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Molecular Cloning and Expression of α-Amylase Gene from *Bacillus stearothermophilus* in *Zymomonas mobilis* ZM4

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In order to broaden the spectrum of substrate utilization of a Gram negative bacterium *Zymomonas mobilis* which has a great potential as an industrial ethanol producing microorganism, cloning of α-amylase gene into *Z. mobilis* ZM4 was tried. The α-amylase gene was isolated from *Bacillus stearothermophilus*. By Southern blot analysis, it was proven that the α-amylase gene fragment was originated from a naturally occuring plasmid of *B. stearothermophilus* ATCC 31195. To place α-amylase gene under the control of *Z. mobilis* promoter, two different *Z. mobilis* expression vectors, pZA26 and pLOI204, were used. The truncated α-amylase gene was then introduced into these vectors. Both qualitative and quantitative activities of α-amylase were observed in *Z. mobilis* cells harboring these plasmids with the α-amylase gene inserted. Gas chromatographic analysis of ethanol showed that one of the *Z. mobilis* transconjugants was capable of producing 67 mM ethanol from rich medium(RM) containing 5% soluble starch as a sole carbon source.

During the last 10 years, *Zymomonas mobilis*, an ethanologenic bacterium, has been given much attention as an alternative microorganism for yeasts in ethanol fermentation. Compared with yeasts, *Z. mobilis* has a number of beneficial characteristics as a potential industrial ethanol producer; anaerobic growth, higher ethanol and sugar tolerances and higher ethanol yield and productivity (17). 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 (23).

Since the substrate is a main cost component for industrial ethanol production (5), it is essential that ethanol production 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 utilizable simple sugars, it is necessary for starch to be hydrolyzed to glucose prior to ethanol fermentation using two exogenously added enzymes, α-amylase and glucoamylase for liquefaction and saccharification, respectively. Otherwise, the microorganism should produce its own enzymes endogenously to carry out starch hydrolysis by itself. To this end, it is of importance to genetically manipulate *Z. mobilis* cells to have the α-amylase and glucoamylase genes cloned and expressed. In this regard, the present investigation deals with the molecular cloning and expression of the α-amylase gene from *Bacillus stearothermophilus* in *Z. mobilis* ZM 4.

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Key words: *Zymomonas mobilis* ZM4, *Bacillus stearothermophilus*, α-amylase gene expression, ethanol production, starch

MATERIALS AND METHODS

Bacterial Strains, Plasmids and Culture Media

All bacterial strains and plasmids used in this study are listed in Table 1. The rich medium(YPG) was used as a non-selective medium for the growth of *Z. mobilis*. 100 μg/ml of chloramphenicol and 100 μg/ml of nalidixic acid were added to the rich medium to be used as a selective medium (4). For the cultivation of *E. coli* and *B. stearothermophilus*, LB (Luria-Bertanii) medium was used.

Table 1. Strains and plasmids used in this study.

Strains or plasmids	Relevant properties	Source
Strains		
Z. mobilis ZM4	prototroph	P.L. Rogers (17)
B. stearothermophilus ATCC 31195	prototroph	ATCC
E. coli		
HB101	F⁻ *recA*13, *lacY*1	
JM109	F′ *lacZ*Δ15	
C600	mobilizing strain	
Plasmids		
pBR322	Amp^r, Tc^r	
pBluescripts (KS^+)	Amp^r, *lacZ*, $M13^+$	
RP4	mobilizing plasmid	P.T. Barth (1)
YEp353	*E. coli*-yeast shuttle vector	A.M. Myers (10)
pLOI204	*Z. mobilis* expression vector	T. Conway (4)
pZA26	*Z. mobilis* expression vector	N. Misawa (13)
pBRB	α-amylase gene	This study
pZAMY38	α-amylase gene	This study
pLOIAMY28	α-amylase gene	This study
pZAMY381	*amy*::*lacZ* fusion	This study

DNA Isolation

Plasmid DNA's were isolated from *E. coli* by the alkaline-SDS method of Birnboim and Doly (2) and from *Z. mobilis* by the modified alkaline-SDS method of Yun *et al.* (25), and purified by CsCl-ethidium bromide equilibrium density gradient centrifugation. Chromosomal DNA from *B. stearothermophilus* was isolated by the method of Raymond and Tait (16).

Transformation and Conjugation of DNA's

Transformation of plasmid DNA into *E. coli* was performed by following the method described by Inoue *et al.* (8). For conjugation of plasmid DNA into *Z. mobilis*, donor strains were first constructed by the supertransformation method (4). Donor and recipient strains were *E. coli* C600 (RP4, non-conjugative plasmid) and *Z. mobilis* ZM4, respectively. Donor and recipient cultures were grown separately to the logarithmic phase in an unshaken culture in a standard medium, and mixed with donor to recipient ratio of 3:1. This mating mixture was centrifuged, resuspended in 5 m*l* YPG broth, transferred to 0.45 μm Millipore filters on an YPG agar plate, and incubated at 30℃ overnight. Next, this was resuspended by placing filters in a tube containing 0.5 m*l* of 0.85% saline and agitating the tube on a vortex. Resuspended cells were then spread on selective plates.

Southern Hybridization

DNA resolved in 0.9% agarose gel was transferred to a nitrocellulose filter by the Southern techniques (21). DNA labelling and detection were done according to the manufacturer's instructions (DNA labelling and detection kit, Boehringer-Mannheim).

DNA Sequencing

The DNA sequence was determined by the Sanger dideoxy chain termination method (19).

Western Blot Analysis

Western blot analysis was performed with proteins extracted from conjugated cells which were resolved by 0.1% SDS-7.5% PAGE using a discontinuous buffer system. The blot was incubated with the first antibody and then followed by the anti-rabbit antibody conjugated by alkaline phosphatase (Promega). Color development was carried out according to the manufacturer's recommendations (Promega).

Gas Chromatography

Ethanol concentration was measured by gas chromatography using a Varian Model 3700. For this process, a stainless steel column (6′ and 1/4″) packed with porapak Q (80~100 mesh) was used. Temperature of the column oven was maintained at 120℃ while injector and detector temperatures were kept at 150℃ and 200 ℃, respectively. The flow rate of carrier gas (N_2) was 30 m*l*/min. The amount of ethanol was quantified by peak heights relative to the known standard.

Analytical Methods

β-galactosidase activity was measured by the method described by Miller (12). The α-amylase activity was determined by the iodine staining method (6). The specific activity of α-amylase was measured as units/mg of pro-

tein. One unit of α-amylase activity was defined as the amount of enzyme which reduces the absorbance value of 0.001 at 700 nm per min.

RESULTS

Isolation and Characterization of α-Amylase Gene

In order to isolate the α-amylase gene, a shot-gun cloning method was employed with chromosomal DNA and natural plasmids of *B. stearothermophilus* ATCC 31195.

Chromosomal DNA and natural plasmids isolated by ultracentrifugation were digested with *Hin*dIII and ligated with the *Hin*dIII-digested selection vector pBR322. *E. coli* HB101 was transformed with a ligation mixture and transformants were selected for ampicillin resistance on agar plates containing starch azure. From the library of natural plasmids, 15% of total transformants showed a clear amylase-positive phenotype. But in the case of genomic library, we could not obtain any amylase-positive phenotypes among 10,000 transformants screened. This fact strongly suggested that the gene coding for α-amylase was present in natural plasmids of *B. stearothermophilus* ATCC 31195. From the library of natural plasmids, 24 colonies showing α-amylase activity were picked up at random and rapid plasmid preparation was performed. These recombinant plasmids were analysed by restriction analysis, and it was found that all recombinant plasmids contained 5.3 Kb internal *Hin*dIII fragments. One of the plasmids containing this DNA fragment was designated as pBRB and further characterized (Fig. 1).

Restriction analysis of the gene encoding α-amylase on pBRB showed that the restriction pattern of this gene was very similar with that of the α-amylase gene reported by Ihara (7). To compare the nucleotide sequences of these genes, partial nucleotide sequencing was carried out by use of several restriction sites. From the sequencing results, it was found that the nucleotide sequence of α-amylase gene on pBRB was identical with that of *B. stearothermophilus* DY-5 in the regulatory and coding regions, except some differences in the 5′ upstream of the regulatory region of the genes (7).

Cloning of α-Amylase Gene

Plasmids pZA26 (13) and pLOI204 (4) were used to express the α-amylase gene in *E. coli* and *Z. mobilis* ZM4. These expression vectors contained the replication origins of *E. coli* and *Z. mobilis*, dual selectable markers (Amp^r and Cm^r), promoter fragment of *Z. mobilis* origin and one BamHI site as a cloning site for a target gene. Plasmid pBRB was digested with *Pvu*II and *Xho*I, and

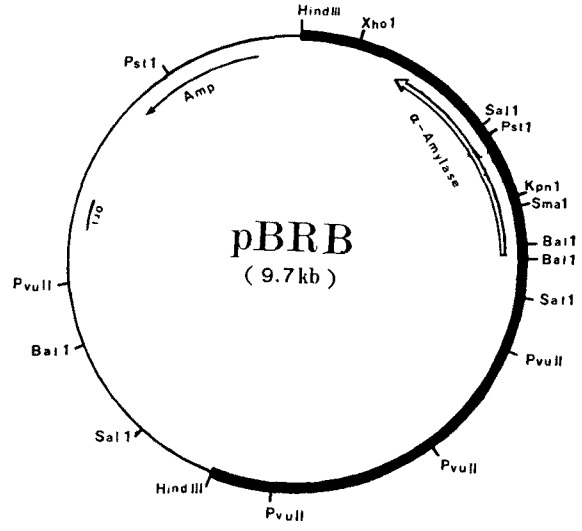

Fig. 1. Restriction map of pBRB.
Restriction sites are indicated, and the black bar represents the cloned fragment. The arrowhead indicates the position of the coding region of α-amylase gene from *B. stearothermophilus*.

3.5 Kb DNA fragment carrying α-amylase gene was recovered from the gel after separation on a 0.8% agarose gel. This was ligated with the vector DNA, pBluescripts (KS^+). The resulting plasmid, pKSAMY, contained *Xba*I and *Sac*I sites at the 5′ end of α-amylase gene and *Xho*I site at the 3′ end which made the deletion very convenient. To obtain the truncated α-amylase gene, pKSAMY was digested with *Sac*I and *Xho*I, treated with exonuclease III and Mung bean nuclease, digested with *Xba*I, and then filled in with Klenow enzyme. The deleted DNA fragment with 400~800 bps missing was purified, followed by ligation with the blunt-ended cloning sites of the *Z. mobilis* expression vectors, pZA26 and pLOI204. The ligation mixtures were transformed into *E. coli* JM109, and after this, halo-forming transformants were selected on LB-plates containing starch azure after incubation overnight (Fig. 2).

Out of 24 recombinants picked at random from these plates, 16 colonies were found to contain the truncated α-amylase gene which missed its own regulatory region. These constructed plasmids were transferred to *Z. mobilis* ZM4 by triparental transconjugation involving the helper plasmid, RP4.

Expression of α-Amylase Gene

Z. mobilis transconjugants harboring one of such plasmids, pZAMY38, which contained the α-amylase gene were found to form halos around their colonies showing the α-amylase activity (Fig. 3).

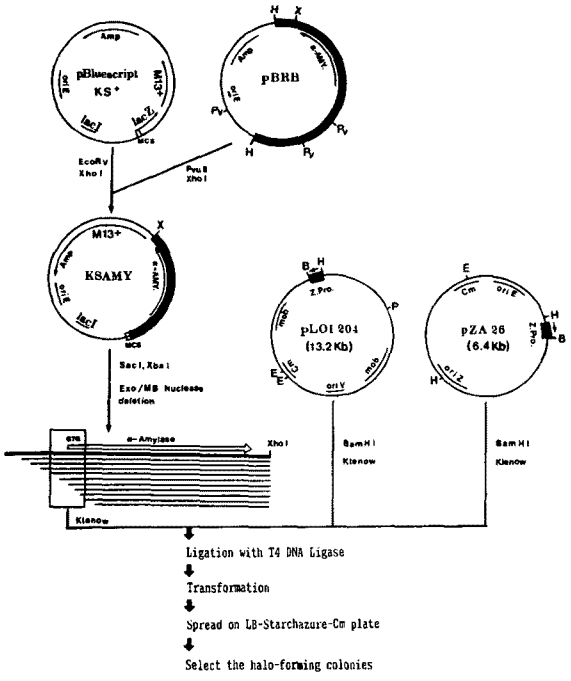

Fig. 2. Strategy for α-amylase gene cloning.
Arrows indicate the transcriptional direction. Sites for restriction endonucleases are: H, *Hin*dIII; B, *Bam*HI; X, *Xho*I; Pv, *Pvu*II; E, *Eco*RI; P, *Pst*I.

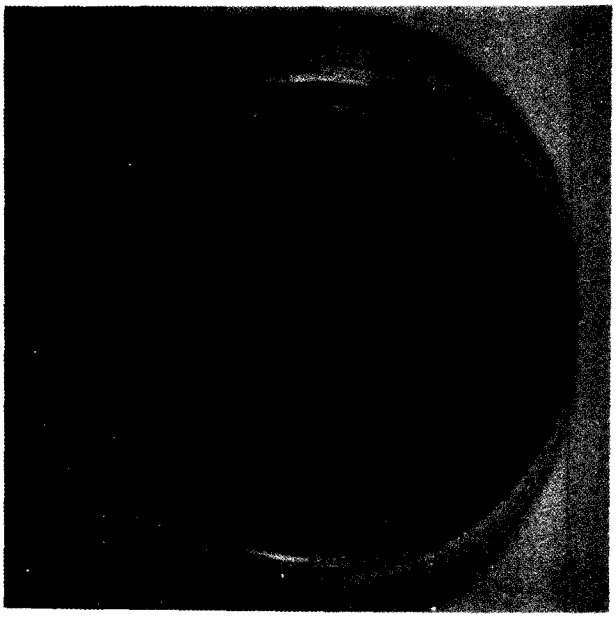

Fig. 3. *Z. mobilis* transconjugants showing α-amylase activity on YPG-starch azure plate.
Z. mobilis transconjugants containing α-amylase gene were toothpicked to YPG-starch azure agar plate with chloramphenicol (100 μg/ml) and nalidixic acid (100 μg/ml), and incubated at 30℃ for 4 days.

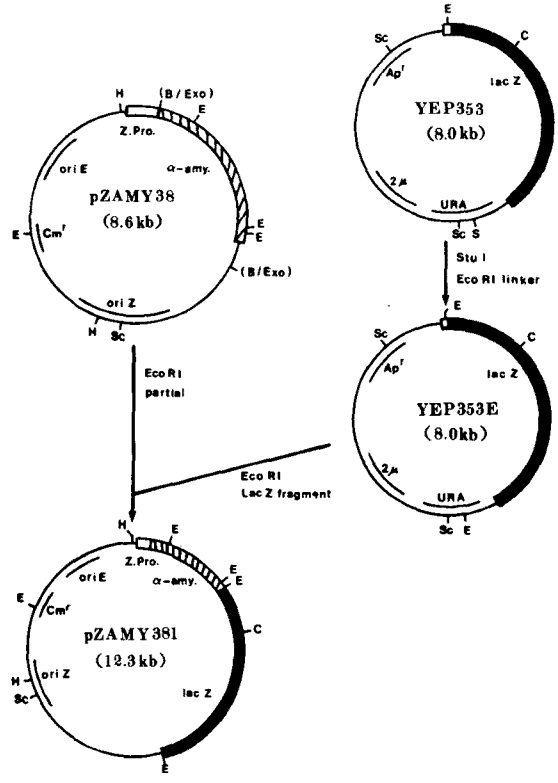

Fig. 4. Construction of *amy::lacZ* fusion expression vector.
The open, dashed and closed boxes represent promoter region, α-amylase gene and β-galactosidase gene, respectively.

When the whole cell extracts of *Z. mobilis* transconjugant harboring pZAMY38 were analyzed by SDS-PAGE, no protein band corresponding to α-amylase could be detected. Consequently, an *amy::lacZ* fusion expression vector was constructed to indirectly demonstrate the α-amylase band produced by *Z. mobilis* transconjugant on SDS-PAGE, as summarized in Fig. 4. YEp353, an *E. coli*-yeast shuttle vector, was used as a donor of β-galactosidase structural gene. YEp353 was digested with *Stu*I, ligated with *Eco*RI linker, and digested with *Eco*RI. The resulting 3.7 Kb DNA fragment carrying the β-galactosidase gene was isolated and then ligated to pZAMY38 which was partially digested with *Eco*RI, and finally the resulting plasmid was transformed into *E. coli* 109. *E. coli* transformants harboring the desired hybrid plasmid, designated as pZAMY381, were screened out for blue and halo-forming colonies which showed β-galactosidase and α-amylase activity simultaneously on an LB agar plate containing X-gal and starch azure. The hybrid plasmid contained the β-galactosidase gene fused in frame to the α-amylase gene with 44 bps deleted in the C'

terminal region. This plasmid was introduced into *Z. mobilis* ZM4 by conjugal transfer. *Z. mobilis* transconjugants showed β-galactosidase and α-amylase activities on RM agar plates containing X-gal, starch azure, chloramphenicol and nalidixic acid(100 μg/ml) in the simultaneous manner. Plasmid pZAlac was also constructed as a control plasmid, which contained a β-galactosidase gene fused in frame to the initiation codon of the promoter in *Z. mobilis* expression vector, pZA26. Cell extracts were prepared from these transconjugants and then subjected to Western blot analysis with 7.5% SDS-PAGE electrophoresis. When the cell extracts of *Z. mobilis* carrying pZAMY381 were analyzed, a band of the 175 Kd *amy::lacZ* fusion protein was identified (Fig. 5), indicating that the α-amylase gene could be efficiently expressed in *Z. mobilis*. It was observed that the fusion protein migrated more slowly than the 116Kd β-galactosidase produced by a transconjugant harboring pZAlac. No corresponding protein band could be detected in the extract of *Z. mobilis* ZM4 which did not harbor the plasmid.

To investigate the formation of ethanol by *Z. mobilis* transconjugant harboring pZAMY38, cells were grown several times in RM supplemented with 4% soluble starch and 1% glucose. Subsequently, the culture was inoculated in the RM containing 5% soluble starch as a sole carbon source. Samples were taken out at intervals and ethanol production was measured by gas chromatography. Fig. 6(A) shows the qualitative evidence of ethanol production by the *Z. mobilis* transconjugant with the α-amylase gene expressed properly. After 28 hours of incubation, 67 mM ethanol was produced in RM/starch medium by the *Z. mobilis* transconjugant harboring pZAMY38 (Fig. 6(B)). In contrast, when *Z. mobilis* ZM4 cells without the plasmid were used as a control strain, no detectable ethanol was found under the same culture conditions.

DISCUSSION

The location of α-amylase gene in *B. stearothermophilus* was not clear. Mielenz (11) observed that the α-amylase gene of *B. stearothermophilus* was contained in a natural plasmid. This was supported by the fact that the gene transfer could take place between related strains. Other research groups suggested, however, that the α-amylase gene was on the chromosome in the other

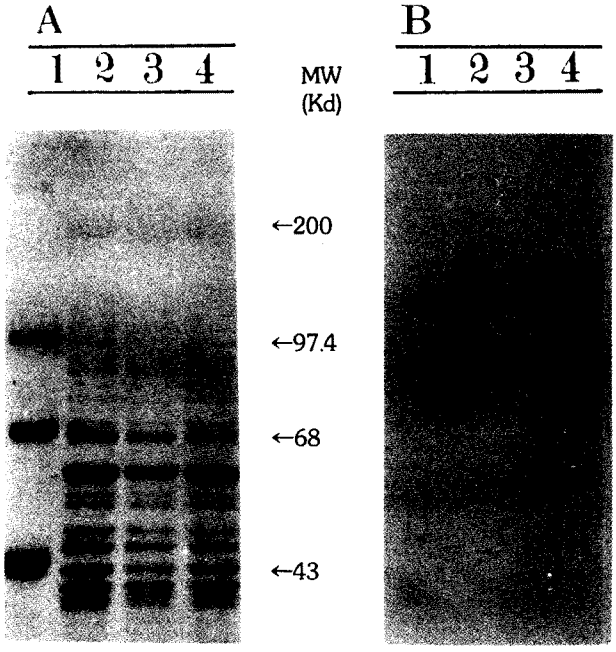

Fig. 5. Analysis of *amy::lacZ* fusion protein in SDS-PAGE and Western blot.
Panel A: Coomassie stained gel, Panel B: corresponding Western blots. Samples are as follows: lane 1, molecular weight standards; lane 2, *Z. mobilis* ZM4 harboring pZAMY 381; lane 3, *Z. mobilis* ZM4; lane 4, *Z. mobilis* ZM4 harboring pZAlac.

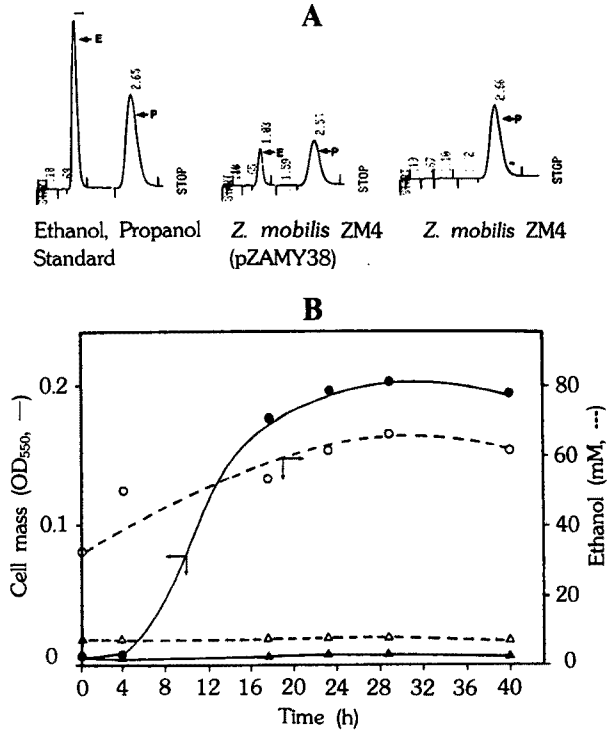

Fig. 6. Ethanol production from soluble starch by *Z. mobilis* transconjugant.
Panel A: by gas chromatographic analysis, Panel B: in a fermentor. pZAMY38 transconjugant was cultured at 30℃ in RM medium containing 5% soluble starch instead of glucose. Symbols: ● and ▲, growth; ○ and △, ethanol.

strains of *B. stearothermophilus* (15, 24). Recently, Sen and Ariel (20) reported on the location of α-amylase gene in two different *B. stearothermophilus* strains. In one strain, the α-amylase gene was present both in two different indigenous plasmids and in the chromosome with multiple copies. In the other strain, multiple copies were found in the chromosome but no indigenous plasmid could be detected. We tested this more systematically by the analysis of the chromosomal DNA and natural plasmids of *B. stearothermophilus* ATCC 31195. Using the cloned α-amylase gene as a probe, Southern hybridization showed signals only with the natural plasmids of *B. stearothermophilus* ATCC 31195 (data not shown). It is not yet clear if there is a mechanism involved in *B. stearothermophilus* which allows α-amylase genes to migrate from chromosomes to plasmids or reversely.

We cloned and expressed the structural gene of extracellular α-amylase from *B. stearothermophilus* ATCC 31195 in *Z. mobilis* ZM4 by use of *Z. mobilis* expression vectors, pZA26 and pLOI204. The *E. coli-Z. mobilis* shuttle vector pZA26 yielded better results as a cloning vehicle of α-amylase gene compared with another shuttle vector pLOI204 in *Z. mobilis*. This is most likely due to the fact that pZA26 has high copy numbers and high stability in *Z. mobilis* (13). On the contrary, the plasmid pLOI204 has low copy numbers due to its broad-host-range replicons (4).

With the hope of providing *Z. mobilis* cells with a capability to convert some cheaper raw materials such as cellulosics directly into ethanol, several heterologous genes such as cellulase gene (13), β-glucanase gene (14, 22), and D-xylose catabolic genes (9) were cloned and expressed in *Z. mobilis*. More practically, however, the substrate most available for the industrial ethanol fermentation is basically starch (e.g. cassava or sargo starch). It would be, therefore, more beneficial to engineer genetically ethanol-producing microbial strains that are capable of using starch as a carbon source for ethanol fermentation. With this in mind, efforts were made to breed yeast (18) or *Z. mobilis* (3) strains. In the present investigation, we have been successful in cloning and expressing the α-amylase gene from *B. stearothermophilus* in *Z. mobilis* ZM4. The practical employment of this genetically engineered *Z. mobilis* strain for ethanol fermentation will, however, require the concomitant cloning of glucoamylase gene, the construction of stable and high copy number expression vectors together with the introduction of an efficient secretion system of these hydrolytic enzymes into the culture medium.

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