# (S4-A) METABOLIC ENGINEERING OF *ZYMOMONAS MOBILIS* FOR ETHANOL PRODUCTION FROM STARCH

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## Introduction

The general scope of metabolic engineering is to redirect the metabolism of microorganism in order to increase the yield or rate of formation of desirable products. This can be achieved by growing the microorganism in appropriate media, by modifying culture method such as cell immobilization or by genetic manipulations(1). From this view, we metabolically engineered an ethanologenic Gram negative bacterium, *Zymomonas mobilis*, at the aim of economical ethanol production from starch.

During last 10 years, considerable efforts were made for the development of new ethanol processes using *Z. mobilis*(2-10). This microorganism has interesting characteristics for being exploited in ethanol fermentation : ability to grow anaerobically, higher ethanol yield and productivity compared with yeasts, and increased potential for genetic manipulation due to its procaryotic nature(11). Despite these advantages of *Z. mobilis*, this

microorganism is only able to utilize glucose, fructose and sucrose as substrates for ethanol production and its growth(12).

Since the substrate is a main cost-component for industrial ethanol production, it is essential that ethanol production should be conducted with inexpensive substrates such as starch or cellulose. In practice, starch is being most used as a main substrate for industrial ethanol fermentation due to its global abundance and comparatively low price.

Owing to the inability of *Z. mobilis* to convert starch into directly untilizable simple sugars, however, starch should be hydrolyzed to glucose prior to ethanol fermentation. This could be achieved by use of two enzymes, $\alpha$-amylase and glucoamylase for liquefaction and saccharification, respectively. In order to increase ethanol yield or productivity by making this multi-stage process into a single one, *Z. mobilis* must be grown in a starch medium together with amylases added exogenously or must produce its own enzymes endogenously. To this end, the redirection of cellular metabolism of *Z. mobilis* was tried in two ways ; by adopting a simultaneous saccharification and fermentation(SSF) processes and by genetic manipulation.

## An Exogenous Approach : SSF Process

SSF process combines enzymatic hydrolysis of starch to glucose with ethanol fermentation into a single operation. Consequently, this process offers a great potential of increased rate of hydrolysis, reduction of fermentation time, and decreased capital cost(13). In this study, various SSF

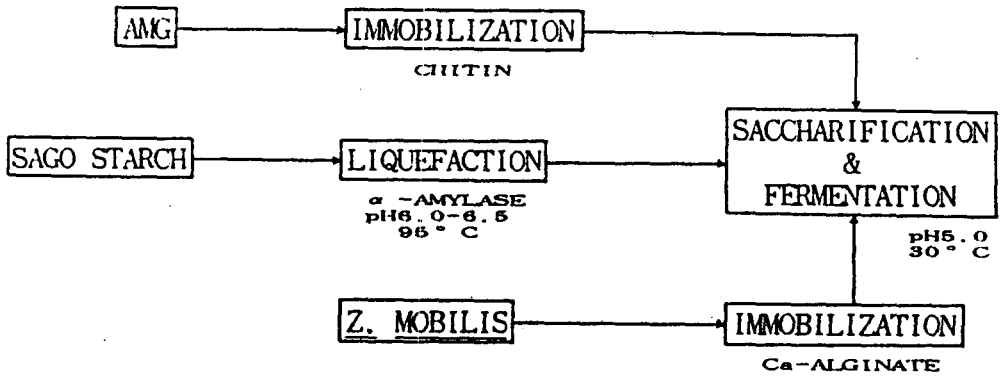
processes using free enzyme and free *Z. mobilis* cells, free enzyme and immobilized cells, and coimmobilized enzyme and cells were evaluated and compared one another with respect to ethanol productivity, operational stability and industrial practicability. SSF processes were performed in batch, semi-batch and continuous modes of reaction in laboratory as well as pilot scale fermentors. Experimental procedures and conditions of each process are shown in Fig. 1.

In a presaccharified process, 15%(w/v) of liquefied sago starch was presaccharified at 60°C and pH 6.5 with 0.15% glucoamylase(AMG 200L ; NOVO Industri) prior to fermentation. The saccharifying reaction proceeded almost completely to the maximum within 25 hrs. This saccharified sago starch was found to be converted to ethanol with 95% of the theoretical yield in 16 hrs. The 40 hrs of total reaction time for saccharification and fermentation yielded 1.7 g/l,h of productivity. In a batch SSF using free enzyme and free *Z. mobilis* cells,the whole reaction was completed in 18 hrs with the final ethanol concentration of 69.2g/l. The ethanol yield and productivity were 97% and 3.5g/l,h, respectively. When *Z. mobilis* cells were immobilized and SSF was carried out with free enzyme at 40°C, the final ethanol concentration attained 64.1g/l and ethanol yield was 90% of the theoretical yield. However, the productivity was slightly increased by 10%(3.8 g/l,h) compared with the SSF using free *Z. mobilis* cells and enzyme (Table 1).

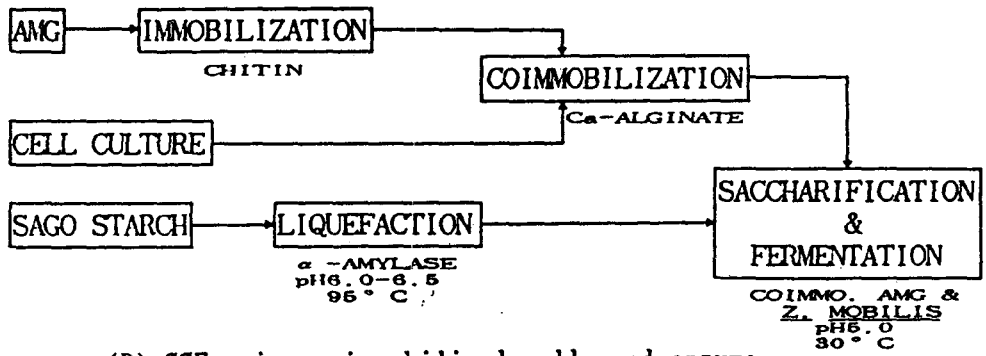
These results suggested that the advantages of SSF process over presaccharified process in general are significant for ethanol production using


(A) Presaccharified system


(B) SSF using free cells and free enzyme




(C) SSF using separately immobilized cells and enzyme




(D) SSF using coimmobilized cells and enzyme

Fig. 1. Schemes showing experimental procedures and conditions of each process

Table 1. Presaccharified and batch SSF processes for ethanol production from sago starch

| Process | AMG <%(v/v)> | Condition | Ethanol Concentration(g/l) | Ethanol Yield (%) | Productivity (g/l,h) |
|---|---|---|---|---|---|
| Presaccharified | 0.15 | 60°C, pH4.5<br>35°C, pH5.0 | 68.1 | 95 | 1.7 |
| Batch SSF | | | | | |
| free enzyme, free cells | 0.5 | 35°C, pH5.0 | 69.2 | 97 | 3.5 |
| free enzyme, immobilized cells | 0.3 | 40°C, pH5.0 | 64.1 | 90 | 3.8 |

*Starch concentration was 15%(w/v) equivalent to 139.5 g/l of reducing sugar.

*Z. mobilis* and sago starch. In terms of productivity, SSF processes showed 2 times higher values than presaccharified process by taking the time required for saccharification into account. There involved, however, a few technical disadvantages in SSF processes. For the batch SSF process, the suboptimal temperature(35°C) for saccharification resulted in an increased AMG requirement(0.5% v/w) compared with 0.15% for presaccharified process. when cells were immobilized and SSF was run at 40°C with a reduced concentration of AMG(0.3% v/w), the reusability of the immobilized cells was quite poor. These shortcomings in SSF could have been overcome by operation of continuoue SSF using coimmobilized enzyme and cells(Table 2). In this system, high densities of enzyme and cells were maintained in a cylindrical type of packed bed reactor and resulted in high ethanol productivity(9.3g/l,h at D=0.21 $h^{-1}$) based on total reactor volume, $V_T$, and low enzyme requirement(0.08% v/w). The continuous operation of this system was maintained stably over 40 days. In a

longer run, however, it was observed that the pressure drop caused by evolving $CO_2$ gas occasionally brought about a breakage of the packed bed.

As shown in Table 2, the use of a tapered column bioreactor packed with coimmobilized *Z. mobilis* cells and AMG alleviated the problems in association with $CO_2$ evolution and provided a significantly better flow pattern for both liquid and gas phases in the bioreactor without channelling. The performance of the tapered column bioreactor was at least equivalent to that of the cylindrical column bioreactor. The productivity of continuous SSF process using flocculent strain of *Z. mobilis* and immobilized AMG(5.2g/l,h) was found to be lower than that of the tapered bioreactor system using coimmobilized *Z. mobilis* and AMG(9.2g/l,h). As shown in Table 3, semi-batch SSF processes by cell recycle with ultrafiltration revealed higher productivity(5.4g/l,h) than that using a settler(4.3g/l,h). Despite the lower ethanol productivity in

Table 2. Continuous SSF processes for ethanol production from sago starch

| Process | Condition | Dilution rate ($h^{-1}$) | Ethanol Concentration(g/l) | Ethanol Yield (%) | Productivity (g/l,h) |
|---|---|---|---|---|---|
| Cylindrical bioreactor | Coimmobilized enzyme and *Z. mobilis* ZM4 | 0.21 | 44.3 | 93 | 9.3 |
| Tapered bioreactor | Coimmobilized enzyme and *Z. mobilis* ZM4 | 0.20 | 46.0 | 97 | 9.2 |
| Fludized-bed bioreactor | Immobilized enzyme and free *Z. mobilis* ZM401 | 0.12 | 44.9 | 94 | 5.2 |

* Starch concentration was 10%(w/v) equivalent to 93.0 g/l of reducing sugar.

semi-batch SSF compared with continuous SSF, the semi-batch process with ultrafiltration system appeared most promising when the scale-up of SSF process is taken into account. This system offers comparatively high ethanol productivity, feasbility of scale-up and simplification of operation.

In batch experiments using pilot scale fermentors, the final ethanol concentrations and yields obtained from 72L stirred tank fermentor, 100L and 500L airlift fermentors were 85.1g/l, 82.5g/l and 82.2g/l, respectively. In semi-batch experiments using ultrafiltration, the average ethanol productivity was 6.8 g/l,h, which was 1.7 times greater than that obtained from batch fermentation(4.0g/l,h) (Table 4). The $CO_2$ recycling for mixing was proven particularly effective in terms of feasibility of scale-up and simplification of operation. In pilot scale fermentation, the similar or even better kinetic results were found than in laboratory scale fermentation. By introduction of cell recycle system using ultrafiltration, ethanol productivity was increased by 80%. Energy consumption could be dramatically reduced by use of evolving $CO_2$ gas for self-mixing. These results suggested that the SSF processes

Table 3. Semi-batch SSF processes for ethanol production from sago starch

| Process | Condition | Ethanol Concentration (g/l) | Ethanol Yield (%) | Productivity (g/l,h) |
|---|---|---|---|---|
| Recycle using settler | Free AMG and *Z. mobilis* ZM401 | 68.2 | 95 | 4.3 |
| Recycle using UF | Free AMG and *Z. mobilis* ZM4 | 65.3 | 91 | 5.4 |

*Starch concentration was 15%(w/v) equivalent to 139.5 g/l of reducing sugar.

developed in this study were technically feasible for the ethanol production in the industrial scale.

Table 4. Pilot scale SSF processes for ethanol production from sago starch

| Fermentor type and process | Ethanol Concentration (g/l) | Ethanol Yield (%) | Productivity (g/l,h) | Calculation Time (h) |
|---|---|---|---|---|
| Jar fermentor (1L) | 88.8 | 96 | 3.7 | 24 |
| Stirred Tank (72L) | | | | |
| Batch | 85.1 | 93 | 4.1 | 21 |
| Semi-batch | 82.3 | 89 | 6.9 | 12 |
| Airlift (100L) | | | | |
| Batch | 82.5 | 90 | 3.9 | 21 |
| Semi-batch | 81.4 | 88 | 6.8 | 12 |
| Airlift (500L) | | | | |
| Batch | 82.2 | 89 | 3.6 | 23 |

*Starch concentration was 20%(w/v) equivalent to 180g/l of reducing sugar.

## An Endogenous Approach : Genetic Manipulation of *Z. mobilis*

The fact that the range of substrate utilization by *Z. mobilis* is confined to only glucose, fructose and sucrose imposes severe limitation of *Z. mobilis* for its industrial use. In this regard, it will be of particular importance to broaden the substrate range of *Z. mobilis* to less expensive substrates such as starch or cellulose through genetic manipulation.

To achieve this goal, we have constructed a number of small size shuttle

vectors containing replication origins of both *Z. mobilis* and *E. coli*. A hybrid plasmid vector of 8.4 Kb in size, designated as pHZ22, contained chloramphenicol resistant gene as a selectable marker and was proven to be conjugally transmissible and stably maintained in *Z. mobilis*(14, 15). By introducing structural region of *lacZ* into pHZ22, a promoter-probe vector of *Z. mobilis* was constructed to screen out the promoters of genes originated from *Z. mobilis*. By use of this vector, a strong promoter containing a translational initiation signal was obtained from the chromosomal DNA of *Z. mobilis* ZM4(ATCC 31821).

Sago starch being used as a substrate in this study, cloning of amylase genes into *Z. mobilis* was tried. The α-amylase gene was isolated from *Bacillus stearothermophilus*. Each *E. coli* clone showing α-amylase activity on starch-azure agar plates contained chimeric plasmids which were composed of pBR322 and a HindIII fragment of foreign DNA. By Southern blot analysis, it was proven that the HindIII fragment was originated from naturally occurring plasmids of *B. stearothermophilus* ATCC 31195. The DNA sequencing data showed that the α-amylase gene cloned was identical to that of the strain DY5 in the structural region.

To place α-amylase gene under the control of *Z. mobilis* promoter, a number of *Z. mobilis* expression vectors were constructed. The truncated α-amylase gene was then introduced into these vectors(Fig. 2). Both qualitative and quantitative activities of α-amylase were observed in *Z. mobilis* cells harboring these plasmids with α-amylase gene inserted. Kinetic

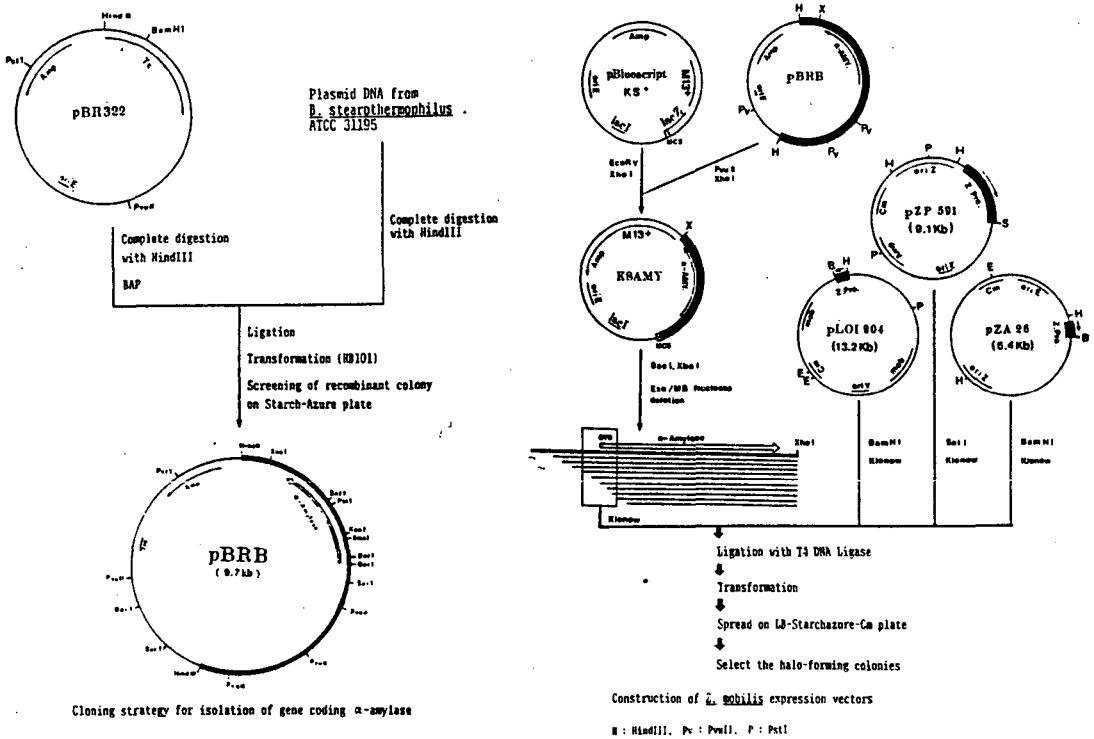


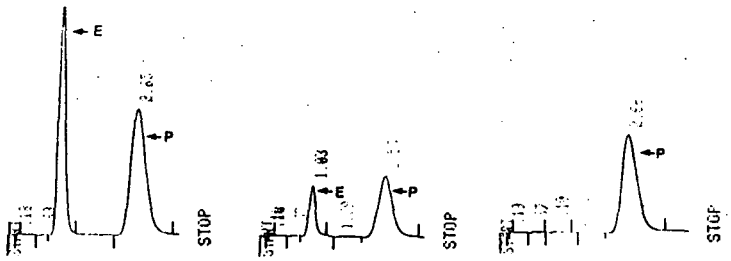

**Fig. 2. Cloning of α-amylase gene from *B. Stearothermophilus* into *Z. mobilis*.**

studies and gas chromatographic analysis of ethanol showed that one of the *Z. mobilis* transconjugants was capable of producing 67mM ethanol from rich medium containing 5% soluble starch as a sole carbon source(Fig. 3). To further improve the efficiency of conversion from starch to ethanol, cloning of glucoamylase gene from *Saccharomyces diastaticus* into *Z. mobilis* is also under study in our laboratory.

## Conclusion

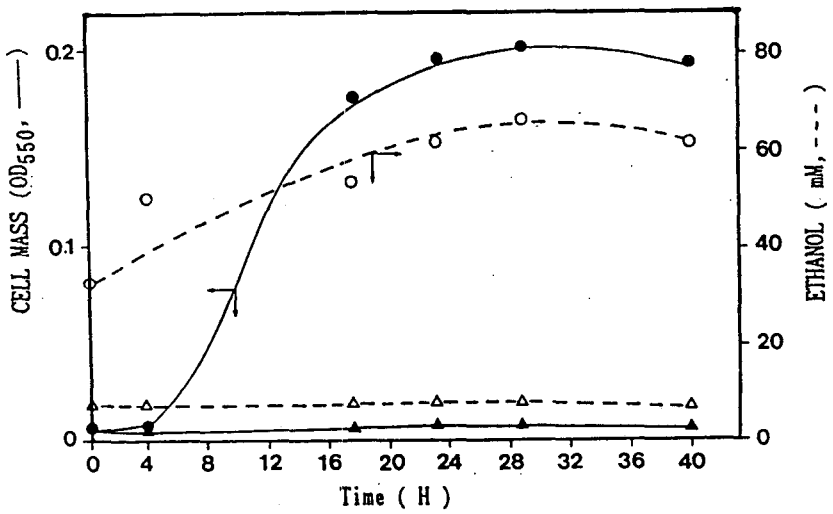
A continuous SSF process using coimmobilized AMG and *Z. mobilis* cells in a tapered column bioreactor was superior to other processes with respect to ethanol productivity and enzyme recovery in a bench scale fermentation. Bearing the large scale production of ethanol in mind, however, a semi-batch SSF process with cell recycle using ultrafiltration was the most favorable of the strategy investigated. Although this process seems technically feasible for the ethanol production in an industrial scale, the cost evaluation must be carefully conducted for the economical feasibility of this process with the unusual fluctuation in recent oil prices taken into account.

The genetic improvement of ethanol producing capability of *Z. mobilis* seems to be most powerful means albeit it is still in early stage for practical use. Apart from the broadening of the range of substrates available to *Z. mobilis*, it will be also beneficial for the industrial ethanol production using this microorganism to develop strains with enhanced tolerance to ethanol, high sugar concentration, temperature and osmotic pressure as well as with the flocculent properties for the facilitation of cell recycle.




Ethanol, Propanol Standard | *Z. mobilis* ZM4 (pZAMY38) | *Z. mobilis* ZM4

Gas chromatographic analysis of ethanol




Comparative kinetics of ethanol production by Z.mobilis ZM4(△) and Z.mobilis ZM4 harboring pZAMY 38 (○) in starch medium.

Fig. 3. Expression of $\alpha$-amylase gene in *Z. mobilis*.

## References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