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Full Length Research Paper

Calcium chloride improve ethanol production in recombinant *Zymomonas mobilis*

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The T7-expression system has been very useful for protein expression in *Escherichia coli*. Here, a T7-expression transposon was constructed, which allowed simple construction of T7-expression *Zymomonas mobilis*. This transposon contained the T7 RNA polymerase being driven by the *gap* promoter from *Z. mobilis*. The T7-expression *fadK* genomes were introduced into *Z. mobilis* ATCC 31821 in order to increase ethanol production. The recombinant bacteria were named as *Z.M.F-1*, *Z.M.F-2*, *Z.M.F-3*, *and Z.M.F-4*. However, *Z.M.F-4* had the highest ability of producing ethanol by selection. Compared with *Z. mobilis* ATCC 3182, there was 7% increase in ethanol production for *Z.M.F-4* with corn hydrolyaztes as fermentation medium. The 16 mmolL^{-1} supplement of calcium chloride could significantly improve the ethanol production. This was also clearly demonstrated by a variety of kinetic parameter values over time in *Z.M.F-4* under high sugar osmotic stress. Calcium chloride not only increased the fermentation ability but also improved the stability of cell membrane.

Key words: Calcium chloride, *fadK*, fermentation, *Zymomonas mobilis.*

INTRODUCTION

Zymomonas mobilis, a gram-negative bacterium has attracted more attention in recent years for fuel ethanol (Alexander et al., 2006; James et al., 2001; Zhiling, 2003) because it is an osmo- and ethanol-tolerant bacterium (Carey and Ingram, 1983) and it has shown higher specific rates of glucose uptake and ethanol production (Dien et al., 2003) via the Entner-Doudoroff pathway (Ingram and Conway, 1988) under anaerobic conditions (Cazetta et al., 2007).

According to the influence of ethanol on the lipid composition of *Z. mobilis*, the presence of long chain fatty acids was detected (Marta et al., 2000). However, pyruvate phosphokinase is a key enzyme in Entner-Doudoroff pathway which is inhibited by long-chain fatty acids. Therefore, it is considered that inserting a *fadK* gene in *Z. mobilis* could utilize or degrade fatty acids under anaerobic conditions (Rachael et al., 2004) and release from long fatty acids inhibition in order to enhance the ethanol yield.

During the fermentation of ethanol, *Z. mobilis* may encounter various environmental stresses which adversely affect the ability of cells to perform efficient and consistent conversion of sugars to ethanol. The major chemical and physical stresses experienced are from metabolic toxicity and stress which retard the growth, viability and fermentation ability of the cell, leading to a reduction of metabolic activity and eventually, death (Pornthap et al., 2007). However, stability and integrity of cell membrane established the soundness of cells, the strength of cell-mediated immunity, physical activity, adaptability and other physiological functions. Calcium chloride is an important electrolyte which would change conformation of peptidoglycan developing cell wall of permeability and stability (Walter et al., 2002; Hiroshi and Marti, 1985). On the other hand, calcium chloride helps to transfer material for maintaining the balance of ions and function of cytoplasmic membrane (Ferguson and Watkins, 1989; Zhonghe et al., 2000). Calcium chloride plays a crucial role in the cellular self-protection and self-recovery from metabolic toxicity and stress. Several reports have also pointed that calcium chloride implicates a positive effect of maintaining construction of plasma membrane to delay senescence (Shen et al., 2008). However, with respect to the ethanologenic *Z. mobilis*, studies on such a protective

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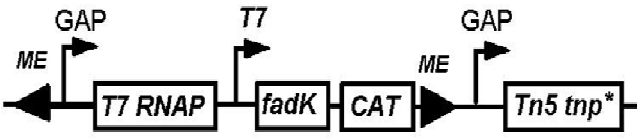

Figure 1. Schematic maps of the T7-expression *fadK transposon.*

effect of calcium chloride have hardly been reported. Here, the effect of calcium chlorides on ethanol production was examined in recombinant *Z. mobilis* constructed previously constructed. These results indicate that calcium chloride improves the fermentation performance and preserves plasma membrane.

In this paper, there are three original points: (1) constructing recombinant *Z. mobilis* by introducing the T7-expression *fadk* genomes into the chromosome. The *fadK* gene could degrade and utilize fatty acids as carbon and energy sources under anaerobic conditions and the T7-expression system has been very useful for protein expression; (2) choosing corn hydrolyaztes as fermentation medium for *fadK*, containing fatty acids; and (3) discussing the effect of calcium chloride on recombinant *Z. mobilis.*

EXPERIMENTAL PROCEDURES

Bacterial strains

Escherichia coli DH5α was used as the host strain for recombinant DNA manipulations and the source of *fadK*, T7RNApolymerase and T7promoter. *Z. mobilis* ATCC 31821 was used as the breeding host strain for the fermentation and the source of promoter genes of glycelaldehyde-3-phosphate dehyrogenase (*GAP* promoter) (Conway et al., 1987; Hideshi et al., 2007). Original strains were deposited in our laboratory. Recombinant *Z. mobilis* (named as *Z.M.F-1*, *Z.M.F-2*, *Z.M.F-3*, and *Z.M.F-4*) were constructed by our laboratory and were conserved in ZM broth with 20% (v/v) glycerol at -80°C.

Plasmids and culture conditions

E. coli DH5α was grown in Luria-Bertani medium(10 g/l tryptone, 5 g/l sodium chloride, and 1 g/l yeast extract; pH 7.2) containing 100 µg/ml ampicillin aerobically under 37°C, 200 r/min. *Z. mobilis* was cultured statically in ZM medium (10 g/l yeast extract, 100 g/l glucose, 1 g/l KH_2PO_4, 0.5 g/l $MgSO_4.7H_2O$, 1 g/l $(NH4)_2S0_4$; pH 6.0) at 30°C at 60 r/min anaerobically (Tao et al., 2005) in a solid media containing 1.5% (w/v) agar.

Construction of recombinant bacteria

The T7-expression system has been very useful for protein expression in *E. coli*. Here, a T7-expression transposon was constructed, which allows simple construction of T7-expression *Z. mobilis*. This transposon contains the T7 RNA polymerase driven by the *gap* promoter from *Z. mobilis*. The nucleotide sequence encoding *fadK* was amplified by PCR from *E. coli* DNA using the primer pairs 5' GAATTCATGAAAGTGACATTAACGTTT 3' (forward primer) 5'CTCGAGTTCAATCTCTTCACAGACATC 3' (reverse primer). This amplified 1.65 kbp fragment was under the control of T7 promoter (Yun et al., 2007) as in Figure 1. These genes were introduced into *Z. mobilis* ATCC 31821 by the transposition method as described in the literature (Foulongne et al., 1999). The transformants thus obtained were purified on an agar plate containing chloramphenicol (170 µg/ml). Large transformants that formed were picked up and inoculated into ZM medium, and then strains that exhibited rapid growth in ZM medium were selected as acclimated recombinant strains, last named as *Z.M.F-1*, *Z.M.F-2*, *Z.M.F-3* and *Z.M.F-4*.

Media, growth condition of fermentation

The strains were activated by an active medium (yeast extract 10 g/l, $MgSO_4.7H_2O$ 0.5 g/l, KH_2PO_4, 1.0 g/l, $(NH4)_2SO_4$ 3.0 g/l, $NaNO_3$ 0.17 g/l, 25 $mmoll^{-1}$ cubic nitre (Rachael et al., 2004), corn hydrolyzates 25% (v/v); pH 6.0) for 24 h before the 96 h fermentation until ferment was complete. The distinctness was corn hydrolyte content between fermentation and active medium. The fermentation medium contained 1 v/v corn hydrolyte (Yan and Shuzo, 2006). Corn hydrolyzates (from Anhui BBCA Biochemical Co., Ltd.) contained 24% (v/v) reducing sugar (the specific ingredients were unknown), an amount of fatty acids and other elements. The culture medium was sterilized at 115°C for 20 min. The strains were grown at 60 rpm at 30°C in 200-ml flask filled with 50 ml or 100 ml of active medium or fermentation medium, respectively, and the flask was equipped with a rubber stopper (Taherzadeh, 1997). 10% (v/v) of the mid-log phase cells in active medium were added into 100-ml fermentation medium. It was important to maintain the coincident concentration of thaliana switching in broth.

Reducing sugar and ethanol analyses

Fermentation cultures were centrifuged at 14000 r/m for 5 min (Anke tabletop centrifuge, TGL-16B, Shanghai, China) at room temperature. The supernatant liquid was removed and filtered from micropore film (size: Φ25 mm; pore diameter: 0.45 µm; Mosu Sci-Equipments Co. Shanghai), then measured and conserved at 4°C.

Ethanol was measured quantitatively by high efficiency gas chromatography (GC) (Youbin, 2005) (Instrument: GC-7900; Part number: 61984806; Detector type: FID; Colum dimension: TM-930 25 m×0.53 mm (ID) ×1.0 µm (df); Colum serial number: 530708-C01; sample size: 0.4 µl; split ratio: 0:1) using isobutanol as an internal standard.

Total reducing sugars were measured by Fehling reagent method (Wei, 2001; Hermann and Ger, 1849).

All experiments were repeated twice. Each experiment was carried out in triplicates and repeated three times. Modal values were selected.

RESULTS AND DISCUSSION

Results of preliminary screening

The recombinant *Z. mobilis* with the highest production ethanol was measured by high efficiency gas chromatography (GC) between recombinant *Z. mobilis* (*Z.M.F-1*, *Z.M.F-2*, *Z.M.F-3* and *Z.M.F-4*) cultured under the same condition simultaneously and *Z. mobilis* ATCC 31821 as a control. Compared with *Z. mobilis* ATCC 31821, ethanol production increased to 101.1% in *Z.M.F-1*, 103.6% in *Z.M.F-2*, 104.2% in *Z.M.F-3* and 107.0% in

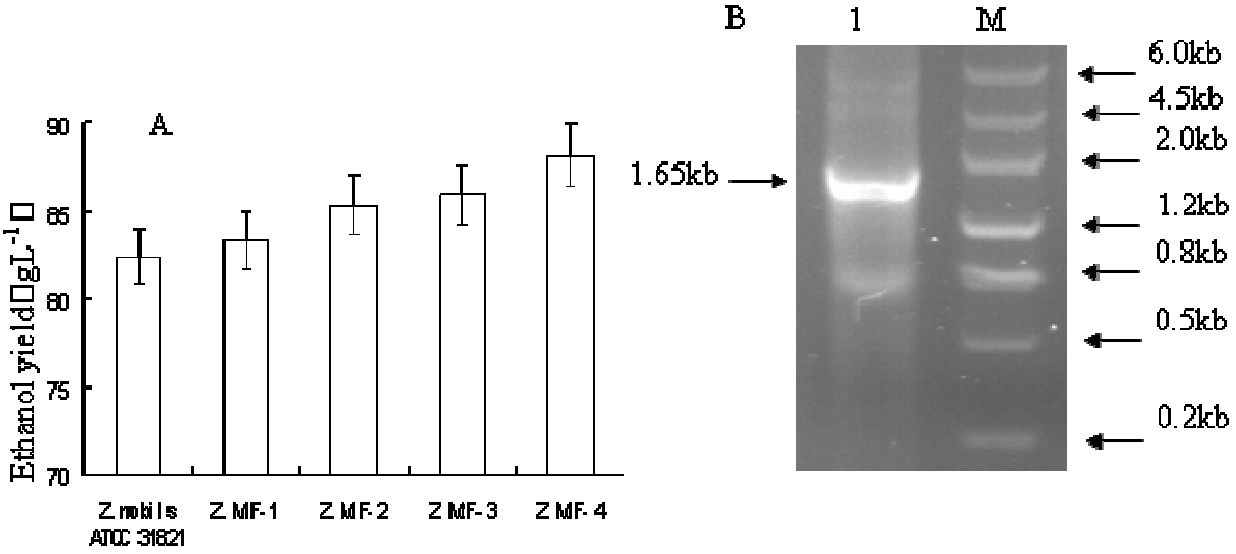

Figure 2. A) Ethanol production at 30°C after 96 h fermentation. B) Result of PCR amplification of *fadK*. M: DNA Marker.1: *fadK* gene — 1.65 kb.

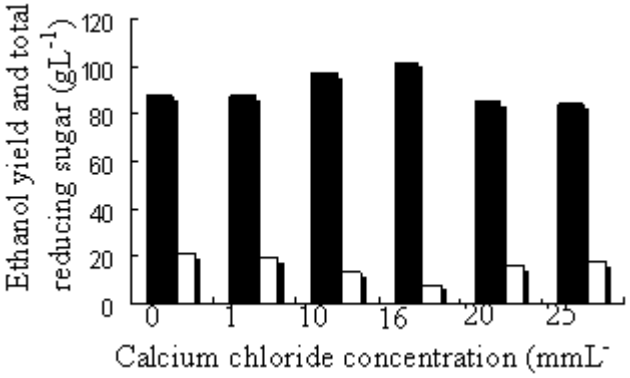

Figure 3. Ethanol yield and total reducing sugar (gL^{-1}) in medium under high sugar stress with different calcium chloride concentration (mml^{-1}) at 0 h: (■) ethanol yield; (□) total reducing sugar.

Z.M.F-4 (Figure 2A). The results suggested that *Z.M.F-4* has the highest ability of ethanol production. Besides, the resultant sequence *fadK* inserted into the recombinant bacterium *Z.M.F-4* was verified by PCR from chromosomal DNA. So, the following experiments focused on *Z.M.F-4* (Figure 2B).

Effect of different calcium chloride concentration on ethanol production

In order to examine the effect of unique calcium chloride on the fermentation ability of *Z.M.F-4*, several changes were made. As for medium, corn hydrolyaztes was replaced by glucose-containing liquid. Active medium contained 10% (w/v) glucose, 1% (w/v) yeast extract, while fermentation medium contained 24% (w/v) glucose, 1% (w/v) yeast extract and the different calcium concentration, according to the experimental design. Culture conditions were described as before. According to the results, it was found that increasing concentration of calcium chloride supplemented had significant impacts on the ethanol yield shown in Figure 3. As calcium chloride increased, ethanol yield progressively increased. Initial concentration of 1, 10, or $16mmolL^{-1}$ calcium chloride in the fermentation medium resulted in increased 0.28, 11.2 or 15.9% in ethanol yield, respectively. But ethanol yield significantly decreased when the initial calcium chloride concentration was higher than $16mmolL^{-1}$. On the other hand, initial concentration of 0, 1, 10, 16, 20 or $25mmolL^{-1}$ calcium chloride resulted in progressive reduction in total reducing sugar from $240gL^{-1}$ to 20.5, 19.2, 13, 7.7, 16, or $17.6gL^{-1}$, respectively. These results suggested that adequate calcium chloride concentration could enhance ethanol yield and that 16 mml^{-1} calcium chloride concentration being optimum for ethanol product and conversion of sugars to ethanol in *Z.M.F-4*.

One possible explanation was that calcium chloride enhanced tolerance by improving the stability of cellular membrane in *Z.M.F-4*, which also stimulated cells to metabolize, enhancing the ethanol production. This point was very important especially for the medium which contains high concentration glucose. The osmotic pressure of fermentation medium was high because it contained high concentration glucose (24%, v/v). The stability of cellular membrane was very important to cell metabolism. Calcium chloride improved its stability by adjusted materials entrance and exit through cell membrane. In other words, calcium chloride enhanced the permeability of membrane which made endocellular pressure being consistent with exocellular pressure to retain stability of cellular membrane.

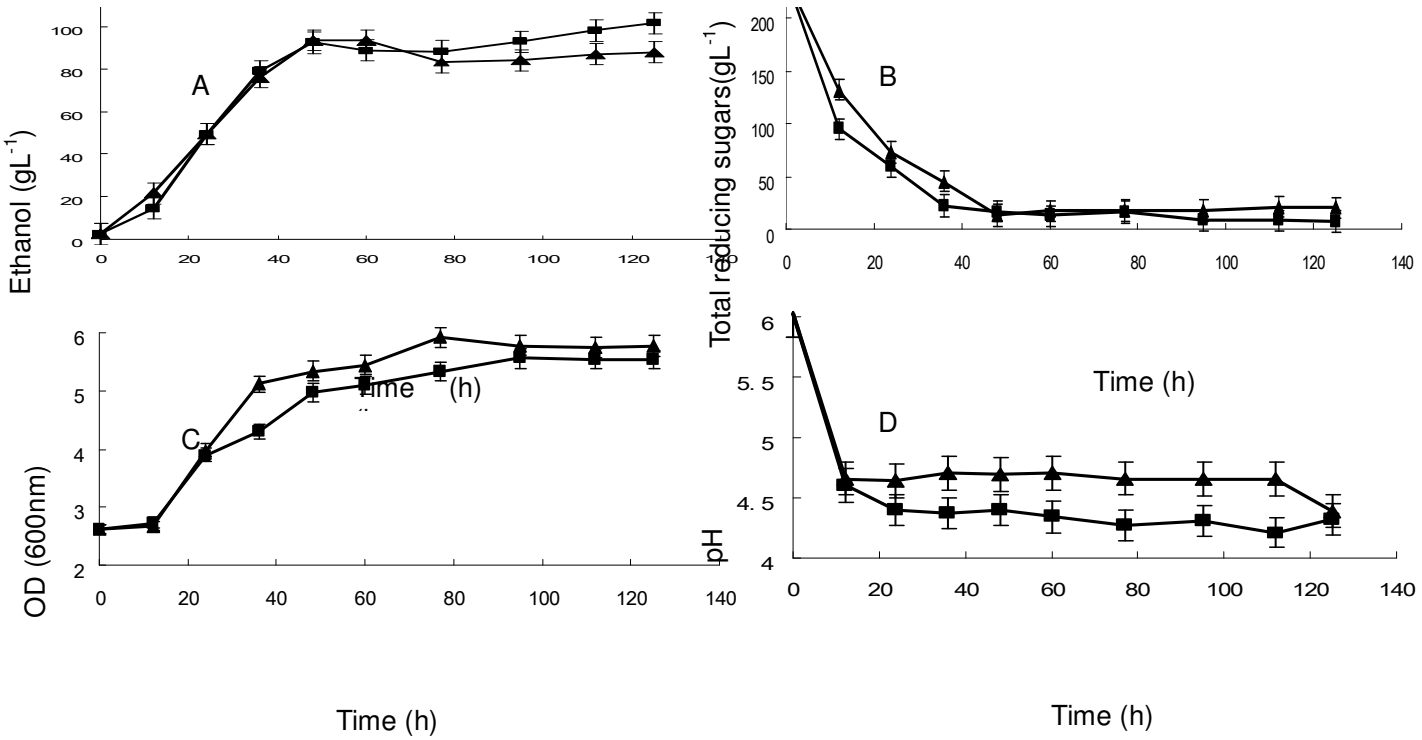

Figure 4. Effect of calcium chloride on parameter values at 0, 12, 24, 36, 48, 60, 77, 95, 112 and 125 h in *Z.M.F-4*. Calcium chloride was added at a final concentration of 0 or16 mmL^{-1} at 0 h. A: Ethanol yield (0 mmL^{-1}: triangles (▲), 16 mmL^{-1}: squares (■)). B: Total reducing sugars (0 mmL^{-1}: triangles (▲), 16 mmL^{-1}: squares (■)). C: Growth (OD_{600nm}) (0 mmL^{-1}: 0 mmL^{-1}: triangles (▲), 16 mmL^{-1}: squares (■)). D : PH (0 mmL^{-1}: 0 mmL^{-1}: triangles (▲), 16 mmL^{-1}: squares (■)).

Effect of calcium chloride on a variety of kinetic parameter values over time in *Z.M.F-4*

The change of various parameters values was extremely quite different. At cell growth prophase (from 0 to 12 h), total reducing sugar and pH strictly decreased from 240 to 132.4 or 95.2 gL^{-1} and 6.0 to 4.66 or 4.6 in 0 or 16 $mmolL^{-1}$ calcium chloride, respectively, while ethanol yield and biomass increased slightly. The cells growth required huge energy provided by glucose in cell growth prophase. *Z.M.F-4* consumed largely glucose and produced a little ethanol. Since most of the cell metabolic products were highly acidic, biomass increased slightly but pH vigorously decreased. At cell growth log-phase (from 12 to 48 h), ethanol yield and growth (OD_{600nm}) strongly increased from 21.7 - 93.4 gL^{-1} or 14.6 - 92.3 gL^{-1} and 2.7 - 5.34 or 2.7 - 5.0 in 0 or 16 $mmolL^{-1}$ calcium chloride, respectively (Figures 4A and C). In contrast, total reducing sugar decreased drastically from 132.4 - 13.7 gL^{-1} or 95.2 - 16.75 gL^{-1} (Figure 4B). However, pH was maintained at about 4.3 - 4.4 during log-phase. All the pH values in 16$mmolL^{-1}$ calcium chloride were lower than in 0$mmolL^{-1}$ from 12 h (Figure 4D). The reason was that calcium ion increased permeability of cell membrane and caused more acidic products penetration into ectodomain at cell growth phase, while calcium ion decreased permeability at cell stable phase. This change perhaps was adjusted by various ion pumps in the cell membrane for calcium chloride. Since it has entered the cell stable phases, changes of a variety of cell metabolism parameters values can proceed stably. Hence, total reducing sugar, cell growth (OD_{600nm}) and pH in 16 $mmolL^{-1}$ calcium chloride medium have been less than these in 0 mmL^{-1} medium, but ethanol production has been higher during stable phases. This change further illustrated that calcium chloride enhance ethanol yield by affecting cell membrane rather than increasing cell biomass. With the extension of fermentation time, various metabolic toxicity and stress have been continuously accumulated in the fermentation broth to repress cell growth even till death. So there was a downward phase of ethanol production between 48 h and 77 h (Figure 4A). The ethanol production reached the 101.5 gL^{-1} maximum in medium with 16 $mmolL^{-1}$ calcium ion at 125 h, but it reached a low level of 87.8 gL^{-1} in medium without calcium ion. Increasing of ethanol production at the final stage of fermentation might be related to calcium ions playing the role of delaying cell senescence.

Conclusions

First, a recombinant bacterium *Z.M.F*-4 had the T7-expression *fadK* genomes, besides its ethanol production had been higher than the control (*Z. mobilis* ATCC 31821) in corn hydrolyzates medium. The fact is that pyruvate phosphokinase is inhibited by long-chain fatty acids and high concentration of ATP; it is also a key enzyme in Entner-Doudoroff pathway. *FadK* utilized fatty acids as a carbon source to degrade the long-chain fatty acids and this process consumed ATP; *fadK* slightly relieved this inhibition in order to enhance ethanol production. On the other hand, under a common control of T7 promoter, the result was that there was 7% increase in ethanol production by *Z.M.F-4*. Taking into account the role of *fadK*, corn hydrolyzates was precisely chosen as a fermentation medium, because it contains small amount of fatty acids, beside 24% (v/v) glucose, growth factors and other substances.

Secondly, the effect of calcium chloride on *Z.M.F-4* was considered. In order to accurately work out the influence of unique calcium chloride on the *Z.M.F-4*, we chose the fermentation medium which only contains 24% glucose, 1% yeast and different concentration of calcium chloride. Lastly, ethanol production of *Z.M.F-4* reached maximum in medium with 16 $mmolL^{-1}$ calcium chloride. From series of time curves of kinetic parameter values in medium containing 16 or 0 $mmolL^{-1}$ calcium chloride, the conclusion about effect of calcium ion on *Z.M.F-4* was that the effect at stable phase is most obvious. To sum up, calcium chloride not only helped cell grow but enhanced the ethanol production. The reasons for the effect of calcium chloride on ethanol production can be concluded as below: firstly, free calcium ions were rightly sensitive to cells especially at the stable phase and largely positively adjusted to the ion pumps in cell membrane to impact on the permeability of cell membranes and maintain stability of membrane. Under the high sugar concentration and stress, stability of plasma membrane or cell membrane was the most important point for normal cell growth and metabolism. Secondly, calcium ions effectively reduce membrane permeability delaying cellular senescence at the stable phase.

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REFERENCES

Alexander EF, Richard JP, Brian TT, Andrew DJ, Michael O'H, Daniel MK (2006). Ethanol Can Contribute to Energy and Environmental Goals. Science, 311(5760): 506-508.

Carey VC, Ingram LO (1983). Lipid Composition of *Zymomonas mobilis*: Effects of Ethanol and Glucose. J. Bacteriol. 154(3): 1291-1300.

Cazetta ML, Celligoi MAPC, Buzato JB, Scarmino IS (2007). Fermentation of molasses by *Zymomonas mobilis*: Effects of temperature and sugar concentration on ethanol production. Bioresour. Technol. doi:10.1016/j.biortech.2006.08.026:1-5.

Conway T, Sewell GW, Ingram LO (1987). Glyceraldehyde-3-phosphate dehydrogenase Gene from *Zymomonas mobilis*: Cloning, Sequencing, and Identification of Promoter region. Bacteriol. 169: 5653-5662.

Dien BS, Cotta MA, Jeffries TW (2003). Bacteria Engineered for Fuel Ethanol Production: Current Status. Appl. Microbiol. Biotechnol. 63: 258-266.

Ferguson IB, Watkins CB (1989). Bitter Pit in Apple Fruit. Horticult. Rev. (11): 289-355.

Foulongne V, Bourg G, Cazevieille C, Michaux-Charachon S, O'Callaghan D (1999). Identification of Brucella suis genes affecting intracellular survival in an in vitro human macrophage infection model by signaturetagged transposon mutagenesis. Infect. Immun. 68: 1297-1303

Hideshi Y, Dai S, Keiko Y, Saori M, Sho Y, Kenji O (2007). Genetic Engineering of *Zymobacter palmae* for Production of Ethanol from Xylose. Appl. Environ. Microbiol. 73(8): 2592-2599.

Hiroshi N, Marti V (1985). Molecular Basis of Bacterial Outer Membrane Permeability. Microbiol. Rev. 49(1): 1-32.

Ingram LO, Conway T (1988). Expression of Different Levels of Ethanologenic Enzymes from *Zymomonas mobilis* in Recombinant Strains of *Escherichia coli*. Appl. Environ. Microbiol. 54(2): 397-404.

James C, Ogbomma, Hiroaki Mashima, Hjideo Tanaka (2001) Scale up of Fuel Ethanol Production from Sugar Beet Juice Using Loofa Sponge Immobilized Bioreactor. Bioresour. Technol. 76(1): 1-8.

Marta IV, Maria del RC, Emilio R, Danley C (2000). Identification of Heptadecanoic and C19 Cyclopropane Fatty Acids in the Lipid Fraction of *Zymomonas mobilis*. Curr. Microbiol. 41: 305-306.

Pornthap T, Pattana L, Kaewta S, Mamoru Y (2007). Magnesium Ions Improve Growth and Ethanol Production of *Zymomonas mobilis* under Heat or Ethanol Stress. Biotechnology, 6(I): 112-119.

Rachael M, Morgan K, John EC (2004). The *Escherichia coli fadK* (*ydiD*) Gene Encodes an Anerobically Regulated Short Chain Acyl-CoA Synthetase. Biol. Chem. 279(36): 37324-37333.

Shen L, Ding Y, Li S-Q, Sheng J-P (2008). Effects of Ca^{2+} on the aging process of post-harvest Pak-choi. Sci. Technol. Food Ind. 29(4): 1-3.

Tao F, Miao JY, Shi GY, Zhang KC (2005). Ethanol Fermentation by an Acid-tolerant *Zymomonas mobilis* under Non-sterilized Condition. Process Biochem. 40: 183-187.

Walter L, Miyoshi H, Leverve X, Bernardi P, Fontaine E (2002). Regulation of the mitochondrial permeability transition pore by ubiquinone analogs. A Progress Rep. 36(4): 405-412.

Wei Z (2001) The Factors of Affecting the Determination of Reducing Sugar in Food. Liaoning Chem. Ind. 30(10): 463-464.

Yan L, Shuzo T (2006). Ethanol Fermentation from Biomass Resources: Current State and Prospects. Appl. Microbiol. Biotechnol. 69: 627-642.

Youbin X (2005). Gas Chromatography Internal Standard Method Analysis Ethanol Content in Beer. Journal of Huainan normal University 7(5): 19-20.

Yun K, Mike SS, Tung TH (2007). One step engineering of T7-expression strains for protein production:Increasing the host-range of the T7-expression system. Protein Exp. Purif. 55(2): 325-333.

Zhiling H (2003). Production and Utilization of Fuel Ethanol. Chem. Technol. 11(4): 44-47.

Zhonghe H, Xizhong W, Mingxiao DB (2000). Cell Biology. Higher Education Press, China, pp. 95-117.