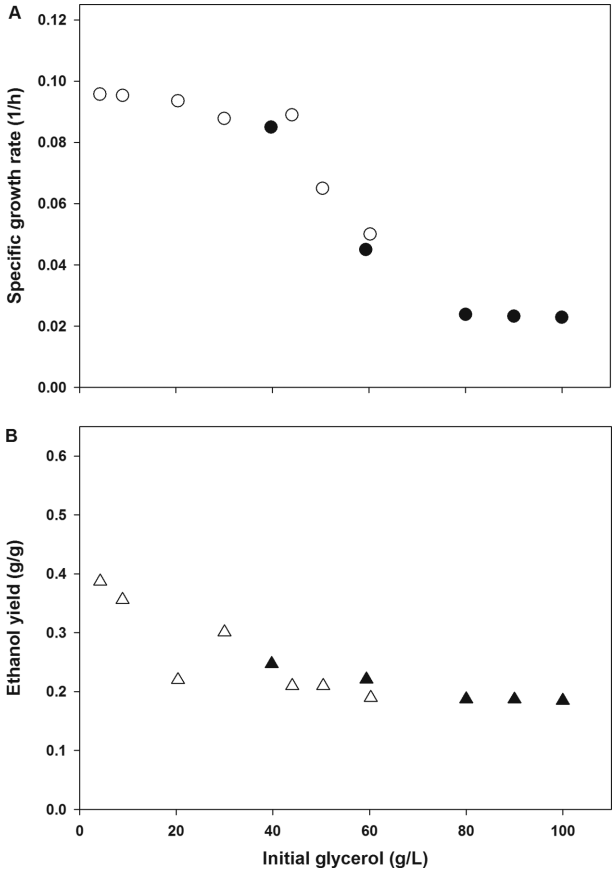

Fig. 2. Specific growth rate and ethanol yield from glycerol in the flask culture of *Pachysolen tannophilus*. White symbols (○, △) indicate specific growth rates and ethanol yields, respectively, using the YCG1 media series. Black symbols (●, ▲) indicate specific growth rates and ethanol yields, respectively, using the YCG2 media series.

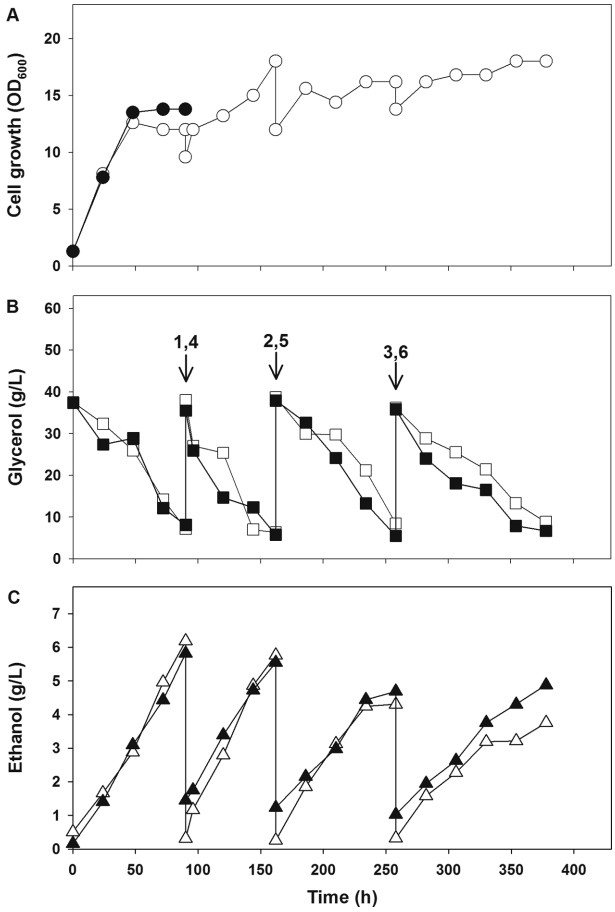

Fig. 3. Repeated-batch flask cultures for ethanol production from glycerol using *Pachysolen tannophilus*. A, Cell growth (OD_{600}) (○, ●); B, Residual glycerol concentration (□, ■); C, Ethanol production (△, ▲). White symbols indicate the repeated-flask culture, where the cells were collected by centrifugation after each repeated-batch culture, with batches represented by the arrows 1, 2, 3 in panel (B). Black symbols indicate the repeated-batch flask culture where 2.0 g Celite was added into the culture broth after the first batch culture was completed, with batches represented by arrow 4 in panel (B). Arrows 5 and 6 in panel (B) indicate the replacement of new medium. Cell growth could not be monitored in the repeated-batch flask culture using Celite after the first batch culture because the added Celite interfered with optical density measurements.

on yeast growth. As shown in Fig. 2, high glycerol concentrations were inhibitory, particularly those > 40 g/L initial glycerol concentration. At concentrations > 40 g/L glycerol, the specific growth rate decreased to < 0.08 1/hr and the ethanol yield decreased by approximately 0.2 g-ethanol/g-glycerol. Therefore, based on the results shown in Fig. 2, to circumvent substrate inhibition in the flask cultures, the maximum initial glycerol concentration used was 40 g/L.

Batch flask cultures using YCG2 medium (40 g/L glycerol) was performed (Fig. 3) by replacing the media three times to produce a series of four batch cultures using the two techniques described above. As shown in Fig. 3A, when the cells were collected by centrifugation, the cell growth increased slowly despite rapid growth in the first batch culture. However, when the cells were immobilized on Celite, the cell growth profile could not be monitored using the OD_{600} because the medium contained Celite, which prevented meaningful optical density measurements. In two repeated-batch cultures, glycerol was consumed until the glycerol concentration was reduced to < 10 g/L over a period of approximately 90 hr during the first batch culture. Afterwards, the glycerol consumption rate decreased gradually (Fig. 3B).

As shown in Fig. 3C, the concentration of ethanol increased to approximately 6.0 g/L over a 90 hr period of the first batch culture. After the first batch culture, ethanol production rates decreased gradually. The maximum ethanol production of the batch cultures were 3.7 to 6.2 g/L when the cells were collected by centrifugation, and 4.7 to 5.8 g/L when the cells were immobilized on Celite. In addition, the average ethanol yield from glycerol with two

repeated-batch cultures was 0.154 g-ethanol/g-glycerol when the cells were collected by centrifugation, and 0.142 g-ethanol/g-glycerol when the cells were immobilized on Celite. A recent report on the results of ethanol production from glycerol by *P. tannophilus* showed that the ethanol yield was 0.28 g-ethanol/g-glycerol [6]. In addition, our previous result showed that the ethanol yield was 0.166 g-ethanol/g-glycerol when YPG medium was used for ethanol production [18]. However, the medium composition and the cultivation methods were very different and therefore, the two results cannot be compared directly with each other. In the present study, the lower ethanol production was a result of incomplete glycerol consumption during the repeated-batch culture, and the lower ethanol yield was considered to be the result of the gradual decrease in the ethanol production observed between repeated-batch cultures.

Liu *et al.* [6] used a chemically defined medium consisting of both yeast nitrogen base without amino acids (Difco, Detroit, MI, USA) and glycerol, while we used CSL, industrial-grade yeast extract, salts, and glycerol for medium ingredients. In other words, Liu *et al.* [6] used an expensive and commercially impractical medium for ethanol production, while we used cost-effective and commercially practical medium ingredients. In this study, we prepared the medium for ethanol production on the basis that a more cost-effective and practical medium could be applicable to the industrial production of ethanol from glycerol.

In a previous study [6], it was observed that a controlled microaeration of 0.05 L/min played an important role in achieving higher levels of ethanol production from glycerol by *P. tannophilus*. Because we were not able to finely control the aeration in the repeated-batch flask cultures, it was anticipated that a lower ethanol production and yield might occur. Therefore, if the repeated-batch cultures were conducted in a fermenter with fine control of the aeration rate, higher levels of ethanol production and yield would be expected.

Interestingly, as shown in Fig. 3, two repeated-batch cultures showed remarkably similar profiles of glycerol consumption and ethanol production. In addition, both ethanol production and ethanol yield from glycerol were shown to be in a relatively similar range. As shown in Fig. 4, photographs and electron micrographs of Celite taken after exposure to the yeast demonstrated that the yeast cells were immobilized on the surface of the Celite, and were present in a configuration somewhat similar to a

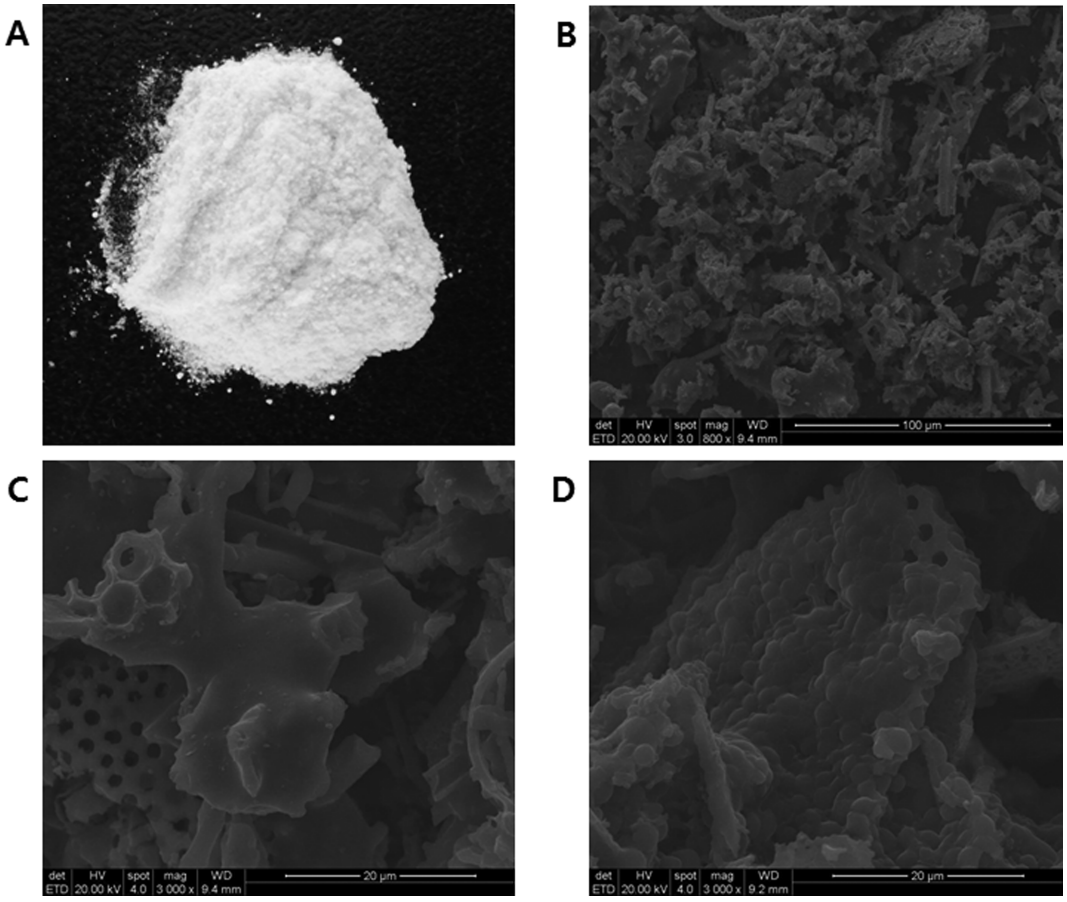

Fig. 4. Photograph of Celite (A), and electron micrographs of Celite (B, ×800; C, ×3,000), and yeast cells immobilized on Celite (D, ×3,000). Samples for observation of immobilized yeast cells were collected after culture of the cells was completed, as shown in Fig. 3.

cluster of eggs.

In conclusion, we developed a novel repeated-batch ethanol production process from glycerol using the yeast *P. tannophilus* immobilized on Celite and industrial-grade medium ingredients (CSL and yeast extract). This process could be a practical approach for use in large-scale commercial ethanol production from glycerol because of the use of inexpensive media ingredients and yeast cells immobilized on Celite. Unfortunately, the levels of ethanol production and yield from glycerol were lower than those previously reported [6]. However, conducting the repeated-batch process in a finely aerated-fermenter would be expected to improve ethanol production and yield.

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