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Fermentation potentials of *Zymomonas mobilis* and its application in ethanol production from low-cost raw sweet potato

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The effects of pH, high concentration of glucose and the initial ethanol content on the fermentation process of ethanol with three strains of *Zymomonas mobilis* were investigated and the strain of ATCC 29191 was chosen for the next study. The optimal parameters for the ethanol fermentation were studied using the sweet potato raw material as feedstock with an orthogonal experimental design. It was found that the condition for ethanol production was optimized to be pH 4, substrate concentration of 20%, inoculum size of 7.5% and time of fermentation of 24 h, resulting in ethanol yield of 66.4 g/L, productivity of 2.77 g/L/h and fermentation efficiency of 93.5%, respectively. In addition, the inoculum size was identified to be the main factor for efficient ethanol production. By adopting the optimized fermentation condition, high concentration fermentation using sweet potato as sole feedstock was achieved with *Z. mobilis* ATCC 29191. The ethanol yield and fermentation efficiency were obtained with 99.1 g/L and 92.4%, respectively, in the presence of 400 g/L of initial content of sweet potato. This work demonstrates that the low-cost sweet potato is a feasible feedstock for ethanol fermentation with *Z. mobilis*.

Key words: Ethanol, *Zymomonas mobilis*, sweet potato, fermentation, orthogonal experimental design.

INTRODUCTION

Fuel ethanol is one of the most important clean fuels and renewable energy resources, which would play an important role in effectively solving the problem of the forthcoming oil shortage. When compared to *Saccharomyces cerevisiae*, a facultatively anaerobic and ethanologenic bacterium, *Zymomonas mobilis*, shows some physiological and metabolism advantages, such as its higher ethanol yield and tolerance, faster sugar uptake rate and lower biomass (Rogers et al., 2007; Jeffries, 2005; Rogers et al., 1982; Swings and Deley, 1977). Therefore, this bacterium has attracted considerable attention in ethanol production over the past decades, and it is thought to be a promising strain for further development.

The establishment of ethanol industry requires sufficient and cheap feedstock in order to reduce the costs of production that has been recognized as a critical point (Cardona and Sanchez, 2007). However, the transformation of some conventional raw materials (like corn, wheat and rice etc) is not feasible, especially in developing countries, because they are mainly used for food. For the sake of the win-win prospect between energy and food security, the development of fuel ethanol industry should be based on the non-grain crops and biomass (Qiu et al., 2008; Ragauskas et al., 2006; Lin and Tanaka, 2006; Mielenz, 2001).

In view of this problem, some starch-rich crops, such as sago, cassava (Rhee et al., 1984), sorghum (Aggarwal et

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al., 2001) and agro-industrial by-products (Cazetta et al., 2007; Davis et al., 2006; Ruanglet et al., 2006) were used as materials to produce ethanol with *Z. mobilis*. Sweet potato, the seventh most important crop in terms of production (Loebenstein and Thottappilly, 2009) is widely cultivated in large parts of China. And China has become the largest producer of sweet potato in the world with an annual yield of more than 100 million tons (Zhang et al., 2009). At present, sweet potato is mainly used for industrial raw material and livestock feed. Along with its low cost and high production, sweet potato has been regarded as a non-grain feedstock and potential source for ethanol production.

This study aims to evaluate feasibilities of the locally available feedstock of sweet potato for ethanol production using *Z. mobilis*. Higher ethanol yields have been achieved with high content of sweet potato as sole substrate.

MATERIALS AND METHODS

Bacterial strains and culture condition

Three strains of *Z. mobilis*: IFFI 10225, ATCC 29191 and CICC 10232 were obtained from the China Center of Industrial Culture Collection (CICC). The bacterial strains were grown at 30°C for 24 h by regularly streaking on the agar plates of the rich medium (RM) containing (per liter): glucose, 20 g; yeast extract, 10 g; $(NH_4)_2SO_4$, 1 g; K_2HPO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g, and with the native pH (approximately 6.2) (Struch et al., 1991).

Evaluation of three strains for ethanol fermentation

For determining the tolerance of the three strains of *Z. mobilis* to the high substrate concentration, 100 ml of RM medium containing 100 to 300 g/L of glucose were added into the 100 ml of Erlenmeyer flasks. After inoculation with 5% (v/v) seed culture, the cultures were incubated at 30°C without agitation. The yield of CO_2 was used to assess the fermentation performance and determined by weight loss (Hayashida et al., 1982; Zhang et al., 2008) at the designated intervals during the whole fermentation procedure.

In order to evaluate effects of the pH values and initial ethanol content on the growth and fermentation of *Z. mobilis*, various concentrations of initial ethanol content and different pH values in the sterilized RM medium were prepared. Aliquots of 50 ml medium were poured into the graduated fermentation tube and inoculated with 8% (v/v) seed culture. The fermentation was performed without agitation at 30°C. Afterwards, the CO_2 gas evolving at different intervals was recorded with the graduated fermentation tube. The above experiments were performed twice with three repeats.

Sweet potato material and pretreatment

The fresh sweet potato from two varieties of XuShu18 and Chuan Shu34 with the starch content of about 27% (w/w) was harvested from a local experimental field in Sichuan, China. The samples were washed with distilled water to remove extraneous matters and dried to a constant weight in a drying oven at 80°C. The dried materials were then ground to powder using a grinding machine attached with a sieve corresponding to 40 mesh. The resulting raw materials were used for preparation of fermentation media.

Liquefaction of the above raw materials of sweet potato was carried out by heating at 100°C for 3 h. The saccharification was performed at 60°C for 2 h in a water bath using the glucoamylase (Beijing Aoboxing Biotech Company Ltd., China) with a dosage of 1000 U per gram of the sweet potato raw material. The resulting slurry was centrifuged at 12,000 rpm for 15 min, and the pH of the supernatant was adjusted to the desired values by the addition of 1 M HCl.

Orthogonal experiment for ethanol fermentation

An orthogonal experimental design L9 (3^4) in triplicate was employed to assess the effects of pH, concentration of sweet potato raw materials, inoculum size and fermenting time on ethanol production. The ranges of parameters set for this analysis were shown in Table 1. The fermentation efficiency [defined as the percentage of the producing ethanol yield (g)/the theoretical ethanol yield (g)] was used to evaluate the fermentation parameters. The optimal parameters can be deduced from the T values, which were the average of the results of the fermentation efficiency corresponding to level 1, 2, and 3, respectively.

Ethanol fermentation with high concentration of sweet potato raw materials

The supernatant derived from the pretreated raw materials of sweet potato without any additive was directly used for ethanol fermen-tation. Each 500 ml of the supernatant from 30 and 40% (w/v) of the sweet potato raw material was poured into the three-neck flasks. The fermentation was conducted under the optimal condition obtained from the orthogonal experiment. During the fermentation procedure, samples were periodically removed from the flasks and used to determine the concentrations of the reducing sugar and ethanol.

Analytical methods

The leftover reducing sugar was estimated by 3,5-dinitrosalicylic acid method (Miller, 1959) and the ethanol content was analyzed by gas chromatography (He et al., 2009).

RESULTS

Effect of glucose concentration

Three strains of *Z. mobilis* were selected to evaluate the ethanol fermentation capacity in RM medium containing high concentrations of glucose. At 100 and 150 g/L of glucose, there was no obvious difference in CO_2 production rate (defined as the rate of the weight of CO_2 emitted to the weight of initial glucose) for those three strains (Figure 1). Nevertheless, the strain of ATCC 29191 required a shorter time to reach the plateau for CO_2 production, indicating that this strain could consume glucose more quickly than the other two strains (Figure 1). When the concentration of glucose was increased to 200 or 300 g/L, the time required to produce CO_2 was delayed for all the three strains, and the amount of CO_2 produced was reduced too. For example, when the concentration of glucose was increased from 100 to 300 g/L, the CO_2 production rates with ATCC 29191 were decreased from 40.5 to 18.1% within 96 h. At the same

Table 1. Range analyses of fermentation efficiency in orthogonal experiments*.

Number	pH	Inoculum size (%, v/v)	Material concentration (g/L)	Time (h)	Fermentation efficiency (%)
1	5.0	5.0	200	16	85.5
2	5.0	7.5	150	20	86.6
3	5.0	10	120	24	83.8
4	4.5	5.0	150	24	90.3
5	4.5	7.5	120	16	88.1
6	4.5	10	200	20	83.2
7	4.0	5.0	120	20	88.6
8	4.0	7.5	150	24	92.0
9	4.0	10	200	16	81.6
T_1 (%)	85.3	88.1	86.9	85.1	
T_2 (%)	87.2	88.9	86.1	86.1	
T_3 (%)	87.4	82.8	86.8	88.7	
R (%)	2.1	6.1	0.7	3.6	

* Fermentation efficiency (%) = Ethanol produced (g)/Theoretical ethanol yield from sugar (g), and the theoretical ethanol yield = 0.51 g of ethanol per gram sugar.
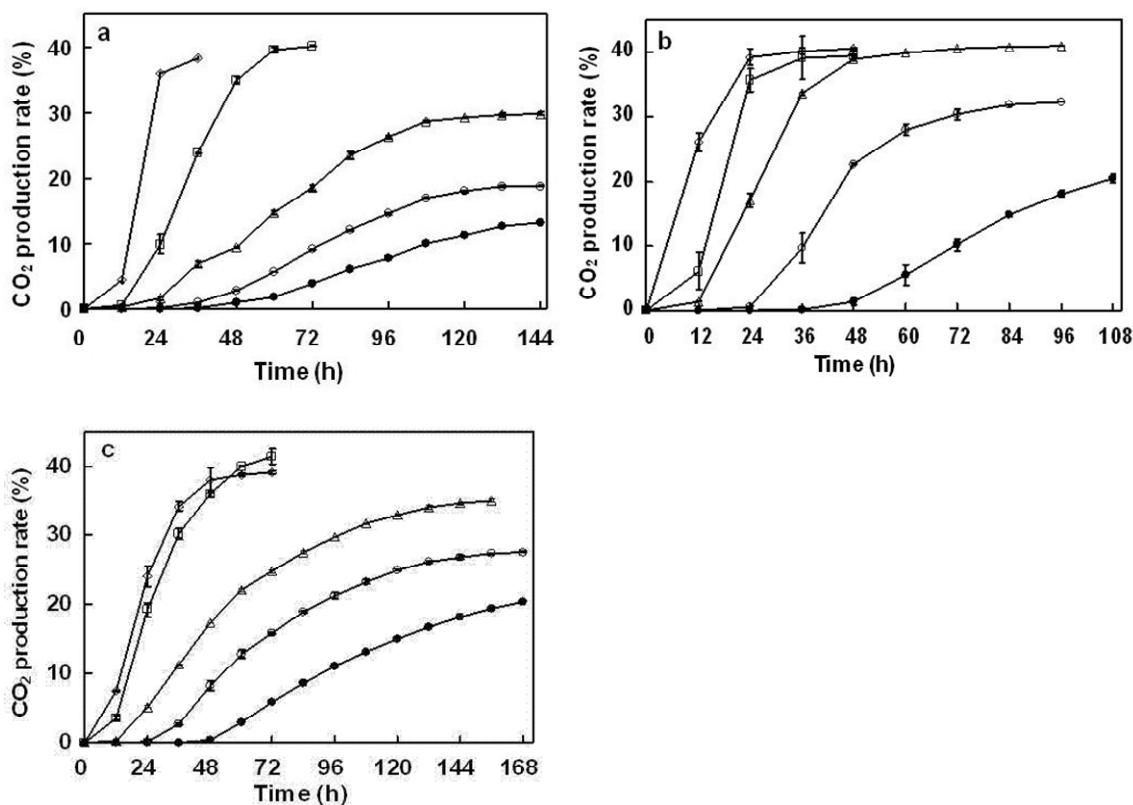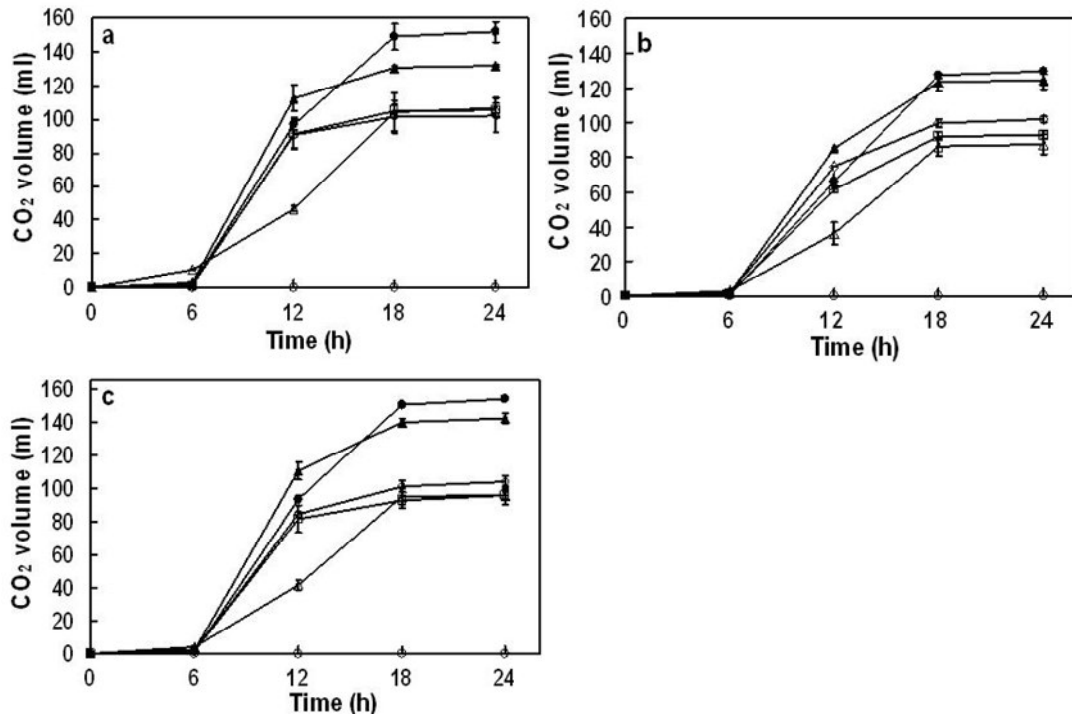

Figure 1. Effect of initial glucose concentration on CO_2 production during ethanol fermentation by *Z. mobilis*. a, IFFI102259; b, ATCC29191 and c, CICC10232. The bacteria was cultivated in the RM medium but containing 100 (◊), 150 (□), 200 (Δ), 250 (○) and 300 (●) g/L of glucose. CO_2 production rate is defined as CO_2 emitted (g)/ initial glucose (g). The data are derived from three independent experiments.

time, the CO_2 production rates with the strains of IFFI 10225 and CICC 10232 were reduced to 7.8 and 11.0%,

respectively.
In brief, these results indicated that *Z. mobilis* ATCC

Figure 2. Effect of initial pH on CO_2 production during ethanol fermentation by *Z. mobilis*. a, IFFI102259; b, ATCC29191 and c, CICC10232. The RM medium was used with the initial pH 6 (◊), 5 (□), 4.5 (▲), 4.0 (●), 3.5 (∆), 3 (○).These are the mean values obtained from three experiments.

29191 had an advantage in glucose utilization over the other two strains.

Effect of pH value

The effects of different initial pH values in RM medium on CO_2 production were presented in Figure 2. In total, there was no big difference in the pattern of CO_2 production with the three strains. It can be seen that the yields of CO_2 gas reached to the highest peak at pH 4, followed by pH 4.5 for each of these three strains (Figure 2). However, CO_2 was hardly produced at pH 3.
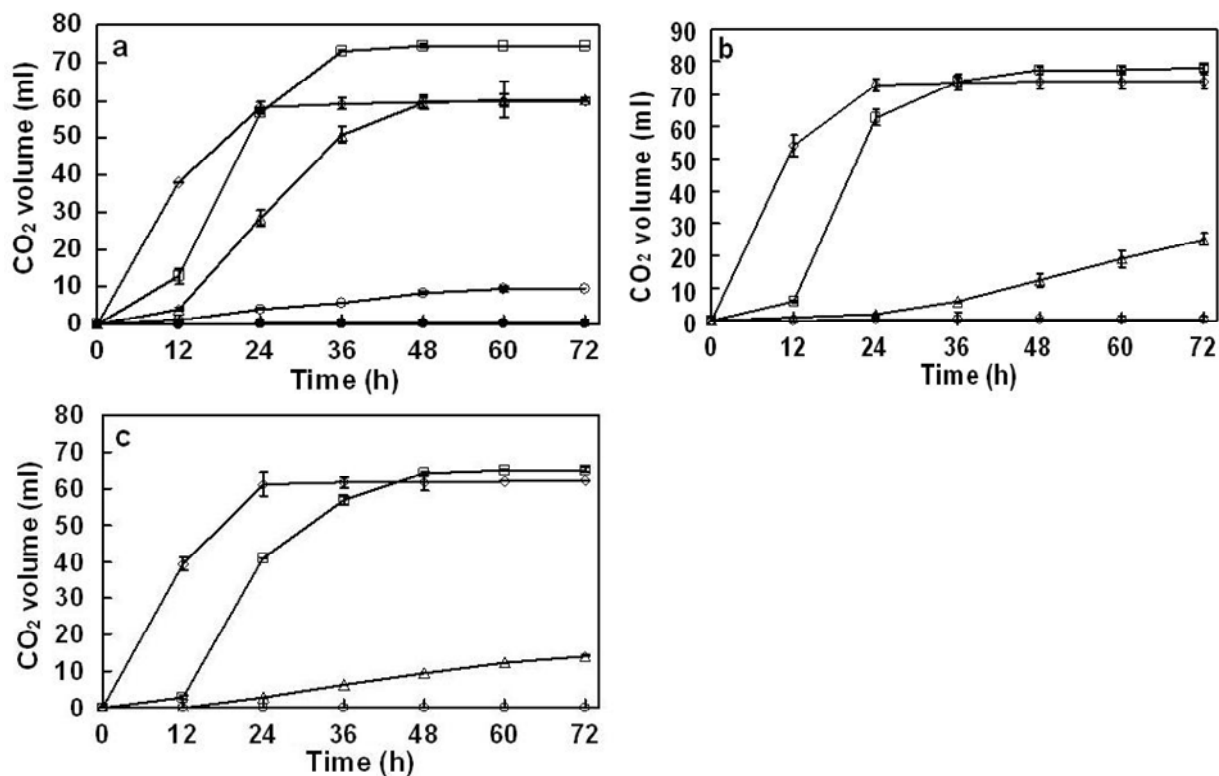
Effect of initial ethanol content

Higher ethanol content in the RM medium caused a great impact on the fermentation in terms of CO_2 production. It is demonstrated clearly from Figure 3 that the volume of CO_2 gas produced in the RM medium containing an initial ethanol content of 10% or more was significantly reduced and delayed. At 0 and 8% of initial ethanol content, the gas production was about the same in amount, but delayed in time for the three strains. Besides, the strain of IFFI 10225 showed higher ethanol tolerance than the other two strains. In the presence of 12% ethanol content, the CO_2 gas produced by the strain of IFFI 10225 was

sharply reduced, while no CO_2 gas could be detected with ATCC 29191 and CICC 10232 (Figure 3).

Optimal fermentation condition via orthogonal design

In order to assess the fermentation potential of sweet potato as raw materials to produce ethanol with *Z. mobilis*, the strain of ATCC 29191 that was demonstrated to utilize glucose most quickly, was selected to optimize the fermentation parameters using the orthogonal design. From the results obtained, the best condition for the highest fermentation efficiency was: pH 4, 7.5% of inoculum size, 20% of substrate concentration and 24 h of fermentation time. According to the values of R in Table 1, it can be deduced that the influence of the factors on ethanol fermentation was $R_{(inoculum\ size)} > R_{(time)} > R_{(pH)} > R_{(material\ concentration)}$, indicating that the major impact came from inoculum size under this condition.

By combining the above optimal parameters, a fermentation experiment was subsequently conducted with 200 g/L of sweet potato raw material as sole fermentation substrate. Kinetic processes of the ethanol production and consumption of substrate (expressed as reduced sugar, g/L) were shown in Figure 4. During the fermentation process, the substrate was consumed slowly within the first 6 h, and followed by a quickly decreasing phase from 9 to 18 h, which was well matched to the ethanol

Figure 3. Effect of initial ethanol content on CO_2 production during ethanol fermentation by *Z.mobilis*. a, IFFI102259; b, ATCC29191 and c, CICC10232. The RM medium was used but amended with ethanol content 0 (◊), 8% (□), 10% (Δ), 12% (○) and 14% (●), respectively. These are the mean values obtained from three experiments.
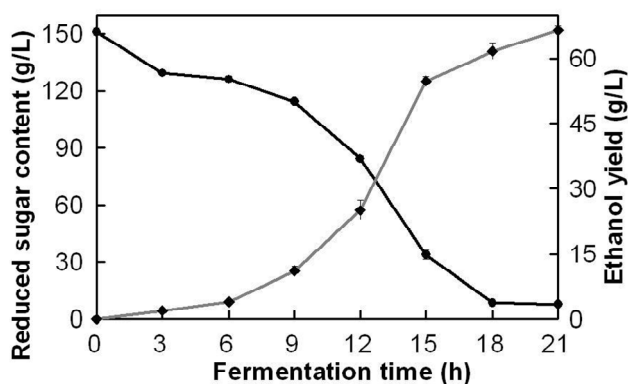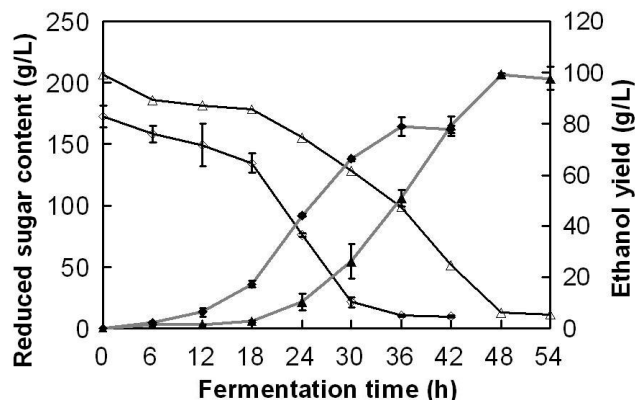

Figure 4. Optimal fermentation by *Z. mobilis* (ATCC 29191) with sweet potato raw material via orthogonal design. The fermentation was performed at 30°C with the concentration of sweet potato raw material of 200 g/L, inoculum size of 7.5%, and pH 4.0. The residual reduced sugar content (●); the ethanol yield (♦).

production. At the end of fermentation (21 h), the ethanol yield of 66.4 g/L and ethanol productivity of 2.77 g/L/h were obtained. The corresponding fermentation efficiency reached to 93.5%, which was higher than 92.0% of the highest fermentation efficiency listed in the above

orthogonal design (Table 1).

Ethanol production at high concentration of raw material

Based on the above experimental results, and to achieve higher production of ethanol, high concentrations of sweet potato raw material (300 and 400 g/L) were used to evaluate the ethanol fermentation with *Z. mobilis* ATCC 29191. The results were presented in Figure 5. A continuous increase in the ethanol yield was accompanied with a decrease in the reduced sugar concentration during the whole fermentation process. Within the first 18 h, a slow increase of ethanol yield occurred at both reduced sugar concentrations of 172 and 207 g/L, which corresponded to the raw material of 300 and 400 g/L, respectively. And then, the production of ethanol was quickly increased until 36 and 48 h for the two substrate concentrations, respectively. However, the whole fermentation time required for fast production of ethanol at high substrate concentrations (Figure 5) was delayed in comparing with that of 200 g/L of raw material (Figure 4). The ethanol yield and the corresponding fermentation efficiency were finally achieved with 78.9 g/L and 93.8%, 99.1 g/L and 92.4%, at the 36th h for the raw material of

Figure 5. Ethanol fermentation by *Z. mobilis* (ATCC29191) with high concentrations of sweet potato raw material. The fermentation was performed with 7.5% inoculum size at 30°C and pH 4.0. The residual reduced sugar content: 400 g/L (△), 300 g/L (◇); the ethanol yield: 400 g/L (▲), 300 g/L (♦).

300 g/L and the 48th h for 400 g/L, respectively. Besides, the residue reducing sugar was only about 5% (w/v) of the initial content after the fermentation ended.

DISCUSSION

Various strains of *Z. mobilis* were obtained and have been used for ethanol fermentation. In fact, different strains exhibited different properties in fermentation potentials. For example, ATCC 29919 shows quick utilization rate of substrate (Figure 1); and IFFI10225 possesses higher tolerance to initial ethanol content (Figure 3). Therefore, it is necessary to select or improve the strains used in efficient ethanol fermentation.

Acid-tolerant strains of *Z. mobilis* have been selected and used in ethanol fermentation with unsterile substrate (Tao et al., 2005). Lower pH in the media is regarded to minimize the occurrences of contamination (Rogers et al., 2007). As shown in this work, the wild-type strain tested here could utilize glucose (Figure 1) and the hydrolysates derived from sweet potato raw material (Figure 4) to achieve higher fermentation efficiency at the pH 4. In addition, the *Z. mobilis* strains (like ATCC 29191) could rapidly metabolize glucose, especially at high density culture (Rogers et al., 2007), thus competitively inhibiting the growth of miscellaneous bacteria. Coupled with the majority of bacteria killed during liquefaction and saccharification pretreatment, the probability of contamination could be markedly reduced. Therefore, the wild type strains of *Z. mobilis* might also be employed to produce ethanol with non-sterile substrate, which could reduce the energy consumption as a result of the autoclaving in industrial processing of ethanol fermentation.

Cheap materials, low-cost processing and high ethanol productivity are the main considerations for most ethanol fermentation (Tao et al., 2005; Aggarwal et al., 2001). By adopting the optimal parameters as described in this work, sweet potato can be used as a cheap and abundant feedstock for ethanol fermentation with high ethanol productivity without any additive. The hydrolytic products of sweet potato alone were demonstrated here to be enough to support the growth of *Z. mobilis* and ethanol production, maybe due to its high starch content and rich nitrogen source. It was also noticed that the components hydrolyzed from sweet potato exhibited no obvious inhibition to the ethanol production by *Z. mobilis* because the fermentation efficiency at different substrate concentrations was easily close to the theoretical value. This is different from the other feedstock, such as the lignocellulosic hydrolysate which has often been shown to cause more or less inhibition to ethanol fermentation (Palmqvist and Hahn-Hagerdal, 2000). Overall, this work demonstrated that sweet potato was an excellent feedstock for ethanol fermentation with *Z. mobilis*.

Recently, fermentation with high concentration of substrates is desirable for the purpose of increasing the ethanol yield. In this study, the fermentation efficiency with high concentrations of sweet potato (300 or 400 g/L) was achieved over 92%, which was quite similar to that of 200 g/L. However, the time required to obtain the highest yield is delayed, resulting in a lower productivity. To solve this problem, some additives have been added to the medium (Zhang et al., 2008; Patil et al., 1998), or the physiological state of the seed culture should be accurately adjusted to shorten the lag phase in the fermentation process so as to enhance the rate of ethanol production.

Besides, the processing of high concentration of raw material is difficult, especially at the large scale of industrial process. The mash from high concentration of raw material possesses high viscosity, which is less efficient in the hydrolysis by amylase or glucoamylase and transported in pipes (Che et al., 2009), thus leading to a decline in the efficiency of ethanol fermentation (Underwood et al., 2004). A previous study has demonstrated that the sucrose hydrolysis and the conversion efficiency simultaneously dropped with *Z. mobilis* 39676 in high concentration fermentation of sucrose (200 - 400 g/L) (Doelle and Greenfield, 1985). The same problems still existed in handling with high concentration of sweet potato. For example, the hydrolysis pretreatment of high concentration of dried sweet potato (400 g/L) only produced 207 g/L of reduced sugar in comparison to 150 g/L of reduced sugar obtained from 200 g/L raw material (Figures 4 and 5). So these obstacles should be given full consideration in the development of the industrial procedure for ethanol production.

In conclusion, owing to its low cost and no inhibition to ethanol production, the sweet potato is a feasible feedstock for ethanol fermentation with *Z. mobilis*. And high concentration fermentation is also achieved with higher fermentation efficiency. Finally, it is important to note that some problems should be overcome prior to application to the industrial scale of ethanol fermentation.

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