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Effect of Ethanol on Selected Enzymes of the Entner-Doudorff Pathway in *Zymomonas mobilis*

Park I.L., S.H. Kwon and K.J. Lee*

Department of Microbiology, Seoul National University, Seoul 151-742, Korea

에탄올이 *Zymomonas mobilis*의 糖代謝 關連 酵素에 미치는 影響

朴仁鈴·權錫欽·李啓準*

서울대학교 자연대학 미생물학과

The aim of the presented paper was to elucidate the physiological background of ethanol inhibition on glucose uptake, ethanol production and cell growth in *Z. mobilis*. Data obtained from batch and continuous cultures showed that the rates of glucose uptake and ethanol production were not affected but growth rate was apparently reduced by ethanol produced. In order to know the effects of ethanol on the anabolism and the catabolism in *Z. mobilis*, enzyme activities of the Entner-Doudoroff Pathway, viz. hexokinase, glucose 6-phosphate dehydrogenase, were analyzed with the cell grown at different concentration of ethanol produced.

As results, it was found that the activities of the glucose kinase and the glucose 6-phosphate dehydrogenase were not affected greatly by the concentration of ethanol where the glucose uptake rates revealed a relatively constant value. However it was very interesting to note that transketolase, which is an essential enzyme to provide the important precursors for cell growth, was affected more apparently to reduce by increasing ethanol levels. Those results might suggest that the apparent reduction of growth rate at ethanol concentration above 20 g/l would be caused by the reduction of the transketolase activity, which in turn provide less precursor for the cell growth.

Different models have been proposed for the effect of ethanol inhibition on the rates of microbial growth and substrate-metabolism with various strains of yeasts. These include models with linear inhibition(10, 12), linear inhibition with a threshold concentration(11), non-competitive enzyme inhibition models(25, 20), exponential inhibition(1) and empirical relationships(4). In the development model with *Zymomonas mobilis*. It was found that linear inhibition kinetics occurred for the effect of ethanol on specific growth rate (μ) and specific ethanol production rate (q_s). Although the threshold concentrations and the maximum ethanol concentrations were different in the two cases, good agreement was found between the model predictions and experimental data(16). However an appropriate explanation for the uncoupling phenomena has not been suggested.

At the present studies the ethanol effects were evaluated in terms of fermentation kinetics and enzyme inhibitions and the nature of the uncoupling between catabolism and anabolism in *Z. mobilis* was discussed.

Materials and Methods

Strain and media used

The strain used in the investigation was *Z. mobilis* ZM 4 (now designated to ATCC 31821) (13-16, 23-24).

It was maintained by transferring to fresh agar slants containing 20g/*l* glucose, 10g/*l* yeast extract

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*Corresponding author

(Difco) and 20g/l agar at pH 5.0 each week and storing at room temperature. The composition of the seed culture and fermentation media was: 20-250g/l glucose, 10g/l yeast extract, 1g/l KH_2PO_4, 1g/l $(NH_4)_2SO_4$, 5g/l $MgSO_4 7H_2O$. For 250g/l glucose media, second seed culture was carried out with the same media of fermentation for adaptation to high glucose concentration.

Experimental procedure

Z. mobilis was first propagated at 30 °C for 24 hr without agitation by transferring single colony from the stock culture slant to 50ml of preseed culture medium. 10 ml of the culture broth were then transferred to 90ml of seed culture medium. After 12-20hr incubation, it was inoculated to 900 ml of fermentation medium in a 1l fermentor. The culture was grown under nonaerated conditions at 30 °C and pH 5.0. To maintain a homogeneous culture, mild agitation was provided by a stirrer.

Analytical methods

Biomass (expressed as dry weight) was determined from the optical density at 660nm using the uninoculated media as a blank. A turbidity dry weight calibration curve was prepared from cells grown under similar conditions. The total glucose concentration was estimated on the supernatant after centrifugation (40000 g, 10 min) using the dinitrosalysilic acid method(18). For ethanol estimation, samples were distillated and analyzed using a procedure developed by Sawer and Dixon(26).

In order to assay the activity of intra-cellular enzymes, cells harvested from exponential growth phase or from steady-state of each dilution rate were disrupted with fine glass beads and then extracted with Tris buffer pH 6.0. Activities of hexokinase(E.C. 2.7.1.1), glucose 6-phosphate dehydrogenase (E.C.1.1.1.44) and transketolase (E.C. 2.2.1.1) in the cell of *Z. mobilis* were measured with the methods described in "Methods of Enzymatic Analysis"(8). One unit of enzyme activity was defined as that amount catalyzing transformation of 1 μM of substrate per minute under given assay condition. The concentration of protein was measured by the method of Lowry(17) and bovine serum albumin was used as a standard protein.

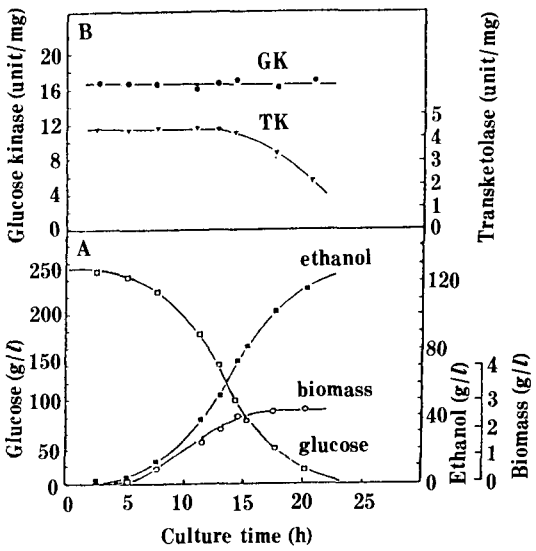

Fig. 1. Batch culture data for glucose uptake, ethanol production, the increase of biomass, and the activities of glucose kinase and transketolase with *Z. mobillis*.

Results and Discussion

Bach culture on a rich medium containing 250 g/l glucose

The activities of glucose kinase, 6-phosphogluconate dehydrogenase, and transketolase were determined with the cells grown in batch and continuous cultures. These enzymes were selected because the enzymes were considered to be essential in the glucose metabolism and also to provide precursors for the synthesis of cell component. As shown in Fig. 1, it was clear that the activity of glucose kinase (GK) were relatively constant throughout the batch culture. 6-Phosphogluconate dehydrogenase (6PGDH) activity were not detected in the extract of *Z. mobilis*. These results suggested that hexose monophosphate pathway was not operated, therefore, there might be other pathway (5), through which some essential precursors would be provided for the synthesis of biomass. It was very interesting to note that the transketolase(TK) activities were apparent in the extract of cell, which indicated that transketolase would play an important role to synthesize the important precursors. It was worth while to note again that the activities of transketolase was reduced with the culture time elapsed where the ethanol concentration was increased steadly. It was considered that transketolase activity might be affected by the ethanol concentration, therefore a more accurate data was necessary to understand the results.

Relationships between anabolism and catabolism at low ethanol concentration

Continuous culture using a rich medium containing 40 g/*l* glucose was carried out in order to know the relationship between cell growth rate and the activities of enzymes involved in glucose catabolic pathway. The experiments were contemplated to reveal the effect of the cell growth rate on the enzyme activity but not to be interfered with other factors, such as ethanol concentration etc.

As can be seen in Fig. 2A, the culture was operated under glucose limited condition until the specific growth rate reached to 0.3 h^{-1}. The further increases in the dilution rate (specific growth rate) gave rise to increase the inflow of glucose resulted in that the cultures were no more controlled by the glucose concentration but controlled by the metabolic activities. Cell growth yield ($Y_{x/s}$), specific glucose uptake rate (q_s), and specific ethanol formation rate (q_p) were calculated as parameters influenced with the specific growth rate (μ) (Fig. 2B.) It was evident that q_s and q_p were partially linked to μ. In other words q_s and q_p were closely related to the rate of glucose inflow where glucose limited condition ($0.3h^{-1} <$). But it was no more related to the rate of glucose addition when there was relatively high concentration of glucose in the culture broth ($0.3h^{-1} >$).

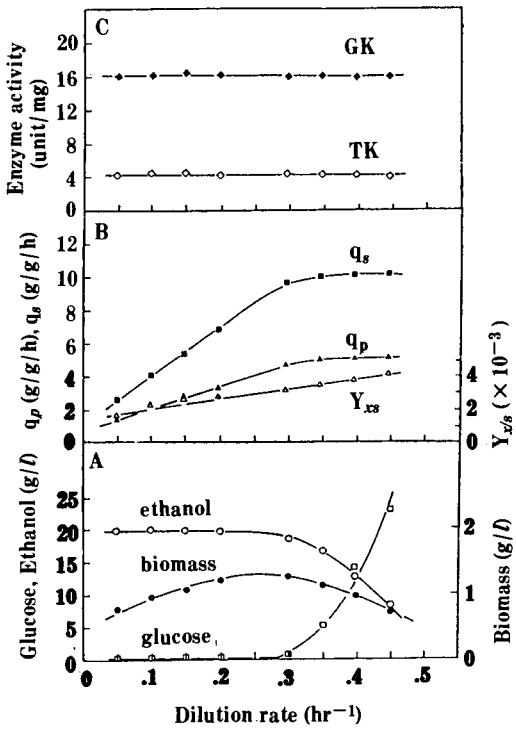

Fig. 2. Steady state data and kinetic parameters for *Z. mobilis* growing on 40 g/*l* glucose medium. (The activities of glucose kinase and transketolase at the steady-states are also shown).

Under the conditions of energy source limited, it was quickly realized that the glucose uptake rate at glucose limited condition was controlled by the external concentration of glucose. Whereat μ is dependent on q_s and the equation postulated by Pirt (24) could be fitted to the present results. And the maintenance energy coefficient (m_s) could be calculated by extrapolating q_s to zero dilution rate. Maximum cell growth yield ($Y_{x/s}^{max}$) could be also obtained to be 0.045 from the interception of the curve. It was found to be independent of the growth rates as far as glucose limited. It was very interesting to note that the value of $Y_{x/y}^{max} = 0.045$ was very close to the value postulated by Bauchop and Elsden (3).

It was apparent that the maximal values of q_s, viz. 10.4-10.7g/g/h, were obtained where the glucose in the culture broth was excessive. The same data have been reported in previous reports (13-16). A similar observation was made by Belaich *et al.*(6) who evaluated the kinetic parameters of *Z. mobilis* ATCC 10988 growing in pantothenate-limited but not limited carbon energy source cultures and reported that catabolic activity in *Z. mobilis* was largely independent of growth rate. In terms of the mechanisms of glucose uptake, Romano *et al.* (22) reported that *Z. mobilis* lack the phosphoenolopyruvate(PEP): glucose phosphotransferase system. An active transport system for glucose is therefore not present. These finding make it likely that glucose uptake by *Z. mobilis* occurs either by passive diffusion or by facilitated diffusion. Their recent paper showed the D-glucose was transported by a constitutive, stereospecific, carrier mediated faciliated diffusion system, whereby its intracellular concentration quickly reached a plateau close to but above the external concentration(9).

The activities of glucose kinase and transketolase of the cells obtained from each dilution rate were determined and shown in Fig. 2C. Since the activity of glucose kinase were not affected greatly by cell growth rate in both conditions of glucose limitation and excess, it was thought that the catabolic activity of cell was independent of

growth rate. It was considered that the q_s was controlled not only by the glucose kinase activity but also by the inflow rate glucose where concentration was very low. It indicated that the concentration of glucose in the glucose limited culture broth could influence the catabolic activity of cell, although the cell had its maximal rate of glucose metabolism.

However as shwon in Fig. 2C, it was very interesting to note that transketolase activity was not affected by the cell growth rate. Whereat higher growth rate would require higher activity of transketolase to provide more precursors. When the activities of transketolase measured at batch and continuous cultures, it was thought that the activity of transketolase was not limiting factor to support cell growth under rich media and low ethanol concentration, such as below 20 g/*l*. In this sense it was necessary, therefore, to confirm using cells growth under ethanol-limited conditions.

Relationship between anabolism and catabolism at high concentration of ethanol

In order to demonstrate more clearly the effect of ethanol concentration on the specific rate of growth (anabolism) and glucose uptake (catabolism), a number of continuous cultures were carried out using rich media containing glucose more 60g/*l*, from which it was possible to reveal the ethanol effect on the both activities. The detailed kinetic analysis were published elesewhere (33, 34).

Figure 3A shows that the specific grown rates (μ) were more strongly influenced by ethanol than the specific rate of glucose uptake (q_s), although there were threshold concentrations of ethanol below which inhibitions on μ and q_s did not occur. It is evident that the growth rate was more apparently inhibited by ethanol at much lower concentration (viz, 20 g/*l*) while the glucose uptake rate (q_s) maintained its maximum rate until ethanol concentration exceeded 60 g/*l*. In modelling studies, good agreement was found between the model predictions and experimental data describing in terms of threshold and linear inhibition function (16). However an appropriate explanation has not made to understand the uncouping phenomena in *Z. mobilis*.

The activities of transketolase and glucose kinase were determined with cells obtained from the continuous cultures and shown in Figure 3B. It was very interesting to compare that the inhibitory ef-

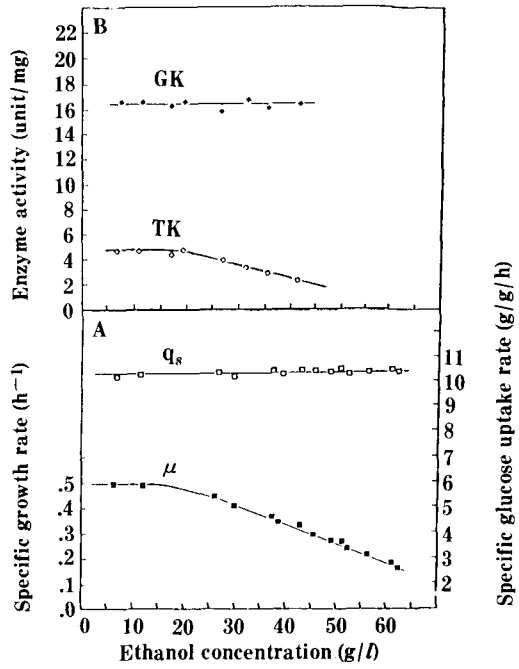

Fig. 3. Effect of steady-state ethanol concentration on the specific growth rate, specific glucose uptake rate, and the activities of glucosekinase and transketolas.

fects of ethanol on μ and transketolase were very similar, while the q_s and glucose kinase activity were relatively unaffected by the concentration of ethanol produced. Millar *et al.*(19) studied the *in vitro* effects of ethanol on the glycolytic enzymes of *S. cerevisiae* as well as the enzymes of the Enter-Doudoroff Pathway in *Z. mobilis*. The most significant inhibitions as far as yeast glycolysis was concerned were of phosphofructokinase, phosphoglycerate kinase and pyruvate decarboxylase. In *Z. mobilis*, the latter two were also concerned to be significantly affected. Interestingly, in *Z. mobilis* glucokinase did not show any significant inhibition even in the presence of 150 g/*l* ethanol *in vitro*(19,2). Rogers *et al.*(25) reported that the uncoupling caused by ethanol would suggest that ethanol has much greater inhibition effect on an enzyme(s) in the anabolic pathways than the enzymes of the EDP. Millar *et al.*(19) have studied the effect of ethanol on the enzymes of the anabolic pathway, but no research results has been reported to identify the way in which ethanol restricts cell growth.

The data in Figure 3B shows that the high concentration of ethanol produced as the result of substrate metabolism inhibited directly the activity of

the enzyme(s) linked to the anabolism of *Z. mobilis*. It was thought that the reduction of growth rate might be caused by the inhibitory effects of ethanol on transketolase, from which some essential growth factors w ·e provided, as illustrated in Figure 4. Although it has not been confirmed whether the precursors for the synthesis of nucleic acids (viz. ribose-5-phosphate) were the limiting factor in such a rich medium containing 10 g/*l* of yeast extract, it was evident that the precursors could be synthesized when it's concentration was not sufficient to allow the cell growth. Because *Z. mobilis* could grow well under chemically defined medium where the precursors were not provided externally(7). Those observation provides further support that uncoupling of growth(anabolism) and ethanol production(catabolism) in *Z. mobilis* at higher concentration of ethanol could result from the apparent inhibition of the transketolase activity. In conclusion, the uncoupling between catabolism and anabolism could be initiated by the different pattern of ethanol inhibition on the glucose kinase and transketolase.

요 약

발효생산된 에탄올의 농도가 *Zymomonas mobilis* 에서 당대사에 관련된 효소의 역가에 미치는 영향을 조사하였다. 그 결과, glucose kinase 및 glucose 6-phosphate dehydrogenase는 큰 영향을 받지 않았으나 transketolase 역가는 에탄올 농도가 증가함에 따라 심하게 저해됨을 알았다. 따라서 에탄올 농도의 증가에 따른 균성장속도의 감소는 transketolase에 의하여 영향을 받는 것으로 생각되었다.

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