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 (Dienie 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
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 hydrolyzates 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 16 mmolL⁻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 16 mmolL⁻¹. On the other hand, initial concentration of 0, 1, 10, 16, 20 or 25 mmolL⁻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 mmolL⁻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.
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\(_{600\text{nm}}\)) 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 16mmolL\(^{-1}\) calcium chloride were lower than in 0mmolL\(^{-1}\) 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\(_{600\text{nm}}\)) and pH in 16 mmolL\(^{-1}\) calcium chloride medium have been less than these in 0 mmolL\(^{-1}\) 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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