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Characterization of *lac*$^+$ *gal*$^+$ Strains of *Zymomonas mobilis* for Ethanol Production from Lactose

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Previously RP1::Tn*951* which is a derivative of RP1 containing the lactose transposon Tn*951* was introduced into *Z. mobilis* strain ZM6100, and RP1::Tn*951* was integrated into its genome to yield ZM6306. The galactose operon was incorporated into ZM6306 to yield ZM6307 for more efficient utilization of lactose. Batch culture study has been carried out on *Z. mobilis* strains, ZM6306 (*lac*$^+$) and ZM6307 (*lac*$^+$, *gal*$^+$), which can convert lactose directly to ethanol. Using a medium containing 80 g·l^{-1} glucose and 40 g·l^{-1} lactose, it was found that ZM6306 and ZM6307 produced maximum ethanol concentration of 40 g·l^{-1} and 42 g·l^{-1},respectively, whereas parent strain ZM6 produced 37 g·l^{-1}.

Recently there has been considerable interest in the genetic manipulation of *Zymomonas mobilis* to extend its substrate range beyond glucose, fructose and sucrose (13). To make large-scale production of ethanol by *Z. mobilis* industrially feasible, this narrow substrate range needs to be extended to include cheap waste substrate such as lactose which is the major organic constituent of whey (8), a waste material produced in large quantities in the dairy industry. Lactose is cleaved to glucose and galactose by the enzyme, β-galactosidase, but only the glucose moiety is fermented to ethanol by *lac*$^+$ *Z. mobilis* strain, since *Z. mobilis* is unable to metabolize galactose (13). Consequently, galactose will accumulate during lactose metabolism and this may be inhibitory (4, 6, 16). To achieve full utilization of intracellular lactose and, consequently to overcome possible galactose inhibition, the *E. coli* galactose operon was incorporated into *lac*$^+$ *Z. mobilis* strain ZM6306 (12) to yield ZM6307. It was found that the Gal$^+$ recombinant plasmid was stably maintained in ZM6307 for at least 100 generations without antibiotic selection. In the present study a detailed kinetic analysis has been carried out in batch culture to characterize the strains more fully and to determine their ability to convert lactose directly to ethanol. Kinetic studies on the parent strain, ZM6 were also carried out as a control since both ZM6306 and ZM6307 were originally derived from this strain.

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MATERIALS AND METHODS

Bacterial Strains

The strains of *Z. mobilis* used for this study were the parent strain ZM6 (ATCC 29191) and ZM6306 derived from ZM6100 (RP1::Tn*951*) following extended subculture in the presence of tetracycline (12). The strain ZM 6100, a methionine-requiring auxotroph, has been derived previously by NTG mutagenesis from ZM6. ZM6307 is a derivative of ZM6306 carrying a Gal$^+$ plasmid (Cho, 1990. Ph.D. thesis, University of New South Wales, Sydney).

Medium Composition

Z. mobilis rich medium (RM) has been described previously (6) and the medium used for batch culture contained 200 g·l^{-1} glucose or 80 g·l^{-1} glucose and 40 g·l^{-1} lactose as well as 10 g·l^{-1} yeast extract, 1 g·l^{-1} $(NH_4)_2SO_4$, 2 g·l^{-1} KH_2PO_4 and 1 g·l^{-1} $MgSO_4 \cdot 7H_2O$ (10).

Experimental and Analytical Procedures

Methods for the preservation of strains, inoculum preparation and the procedures for the estimation of dry weight, ethanol and glucose concentrations have been described earlier (10). Fermentation was performed using a 1 *l* fermentor. Lactose concentrations were estima-

using a YSI enzyme electrode with correction for interference by galactose. Galactose concentrations were determined enzymatically, using a Boehringer Mannheim Lac/Gal food analysis kit.

RESULTS

Compartive Batch Culture Kinetics of ZM6, ZM 6306 and ZM6307 in Medium Containing Glucose

Z. mobilis strains ZM6, ZM6306 and ZM6307 were grown in batch culture with 200 g·l^{-1} glucose medium at 30℃ and pH 5.0. All inocula were prepared under the same conditions. Data for the increase in biomass, utilization of glucose and the production of ethanol are shown in Fig. 1. The profiles of biomass and ethanol formation were consistent with sugar consumption. The biomass concentration of ZM6 increased to 3.5 g·l^{-1} in 20 h and then remained constant. Both ZM6306 and ZM6307 showed longer lag phase compared to ZM6 and the maximum biomass concentrations obtained were 2.1 g·l^{-1} for ZM6306 and 2.0 g·l^{-1} for ZM6307. Glucose uptake by ZM6306 and ZM6307 was slower than that of ZM6. Complete consumption of glucose by ZM6 occurred 12 h prior to that of ZM6306 and ZM6307. It is apparent that a high concentration of glucose can be efficiently and rapidly converted to ethanol by the parent strain, ZM6. The ethanol concentration for ZM6 increased up to 98 g·l^{-1} in 24 h. However, the maximum ethanol concentrations attained with ZM 6306 and ZM6307 were, respectively, 91 g·l^{-1} and 89 g·l^{-1} in 36 h by which time the glucose was exhausted. As can be seen in Fig. 1, there was little difference in the ethanol production rates betweeen strains of ZM 6306 and ZM6307. However, both strains showed a slower rate of ethanol formation than ZM6. To compare the kinetics of ZM6, ZM6306 and ZM6307, various kinetic parameters have been calculated for the exponential phase of growth. The kinetic parameters calculated from these batch culture data are shown in Table 1.

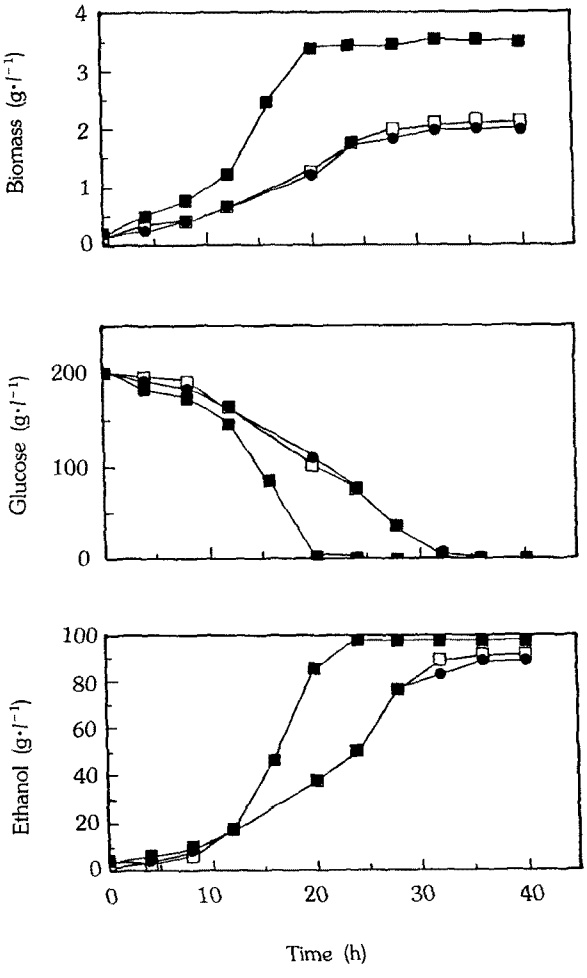

Fig. 1. Batch culture data for ZM6(■), ZM6306(□) and ZM6307 (●) in 200 g·l^{-1} glucose RM medium.

Comparative Batch Culture Kinetics of ZM6, ZM 6306 and ZM6307 in Medium Containing Glucose and Lactose

For comparison of kinetic parameters of ZM6306 and ZM6307 on lactose medium, a batch culture study was carried out in medium which contained both glucose and lactose. It was necessary to have glucose present as a carbon and energy source as well as lactose because in the absence of glucose, growth was undetectable and ethanol production was very slow (4, 6). Results for batch culture studies with ZM6, ZM6306 and ZM6307 grown on 80 g·l^{-1} gluocse plus 40 g·l^{-1} lactose medium are shown in Fig. 2. As ZM6 does not contain the *lac* operon it did not utilize any lactose. With ZM6306, which contains the *lac* operon, complete utilization of glucose occurred within 16 h. But even before glucose was exhausted, some decrease in lactose was observed together

Table 1. Comparison of kinetic parameters of *Z. mobilis* strains ZM6, ZM6306 and ZM6307 grown in batch cultures on 200 g·l^{-1} glucose medium at 30°C and pH 5.0

Kinetic parameters	ZM6	ZM6306	ZM6307
Specific growth rate (μ) (h^{-1})	0.13	0.08	0.083
Specific ethanol production rate (q_p) (g·g^{-1}·h^{-1})	4.04	2.42	2.48
Specific glucose uptake rate (q_s) (g·g^{-1}·h^{-1})	8.66	7.27	6.38
Cell yield ($Y_{X/S}$) (g·g^{-1})	0.015	0.013	0.013
Ethanol yield ($Y_{P/S}$) (g·g^{-1})	0.47	0.40	0.39
Maximun ethanol concentration (g·l^{-1})	98	91	89

with an increase in galactose. After complete utilization of glucose, lactose uptake and galactose release continued until lactose uptake ceased after 20 h. This resulted in a higher ethanol concentration for ZM6306 compared to ZM6. The final ethanol concentration was 40 $g \cdot l^{-1}$ for ZM6306 compared to 37 $g \cdot l^{-1}$ for ZM6. The corresponding increase in galactose for ZM6306 was 5 $g \cdot l^{-1}$. With ZM6307, which contains both the *lac* and *gal* operons, complete utilization of glucose occurred within 15 h. As was observed with ZM6306, some decrease in lactose occurred during this time and was accompanied by an increase in galactose. Following glucose depletion, lactose uptake and galactose release continued until lactose uptake was ceased after 20 h. The amount of galactose released by ZM6307 was slightly less than for ZM 6306. This probably accounts for the slightly higher ethanol concentration produced by ZM6307 compared to ZM6306. Kinetic parameters calculated for the exponential phase of growth are shown in Table 2. No substantial difference in kinetic parameters were observed between ZM6306 and ZM6307 except that ZM6307 seemed to take up some galactose, although at a low rate.

DISCUSSION

During growth of the parent strain ZM6 and its derivatives, ZM6306 and ZM6307, in batch culture on glucose medium, lower biomass, ethanol yields and specific rates (μ, q_p, q_s) were observed with the derivatives. From the results it is apparent that the specific growth rate (μ), the specific ethanol production rate (q_p) and the specific glucose uptake rate (q_s) of ZM6306 and ZM6307 were affected by the high sugar concentration, whereas parent strain ZM6 did not appeared to be influenced to the same extent. Especially, the effect of high glucose concentration on decreasing the growth rate of the derivative strains (ZM6306 and ZM6307) was greater than for the parent strain ZM6.

It is conceivable that the genetically engineered *Z. mobilis* strains which carry more plasmids in addition to the native plasmids (ZM6306 contains RP1::Tn*951* in its chromosome, ZM6307 contains RP1::Tn*951* in its chromosome together with a Gal^+ recombinant plasmid) may grow slower than the parent strain. Zund and Lebek (17) studied the correlation between increase in the generation time of *E. coli* caused by R plasmids and the molecular size of the plasmids. They found that some R plasmids increase the generation time of the host strains into which they were introduced.

The origin of ZM6306 is ZM6100 and ZM6100 is methionine requiring mutant of ZM6 (12). It is possible that the biochemical and physiological characteristics of ZM6 might have changed by NTG mutagenesis, possibly resulting in the lower metabolic activities in mutant strains. The auxotrophic requirements of ZM6306 (*met*$^-$, *asn*$^-$) which are the result of NTG mutagenesis and integration of RP1::Tn*951* into the chromosome (12), might have also caused nutritional limitations for biomass formation in the fermentation medium which contains 1% (w/v) yeast extract.

The main aim of the investigation carried out in this work was to compare the kinetic parameters of ZM6306 and ZM6307, grown in glucose plus lactose medium, and their ability to produce ethanol from lactose. There was little difference in the kinetic parameters betweeen ZM6306 and ZM6307, except that galactose liberated from lactose seemed to be utilized at least in part by ZM6307 as indicated by its specific galactose uptake rate (0.14 $g \cdot g^{-1}h^{-1}$; Table 2). This could explain the slightly higher maximum concentration of ethanol produced by ZM6307 compared to ZM6306. Initially ZM 6307 was expected to produce twice as much ethanol from lactose as ZM6306, as the cloning of the three enzymes of the galactose operon (*galK, galT* and *galE*) was considered to be sufficient for metabolism of intracellular galactose (conversion of galactose to glucose) by *Z. mobilis*. Compared to the parent strain, ZM6306 utilized 10 $g \cdot l^{-1}$ lactose, releasing 5 $g \cdot l^{-1}$ galactose and producing an extra 3 $g \cdot l^{-1}$ ethanol in 30 h (Fig. 2). However, ZM6307 utilized only some of this galactose (2 $g \cdot l^{-1}$) and produced the slightly higher level of ethanol than ZM6306. This could be due to the poor metabolism of galactose in *Z. mobilis* possibly caused by the low levels of phosphoglucomutase and UDPG (Uridine

Table 2. Comparison of kinetic parameters of *Z. mobilis* strains ZM6, ZM6306 and ZM6307 grown in batch cultures on 80 $g \cdot l^{-1}$ glucose plus 40 $g \cdot l^{-1}$ lactose medium at 30°C and pH 5.0

Kinetic parameters	ZM6	ZM6306	ZM6307
Specific growth rate (μ) (h^{-1})	0.218	0.168	0.173
Specific ethanol production rate (q_p) ($g \cdot g^{-1} \cdot h^{-1}$)	2.06	2.23	2.37
Specific glucose uptake rate (q_s) ($g \cdot g^{-1} \cdot h^{-1}$)	4.27	5.41	5.58
Specific lactose uptake rate ($q_{s\text{-}lac}$) ($g \cdot g^{-1} \cdot h^{-1}$)	–	0.56	0.42
Specific galactose uptake rate ($q_{s\text{-}gal}$) ($g \cdot g^{-1} \cdot h^{-1}$)	–	–	0.14
Cell yield ($Y_{X/S}$) ($g \cdot g^{-1}$)	0.051	0.031*	0.031*
Ethanol yield ($Y_{P/S}$) ($g \cdot l^{-1}$)	0.48	0.41*	0.42*
Maximun ethanol concentration ($g \cdot l^{-1}$)	37	40	42

*Calculations based on total glucose utilized, including that derived from lactose hydrolysis.

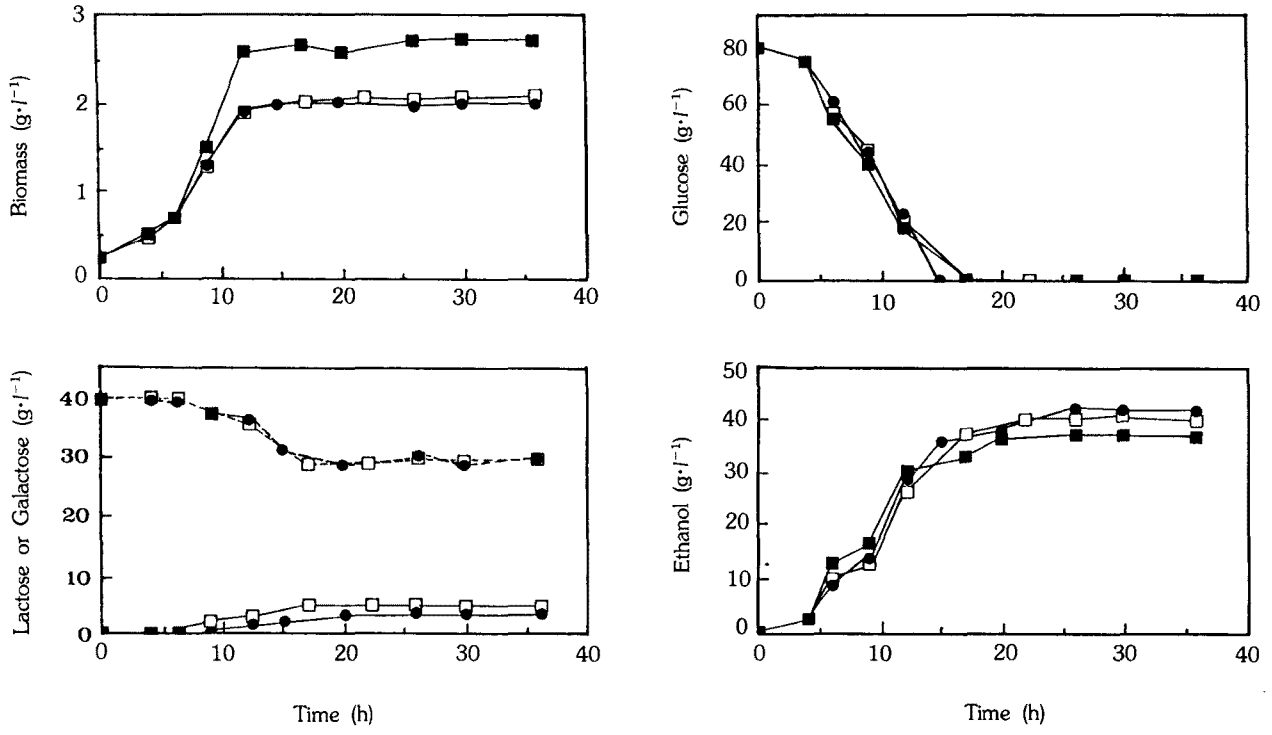

Fig. 2. Batch culture data for ZM6(■), ZM6306(□) and ZM6307(●) in 80 g·l^{-1} glucose plus 40 g·l^{-1} lactose RM medium.

diphosphoglucose). Glucose-1-phosphate, the product of galactose metabolism catalyzed by the enzymes of the *gal* operon, is converted to glucose-6-phosphate, a glycolytic intermediate, by phosphoglucomutase (1). Conversion of glucose-1-phosphate to glucose-6-phosphate is also necessary in *Z. mobilis* in order to feed gluocse into the Entner-Doudoroff pathway, the glycolytic pathway of *Z. mobilis*. UDPG is a necessary participiant in the enzymic conversion of galactose-1-phosphate to glucose-1-phosphate (7). It is possible that UDPG might not be as freely available in *Z. mobilis* as it is in *E. coli* and this could limit galactose catabolism in *Z. mobilis*.

Poor lactose uptake poses another problem for efficient ethanol fermentation by ZM6306 and ZM6307, since rapid lactose uptake would require a fully functioning lactose permease. Lactose permease is a membrane associated enzyme which includes strereospecific and functionally-specialized protein components in the cytoplasmic membrane (9) but the membrane composition of *Z. mobilis* is known to differ from that of *E. coli* (2, 3, 11, 14, 15). Thus, a high lactose permease activity might not occur in *Z. mobilis*.

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