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Ethanol Production from Sago Starch Using *Zymomonas mobilis* Coentrapped with Amyloglucosidase

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동시고정화된 Amyloglucosidase와 *Zymomonas mobilis*를 이용한 전분으로 부터의 Ethanol 생산

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A chitin-immobilized enzyme amyloglucosidase(AMG) and a bacterium *Zymomonas mobilis* were coentrapped in alginate gel beads. Ethanol production was performed in a packed bed column reactor in a simultaneous saccharification and fermentation(SSF) mode using liquefied sago starch as a substrate. It was found that this process eliminated product inhibition and reverse reaction of glucose enhancing the rate of saccharification and ethanol production. At a low dilution rate of $D = 0.11\ hr^{-1}$, the steady-state ethanol concentration was 46.0g/*l* (96.8 % of theoretical yield). The maximum ethanol productivity was 17.7g/*l*,h at $D = 0.83\ hr^{-1}$ when the calculation was based on the total working volume. The continuous production of ethanol was maintained stably over 40 days without problems in this reactor system.

In order to produce ethanol from industrially cheap substrates such as cellulose or starch, a simultaneous saccharification and fermentation (SSF) has been adopted as a means of one-step process. Enzymes, β-glucosidase (EC3.2.1.21, β-D-glucoside glucohydrolase) and amyloglucosidase (EC3.2.1.3, α(1,4)-glucan glucohydrolase) have been employed together with ethanol producing microorganisms such as yeast or a bacterium *Zymomonas mobilis* to produce ethanol directly from cellulosics (1-3) and starches (4-6), respectively, without presaccharification step. When these are used in practice, however, it is necessary that the efficiency of the SSF process should be strengthened to attain high ethanol productivity. The use of immobilized enzymes and/or microbial cells may be one way to achieve this goal.

From this view, we have developed various SSF processes to produce ethanol from starches using AMG and *Z. mobilis* cells by immobilizing only cells (7), immobilizing cells and the AMG enzyme separately (8) or coimmobilizing both cells and the enzyme (9) using various supporting matrices such as sodium alginate (7,8) and κ-carrageenan (9). It was reported that the continuous SSF system where *Z. mobilis* cells were immobilized in alginate beads and the AMG enzyme on chitin separately was most effective with respect to ethanol productivity (8).

In the present investigation, however, we coimmobilized two biocatalysts by coentrapping the chintin-immobilized AMG with *Z. mobilis* cells in alginate beads to further improve ethanol productivity as well as an operational stability.

Key words: Ethanol, coimmobilization, sago starch, chitin, amyloglucosidase
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Materials and methods

Microorganism and materials

The bacterium used throughout the study was *Z. mobilis* ZM4 (ATCC31821). It was maintained on yeast extract-glucose agar slants by subculturing at weekly intervals as already described (10). Sago starch supplied by SIRIM of Malaysia was used as a substrate for fermentation. The total sugar content was 0.93g/g of sago starch (93% expressed as dextrose equivalent). The α-amylase (EC3.2.1.1, α(1,4)-glucan glucanohydrolase) and amyloglucosidase (AMG) were supplied from Novo Industri, A/S Korea. The enzymatic activities of α-amylase and AMG were 120KNU/g and 300AGU / m*l*, respectively, as defined by the manufacturer (11). Sodium alginate was purchased from Junsei Chemical Co. (Tokyo, Japan). Powdered-chitin from crab shells and glutaraldehyde were products of Sigma Chemical Co. (St. Louis, U.S.A).

Immobilization methods

The liquid AMG was added to moistened chitin (water content about 50%) at a proper ratio. The mixture was soaked wet with 1% glutaraldehyde and allowed to stand at room temperature with gentle stirring for 1hr and stored overnight at 4 °C. The product was washed on a sintered glass funnel with distilled water, 3M NaCl and 0.1M acetate buffer, pH4.4.

Z. mobilis cells harvested in the late exponential phase were coentrapped and resuspended in saline solution. Chitin-immobilized AMG and cell suspension were mixed with 4% sodium alginate solution. The mixture was then extruded as small drops using a 5 m*l* disposable tip of a semiautomatic pipette into cold solution containing 0.05M $CaCl_2$. Small beads with ca. 4mm diameter were formed and then allowed to harden in the solution. The immobilized cell concentration was 92g dry cell weight per liter bead volume.

Experimental procedures

Experimental procedures are shown in Fig. 1. After sago starch was mixed with tap water, liquefaction was carried out by adding 0.2% (v/w)

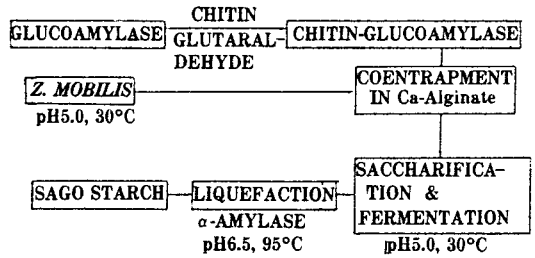

Fig. 1. Schematic diagram showing experimental procedures and conditions.

α-amylase at pH6.5 and 95 °C for 1hr. The fermentation medium was composed of liquefied sago starch, 1.0% yeast extract and 0.3% $CaCl_2$. The midium was sterilized at 121 °C for 20 min and then the pH was adjusted to 5.0 with 0.1N HCl.

The scheme of packed bed reactor was shown in Fig. 2. A column (2.2 cm i.d. × 32 cm long) was used for continuous production of ethanol. Temperature was controlled at 30 °C by water jacket. The beads were aseptically transferred to the column which was previously sterilized. The substrate was continuously fed from the bottom of the column with a peristaltic pump.

Analytical methods

Ethanol concentration of the broth was determined using gas chromatography (12). Reducing

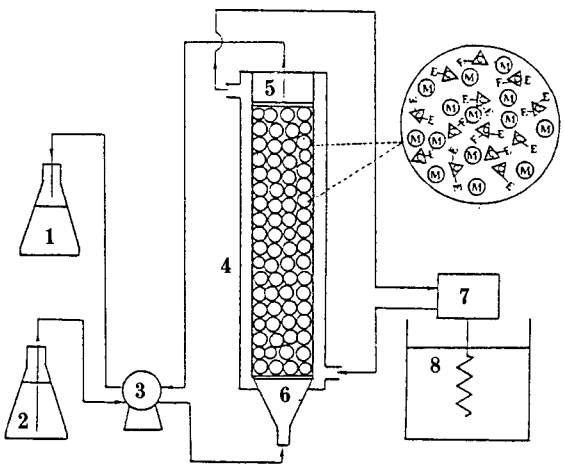

Fig. 2. Diagram of packed bed reactor used for continuous SSF.

1; product reservoir, 2; substrate reservoir, 3; peristaltic pump, 4; glass column, 5; stainless sieve, 6; sintered glass, 7; water circulator, 8; thermostat, C; chitin, E; enzyme, M; microbial cells

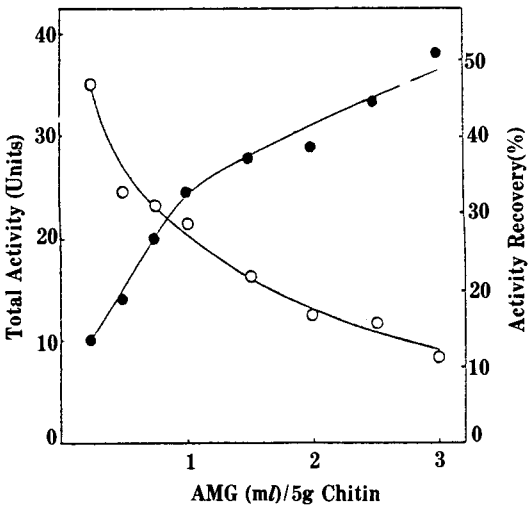

Fig. 3. Effect of AMG concentration on activity of chitin-immobilized enzyme.

● – ●; total activity, ○ – ○; activity recovery

sugars were estimated by dinitrosalicylic acid (DNS) method (13).

AMG activity was measured by determining the amount of reducing sugar released from 10% partially hydrolyzed starch in 0.1M acetate buffer, pH4.4, at 60°C. One unit of AMG activity was defined as the production of one gram of free sugar per hr per volume unit of enzyme (m*l*) at 60°C.

Calculations

The dextrose equivalent (D.E.) value was expressed as percentage of reducing sugars released from starch against the amount of total sugars.

The conversion yield of starch to ethanol was expressed as percentage of sugars converted to ethanol against the amount of total sugars. Ethanol productivity was calculated based on the total reactor volume (V_t=87 m*l*).

Results

Optimum loading of AMG enzyme for immobilization

Experiments were performed in order to determine the optimum loading ratio of AMG and chitin. Various amounts of AMG were added to 5g of chitin. As shown in Fig. 3, the total activity of immobilized AMG on chitin was slightly increased by increasing the amount of the enzyme initially added to the support. Up to 1 m*l* addition of the enzyme, the increasing rate of activity was constant while it was reduced beyond this concentration. The activity recovery was 22.5% at this point but was gradually decreased with an increasing concentration of AMG. To extend the half-life of this immobilized enzyme, chitin was treated with 1% glutaraldehyde for the long term operation.

To determine the optimum amount of chitin in alginate beads, various amounts of AMG-bound chitin was entrapped into alginate gel beads. When the amount of AMG-chitin was increased to 5g, the total enzyme activity linearly increased to 4 enzyme units and was kept nearly constant thereafter (Fig. 4). With chitin concentrations of more than 5 g, the strength of alginate beads became too weak to keep the gel structure intact. In favor of activity retention of AMG, mechanical property of beads and a few economical reasons, the mixing ratio of sodium alginate and chitin was fixed at 10 m*l* of 4% sodium alginate per 1g of chitin bound with 0.2 m*l* of AMG.

Ethanol production using *Z. mobilis* coentrapped with AMG enzyme

Prior to ethanol fermentation, the conversion of sago starch to glucose using chitin-bound AMG en-

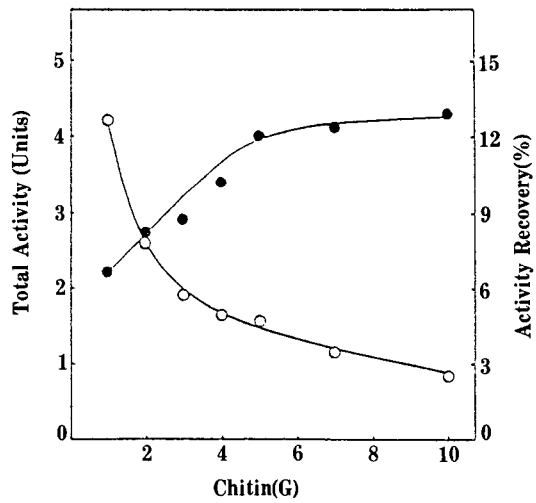

Fig. 4. Effect of chitin concentration on activity of gel-entrapped AMG.

● – ●; total activity, ○ – ○; ativity recovery

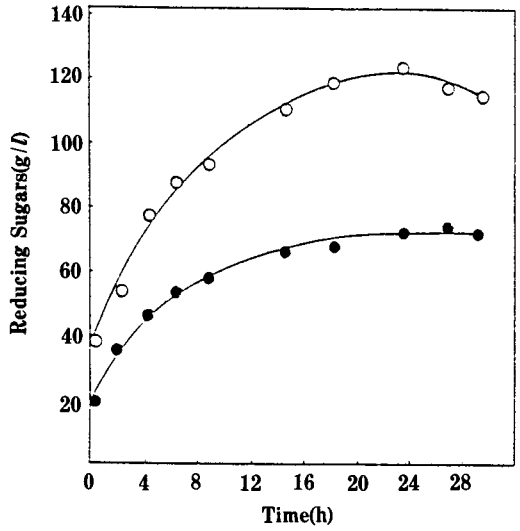

Fig. 5. Effect of substrate concentration on saccharification of sago starch by gel-entrapped AMG.
● – ●; 10% sago starch,○ – ○; 15% sago starch

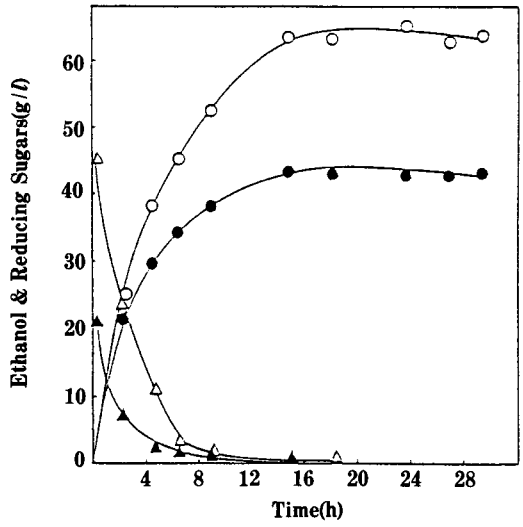

Fig. 6. Batch fermentation of starch to ethanol using AMG coentrapped with *Z. mobilis*.
10% sago starch (● – ● ethanol, ▲ – ▲ reducing sugars), 15% sago starch (○ – ○ ethanol, △ – △ reducing sugars)

trapped in alginate beads was tried. Enzyme reaction was carried out at pH 5.0 and 30 °C with mild shaking. When 10 and 15% of sago starch were used, the maximum D.E values obtained were 70% and 88% respectively in 27 hrs of incubation (Fig. 5).

The ethanol fermentation was carried out in an SSF mode. Batch-run fermentations were performed in flask using a shaking water bath fixed at 30 °C and the initial pH5.0. This was due to the difficulties in keeping alginate beads unfloated without breakage in a fermenter. As shown in Fig. 6, the fermentations were completed in 15 hrs using 10% and 15% liquefied sago starch as a substrate. The conversion yields of total available sugars to ethanol were about 90% of theoretical yield independent of substrate concentration.

For the continuous ethanol production, a packed bed column reactor was filled with 65m*l* beads containing 92g cells, and 15.4m*l* AMG enzyme per liter of beads. These amounts were equivalent to 69g cells and 11.5m*l* AMG per liter of the total reactor volume. In Fig. 7, results of continuous SSF processes are shown. Using 10% sago starch as a substrate, the maximum ethanol concentration of 46g/*l*(96.8%, of theoretical yield) was achieved at a dilution rate $D=0.11h^{-1}$. The maximum ethanol productivity of 17.7g/*l*,h was obtained at $D=0.83h^{-1}$ with 46% of the conversion yield of starch to ethanol. The conversion yield gradually decreased from 97% at $D=0.11h^{-1}$ to 62% at $D=0.47h^{-1}$ while the ethanol productivity increased from 4.9 to 13.7g/*l*, h.

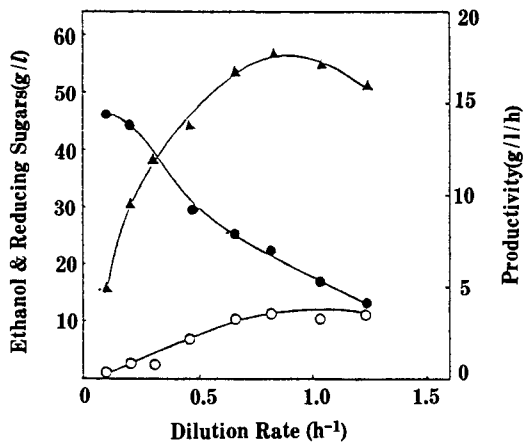

Fig. 7. Continuous fermentation of starch to ethanol using AMG coentrapped with *Z. mobilis*.
▲ – ▲productivity, ● – ● ethanol, ○ – ○ reducing sugars

Discussion

For the immobilization of *Z. mobilis*, κ-carrageenan and sodium alginate have been used as effective supporting matrices (7-9, 14). However, it was reported that κ-carrageenan was not easy to handle in bulk for the coimmobilization of cells and enzymes and the continuous performance of immobilized reactor with this support was poor (9). On the other hand, alginate gels are also too coarse to entrap small molecules such as enzymes. When the AMG enzyme was immobilized within calcium alginate gel beads, about 81% of AMG activity leaked out (8). Although this leakage could be reduced to 30-76% by pretreatment of the enzyme with coupling agents such as carbodiimide or cyanogen bromide, the immobilization methods were still complex and the coupling agents were toxic to cells. Accordingly, AMG was immobilized by crosslinking to chitin prior to entrapment into alginate gels. Chitin has been know particularly suitable for the immobilization of AMG because it contains a specific binding sites for the enzyme (15). It was reported that treatment of chitin with hexamethylenediamine (HEMDA) revealed the improved binding of AMG (16). Considering its toxicity and economical reasons, however, the HEMDA treatment was eliminated in this study.

It was desirable that AMG enzyme could be immobilized as much as possible for the good performance of the coimmobilized cell enzyme reactor. Since the mechanical property of alginate beads was seriously affected by the quantity of chitin entrapped, the volume of the enzyme and chitin was subject to restriction (Fig. 5). When the chitin-bound AMG was entrapped in alginate beads, the activity recovery was only 6%. As shown in Fig. 5, it was also observed that the conversion yield of sago starch into glucose using the re-entrapped AMG was comparatively lower than the case when free AMG was used (D.E. = 95%) (7). It was suggested that the loss of activity and subsequently low conversion rate of the immobilized enzyme resulted from the diffusional limitation of substrate and product. The low D.E. values obtained in the present investigation are likely due to the fact that starch has a large molecular weight. This might have resulted in low diffusion rate of starch within alginate beads. The diffusion limitation, however, could be reduced by increasing the substrate concentration to 15% yielding 88% of conversion.

Ethanol fermentation was carried out in an SSF mode. Since SSF process combines enzymatic hydrolysis of starch into glucose with ethanol fermentation into a single operation, it offers a great potential of increased rate of hydrolysis, reduction of fermentation time and decreased capital cost (5, 17). In batch SSF fermentation, the conversion yields of sugars to ethanol were as high as 90% of theoretical (Fig. 6). This result was prominent as compared with low rate of saccharification by the AMG enzyme in alginate beads (Fig. 5). It suggested that glucose produced from starch was immediately converted to ethanol by *Z. mobilis* cells. As a consequence, AMG would not be subject to product inhibition and a reversion reaction of glucose would not be allowed.

In a continuous SSF process using a packed bed column reactor, it was found that the system was particularly superior to other systems in terms of productivity. The maximum productivity based on total reactor volume (V_t = 87m*l*) was 17.7g/*l*,h at $D = 0.83h^{-1}$. When the calculation was based on void volume of the reactor (V_v = 22m*l*), it increased to 72.6g/*l*,h at $D = 3.3h^{-1}$. This value was twice as much as that obtained in the system with separately immobilized AMG and *Z. mobilis* cells (8) and about 7 times with coimmobilized AMG and cells in κ-carrageenan (9).

The operational stability of the present system was also prominent compared with other systems. It was observed that the CO_2 evolution from beads was quite vigorous in the reactor and the channelling of the substrate solution could be developed. For more than 40 days, however, the reactor could be operated without the breakage of the packed bed which was an occasional phenomenon due to CO_2 gas (8).

Until now, the coentrapment of an enzyme and microbial cells has been tried mainly with substrates which have small molecular weights such as cellobiose (1-3) and lactose (18). It is obvious from this

investigation, however, that the coimmobilized AMG and *Z. mobilis* system also offers a great feasibility of application with starch as a substrate.

요 약

전분으로부터 동시당화 발효법에 의한 에탄올 생산공정의 개발을 위하여 amyloglucosidase를 chitin에 결합시킨 후 *Zymomonas mobilis*와 함께 sodium alginate젤에 재고정화 시킨 다음 에탄올 생산실험을 행한결과 전분의 당화 및 발효속도를 증가시킬 수 있음이 발견되었다. 충진층 발효조를 사용한 연속식 에탄올 생산결과 40일 이상 안정하게 유지할 수 있었고, 최대 에탄올 생산성은 희석배율 0.83hr^{-1}에서 17.7$g/l, h$였다.

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