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Full Length Research Paper

Ethanol production from Jerusalem artichoke (*Helianthus tuberosus* L.) by *Zymomonas mobilis* TISTR548

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The selection and characterization of *Zymomonas mobilis* for ethanol production from Jerusalem artichoke (*Helianthus tuberosus* L.) juice was investigated. Growth and ethanol production of four *Z. mobilis* strains isolated in Thailand, that is, TISTR 405, TISTR 548, TISTR 550 and TISTR 551, were compared with those of the type strain *Z. mobilis* ZM4 (NRRL B-14023) at different temperatures. Among the strains tested, TISTR 548 gave the highest ethanol concentration at 30 to 35°C, as compared to the others. Therefore, this strain was chosen for ethanol production from Jerusalem artichoke juice after acid hydrolysis. The influence of some fermentation factors such as sugar concentration, pH of the fermentation medium, inoculation size and nitrogen source on ethanol production from Jerusalem artichoke juice was determined. The results show that the maximum ethanol concentration (95.9 g/L) with 98% of the theoretical ethanol yield was obtained when the fermentation was carried out in a medium containing 250 g/L total sugars, pH 5.0, inoculation size at 10% and using 0.5 g/L diammonium phosphate as nitrogen source. The maximum theoretical ethanol yield obtained in this study was higher than those previously reported.

Key words: Ethanol production, *Zymomonas mobilis*, thermotolerant microorganism, Jerusalem artichoke.

INTRODUCTION

Among the various sources of biomass for fuel ethanol production, Jerusalem artichoke (*Helianthus tuberosus* L.) is one of the most interesting raw materials (Szambelan et al., 2005). It is a plant native in temperate regions of North America (Margaritis and Bajpai, 1983). The plant contains 11 to 20% (w/w) carbohydrates, of which 70 to 90% is inulin and inulids. Inulin consists of linear chain of D-fructose units in the β (2→1) position (inulids consist of less than 30 residues). The chain is terminated by a D-glucose residue linked to fructose by an $\propto$ (1→2) bond (Niness, 1999; Kays and Nottingham, 2008). The potential advantages of Jerusalem artichoke over the traditional agricultural crops include the following: (a) minimal fertilizer requirement, (b) very resistant to frost, pests and plant diseases, (c) can grow in poor and polluted soils such as salt-affected soils, oil-polluted soil and coal-mining soils, (d) very high carbohydrate yields per acre and (e) has potentially reduce weed interference with the crop, allowing a reduction of mechanical or chemical input required for weed management (Chubey and Dorell, 1974; Dorell and Chubey, 1977; Swanton et al., 1992; Tesio et al., 2010; Ma et al., 2011).

Zymomonas mobilis, the Gram-negative facultative anaerobic bacterium, is considered as an alternative organism in large-scale fuel ethanol production (Roger et al., 1980). It is unique in employing the Entner-Doudoroff (ED) (2-keto-3-deoxy-6-phosphogluconate, KDPG), glyceraldehydes-3-phosphate-to-pyruvate (GP) pathway

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for sugar catabolism and produces ethanol and carbon dioxide as dominant fermentation products (Sprenger, 1996). This organism may be mostly dependent on the substrate-level phosphorylation for energy acquisition and obtains about one mole of ATP per one mole of glucose utilized through the ED-GP pathway. As a consequence, it shows less biomass production than yeast and thus provides a more efficient conversion to fermentation products (Barnell et al., 1990). *Z. mobilis* has a relatively compact genome with a small number of genes, about 2,000 (Seo et al., 2005). It possesses incomplete Embden-Meyerhof-Parnas pathway and incomplete TCA cycle due to a lack of genes for 6-phosphofructokinase, 2-oxoglutarate dehydrogenase complex and malate dehydrogenase (Barnall et al., 1990; Sprenger, 1996; Seo et al., 2005), but possesses strong activities of ED-GP pathway (Fuhrer et al., 2005). Comparative laboratory- and pilot-scale studies on kinetics of batch fermentation of *Z. mobilis* versus a variety of yeast have indicated the suitability of *Z. mobilis* over yeasts due to the following advantages; (a) its higher sugar uptake and ethanol yield, (b) its lower biomass production, (c) its higher ethanol tolerance, (d) it does not require a controlled addition of oxygen during the fermentation and (e) its amenability to genetic manipulations (Sprenger, 1996; Gunasekaran and Chandra Raj, 1999).

Many investigations on ethanol production from Jerusalem artichoke by *Z. mobilis* have been reported, but the maximum ethanol yield (expressed as percentage theoretical yield) obtained by those investigations was almost less than 90%. For example, Szambelan et al. (2004) examined the ethanol production from acid and enzymatic hydrolysis of Jerusalem artichoke tubers and juices by *Z. mobilis*. They found that the maximum ethanol yield after enzymatic hydrolysis was 78.3 to 90.0% in tubers and 78.3 to 88.1% in juices, which was 2.0 to 9.2% higher than those after acid hydrolysis. Bekers et al. (2008) investigated the ethanol production from Jerusalem artichoke powder by co-fermentation of Fructozyme L. and *Z. mobilis* and obtained the maximum ethanol yield of 76.30%. To reach industrial application, further studies on improvement of ethanol production efficiency from Jerusalem artichoke fermentation should be carried out. In this study, selection of the high-ethanol producing *Z. mobilis* isolated in Thailand for ethanol production from Jerusalem artichoke juice was investigated. The influence of some fermentation factors such as sugar concentration, pH of the fermentation medium, inoculation size and nitrogen source on ethanol production by the selected strain was also described.

MATERIALS AND METHODS

Biological materials

Jerusalem artichoke tubers (cultivar KKUAC001) were obtained from the Plant Breeding Research Center for Sustainable Agriculture, Faculty of Agriculture, Khon Kaen University, Thailand. The whole tubers were washed with water and ground into a mash using a food grinder. The juices obtained after pressing the mashed tubers through cloth bags were used in classical batch fermentation processes (Onsoy et al., 2007). The *Z. mobilis*, strain TISTR 405, TISTR 548, TISTR 550 and TISTR 551, obtained from the Thailand Institute of Scientific and Technological Research (TISTR) Culture Collection, Bangkok, and the type strain, *Z. mobilis* ZM4 (NRRL B-14023) provided by E. Yanase, Tottori University, Japan, were used in this study.

Inoculum preparation

All strains of *Z. mobilis* were cultured in a YPD medium consisting of 0.3% (w/v) yeast extract, 0.5% (w/v) peptone and 3% (w/v) glucose (Swings and De Ley, 1977) at 30°C with a shaking speed of 100 rpm until the cell density reached 0.8 to 1.0 (1×10^8 CFU/ml) (Onsoy et al., 2007). These cultures were then used as an active inoculum for batch ethanol fermentation.

Medium for ethanol fermentation

Jerusalem artichoke juices were amended to acid hydrolysis before fermentation by *Z. mobilis* as described by Onsoy et al. (2007). The acid hydrolysis was conducted at pH 2.0 adjusted with sulfuric acid (H_2SO_4) and held at 80°C for 40 min. The pH was re-adjusted to 5.0 unless otherwise stated before fermentation.

Comparison of growth and ethanol production by *Z. mobilis* at high temperatures

All strains of *Z. mobilis* were streaked on agar plates containing YPD agar medium and incubated at different temperatures from 30 to 42°C for 72 h. Batch experiments were carried out in 250 ml Erlenmeyer flasks containing 100 ml YPD medium as described by Thanonkeo et al. (2007). The flasks were inoculated with 10% (v/v) active inoculum (10^8 CFU/ml) and statically incubated at various temperatures. During fermentation, samples were periodically withdrawn and analyzed for cell growth and ethanol content. The relatively high-ethanol producing strain of *Z. mobilis* was selected based on its growth and ethanol production performances at different temperatures in the YPD medium.

Ethanol fermentation and optimization conditions

Sterilized acid hydrolysate of Jerusalem artichoke juices at 110°C for 15 min was directly used as the medium for ethanol fermentation by *Z. mobilis*. All batch fermentations were carried out in 500 ml Erlenmeyer flask equipped with the air-lock, each containing 360 ml of acid hydrolysate. A study into the effect of inoculation size on ethanol fermentation was carried out at 1, 5 and 10% (v/v). The effect of pH on ethanol fermentation was carried out by adjusting the initial pH of the acid hydrolysate of Jerusalem artichoke juices to 4.0, 5.0, 6.0 and 7.0. The effect of nutrient composition on ethanol fermentation, including sugar concentrations (250, 275 and 300 g/L) and nitrogen sources (diammonium phosphate, ammonium nitrate, ammonium sulfate and urea) were also examined. During fermentation, samples were periodically withdrawn and analyzed. All the fermentation experiments were replicated twice and the average values ±SD are presented in this study.

Analytical methods

The growth of *Z. mobilis* was monitored by measuring optical

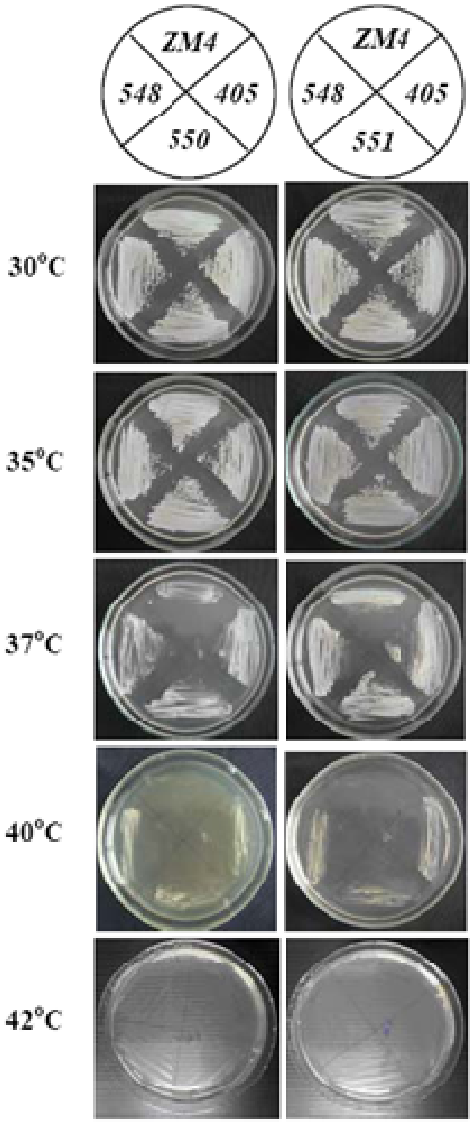

Figure 1. Growth of the Z. mobilis strains isolated in Thailand (TISTR405, TISTR548, TISTR550 and TISTR551) and the type strain (ZM4) at different temperatures on YPD agar plates.

density at 550 nm with a Shimadzu UV-1601 spectrophotometer. The fermentation broths were centrifuged at 13,000 g for 10 min to remove cells. The supernatant was then determined for total residual sugars by phenol sulfuric acid method (Dubois et al., 1956). Ethanol concentration (*P*, g/L) was analyzed by gas chromatography (Shimadzu GC-14B, Japan) using polyethylene glycol (PEG-20M) packed column, nitrogen as carrier gas and a flame ionization detector, as described previously (Thanonkeo et al., 2007). Ethanol was quantified by using 2-propanol as an internal standard. The ethanol yield (*Yps*) was calculated as the actual ethanol produced and expressed as g ethanol per g sugar utilized (g/g). The percentage of conversion efficiency or yield efficiency (*Ey*) was calculated by the following equation: Ey = (*Yps* X 100)/0.51 (Onsoy et al., 2007) and the volumetric ethanol productivity (*Qp*, g/L.h) was calculated by the following equation:

$Qp = P/t$; where, P is the ethanol concentration (g/L) and t is the fermentation time (h) giving the highest ethanol concentration.

RESULTS AND DISCUSSION

Comparison of growth and ethanol production at high temperatures

The comparative studies on growth of *Z. mobilis* isolated in Thailand, that is, TISTR 405, TISTR 548, TISTR 550 and TISTR 551, and a type strain, ZM4 (NRRL B-14023), which is known to be efficient for ethanol production, at various temperatures were carried out and the results are summarized in Figure 1. When grown in YPD agar plates at 30, 35, 37, 40 and 42°C, strains TISTR 405, TISTR 548, TISTR 550 and TISTR 551 were capable of growing up to 40°C, but the type strain did not. The maximum temperature for growth of the type strain was 37°C. At 42°C, no growth was observed in all strains tested. Their abilities for growth and ethanol production in YPD medium containing 16% (w/v) glucose were then compared and the results are summarized in Table 1. At 30°C, cell growth and ethanol production between the TISTR strains and the type strain were not significantly different. However, at 35 to 40°C, the TISTR strains showed higher cell growth and ethanol production than those of the type strain. These results suggest that the TISTR strains had relatively higher thermotolerance abilities than the type strain. Based on the description of thermotolerant microorganism given by Sree et al. (2000), the TISTR strains were not classified as thermotolerant bacteria since their maximum temperatures for growth were not above 40°C.

Among the TISTR strains, TISTR 548 gave relatively high optical density and ethanol concentration at 30 and 35°C, while TISTR 551 gave the lowest. The results from this study seem to indicate that increasing the fermentation temperature of all strains from 35 to 40°C will result in drastically decreased maximal cell growth and ethanol concentrations. Based on the maximum ethanol concentrations at 30 to 35°C, the TISTR 548 was chosen as a high-ethanol producing strain for further studies.

Optimization for ethanol fermentation by *Z. mobilis* TISTR 548

Since the TISTR 548 produced the highest ethanol concentration at 30°C, the study into the ethanol production of Jerusalem artichoke juice was performed at this temperature. Examining the ethanol fermentation by the strain TISTR 548 in the Jerusalem artichoke juice medium containing 250, 275 and 300 g/L total sugars revealed that increasing the sugar concentration resulted in a decrease in final ethanol concentration, ethanol yield and volumetric ethanol productivity (Table 2). This might be attributed to the high osmotic pressure leading to cell

Table 1. Comparison of growth and ethanol fermentation of *Z. mobilis* strains isolated in Thailand with the type strain in YPD medium containing 16% (w/v) glucose at different temperatures after 48 h.

Strain	Temperature (°C)							
	30		35		37		40	
	OD	Ethanol (% w/v)	OD	Ethanol (% w/v)	OD	Ethanol (% w/v)	OD	Ethanol (% w/v)
Z. mobilis ZM4	2.5	6.70±0.45	1.2	6.06±0.56	0.8	2.53±0.13	0.3	0.13±0.06
TISTR 405	2.8	6.96±0.36	2.0	6.68±0.50	1.4	4.48±0.34	1.0	2.98±0.30
TISTR 548	2.7	7.08±0.44	2.1	6.70±0.49	1.4	4.44±0.43	1.0	2.78±0.27
TISTR 550	2.7	7.02±0.52	1.9	6.51±0.48	1.2	4.06±0.40	0.7	1.90±0.10
TISTR 551	2.5	6.74±0.37	1.4	6.32±0.40	1.0	3.55±0.29	0.6	1.64±0.12

Reported values are the mean (±SD) of two independent experiments and each experiment consisted of two replicates.

Table 2. Ethanol production by *Z. mobilis* TISTR 548 in a Jerusalem artichoke juice medium containing different total sugars, initial pHs and inoculation sizes at 30°C.

Condition	Ethanol parameter			
	P **(g/L)**	***Yps*** **(g/g)**	***Ey*** **(%)**	***Qp*** **(g/L.h)**
Total sugar (g/L)				
250	93.28±2.87	0.45±0.12	88.24	1.28±0.01
275	64.04±1.38	0.35±0.05	68.63	0.72±0.04
300	16.44±1.49	0.27±0.03	52.94	0.16±0.02
pH				
pH 4.0	87.37±2.02	0.40±0.06	78.43	1.20±0.12
pH 5.0	94.50±2.98	0.46±0.10	90.20	1.31±0.08
pH 6.0	92.44±1.51	0.42±0.04	82.35	1.30±0.10
pH 7.0	50.71±1.75	0.30±0.01	58.82	0.58±0.05
Inoculation size (% v/v)				
1	83.18±2.22	0.45±0.02	88.24	1.14±0.02
5	86.56±1.10	0.46±0.09	90.20	1.28±0.06
10	90.40±1.90	0.47±0.05	92.16	1.30±0.03

P, ethanol concentration produced (g/L); *Yps*, ethanol yield; *Ey*, conversion efficiency or yield efficiency; Q_p, volumetric ethanol productivity (g/L.h).
Reported values are the mean (±SD) of two independent experiments and each experiment consisted of two replicates.

disruption as described by Grubb and Mawson (1993).

Another possibility is that high sugar concentrations may cause substrate inhibition resulting in stuck fermentation (Thomas, 1996). The highest ethanol concentration (93.28 g/L), ethanol yield (0.45 g/g) and volumetric ethanol productivity (1.28 g/L.h) were obtained in a medium containing 250 g/L total sugars after 72 h, whereas the lowest values were obtained in a medium containing 300 g/L. The maximum ethanol yield (expressed as percentage theoretical yield) in a medium containing 250 g/L was 88.24%. These results are in agreement with those reported by Szambelan et al. (2004). Based on the maximum ethanol yield obtained in this study, the Jerusalem artichoke juice medium containing 250 g/L, the average total sugars found in the raw materials, was chosen for next experiments.

The pH of the medium is one of the important factors influencing cell growth and ethanol yield. It also determines the process of fermentation due to the characteristic of the enzyme that only works in certain pH level. In this study, the effect of the initial pH at 4.0, 5.0, 6.0 and 7.0 on ethanol fermentation by TISTR 548 was examined in a Jerusalem artichoke juice medium containing 250 g/L total sugars. As shown in Table 2, the highest ethanol concentration (94.50 g/L), ethanol yield (0.46 g/g or 90.20% of theoretical yield) and volumetric ethanol productivity (1.31 g/L.h) were obtained when the fermentation was carried out at pH 5.0. These values were only slightly higher than those obtained from fermentation at pH 6.0. The lowest values were obtained from fermentation at pH 7.0. Our results are in agreement with those reported by Swings and De Ley (1977) and Onsoy et al. (2007), who reported the optimum pH for cell growth of *Z. mobilis* at 5.0 to 6.0. Mustofa and Suranto

Table 3. Ethanol production at 30°C by *Z. mobilis* TISTR 548 in a Jerusalem artichoke juice medium supplemented with various nitrogen sources at different concentrations.

Nitrogen source	Nitrogen concentration (g/L)	Ethanol parameter			
		P **(g/L)**	***Yps*** **(g/g)**	***Ey*** **(%)**	***Qp*** **(g/L.h)**
Control	0	90.2±2.28	0.39±0.02	76.47	1.82±0.51
Diammonium phosphate	0.25	91.9±4.05	0.42±0.11	82.35	1.91±0.23
	0.50	95.6±3.52	0.44±0.20	86.27	1.99±0.32
	0.75	88.5±3.09	0.43±0.06	84.31	1.84±0.09
	1.00	86.2±2.98	0.40±0.05	78.43	1.80±0.12
Ammonium nitrate	0.25	92.4±1.27	0.44±0.13	86.27	1.92±0.22
	0.50	95.9±3.02	0.50±0.08	98.04	1.93±0.13
	0.75	93.6±4.50	0.50±0.20	98.04	1.95±0.40
	1.00	92.8±2.01	0.46±0.12	90.20	1.92±0.06
Ammonium sulfate	0.25	75.6±1.16	0.27±0.09	52.94	1.57±0.13
	0.50	80.0±2.09	0.47±0.16	92.16	1.67±0.22
	0.75	82.7±1.87	0.43±0.06	84.31	1.72±0.09
	1.00	81.1±2.00	0.44±0.17	86.27	1.69±0.18
Urea	0.25	79.4±2.45	0.41±0.21	80.39	1.65±0.34
	0.50	76.0±0.95	0.38±0.18	74.51	1.58±0.19
	0.75	75.6±1.07	0.37±0.08	72.55	1.57±0.11
	1.00	70.7±2.24	0.37±0.02	72.55	1.47±0.06

P, ethanol concentration produced (g/L); *Yps*, ethanol yield; *Ey*, conversion efficiency or yield efficiency; Q_p, volumetric ethanol productivity (g/L.h).
Reported values are the mean (±SD) of two independent experiments and each experiment consisted of two replicates

(2009) also showed that during ethanol fermentation from cashew nut by *Z. mobilis*, the pH of the fermentation medium was ranged from 5.8 to 5.9.

The amount of bacterial cells gives huge impact of the success of fermentation. The more amounts of cells, the better fermentation will get (Mustofa and Suranto, 2009). In this study, the effect of initial cell concentration of *Z. mobilis* at inoculation size of 1, 5 and 10% (v/v) on ethanol fermentation was determined in a Jerusalem artichoke juice medium containing 250 g/L total sugars and adjusted pH to 5.0. As showed in this study, the initial cell concentration of *Z. mobilis* had an effect not only on the final ethanol concentration, ethanol yield and volumetric ethanol productivity, but also on the substrate consumption rate and ethanol fermentation rate, coinciding with that reported by D'Amore et al. (1989). Using 5 or 10% inoculation size, total sugars were consumed rapidly within 36 to 48 h after fermentation, whereas it took about 72 to 78 h when using 1% inoculation size (data not shown). The maximum ethanol concentration (90.40 g/L), ethanol yield (0.47 g/g) and volumetric ethanol productivity (1.30 g/L.h) were obtained when the fermentation was carried out using 10% inoculation size, whereas the lowest values were obtained from fermentation using 1% inoculation size (Table 2). The maximum ethanol yield (expressed as percentage theoretical yield) obtained in this study was 92.16%, which was 1.23-fold higher than that reported by Bekers et al. (2008).

Nitrogen is an important constituent for cell growth and metabolism production. A wide range of substances are able to serve as the nitrogen sources, however, inorganic nitrogen are commonly used as nitrogen source for ethanol production by *Z. mobilis*. The effect of different nitrogen sources (diammonium phosphate, ammonium nitrate, ammonium sulfate and urea) at various concentrations (0 to 1.0 g/L) on ethanol fermentation by TISTR 548 was investigated in a Jerusalem artichoke juice medium containing 250 g/L total sugars, adjusted pH to 5.0 and using 10% inoculation size. The different nitrogen sources gave different impact toward the ethanol produced by the fermentation of Jerusalem artichoke juice with *Z. mobilis*. As shown in Table 3, diammonium phosphate and ammonium nitrate gave higher ethanol concentrations, ethanol yields and volumetric ethanol productivities than those of ammonium sulfate and urea. This is in contrast with Torres and Barrati (1990) that the best nitrogen source for ethanol production from wheat flour by *Z. mobilis* is ammonium sulfate. This finding suggests that the optimal nitrogen source for ethanol

production by *Z. mobilis* depended on raw materials used in the process. It should be noted that increasing the concentration of nitrogen sources resulted in a decrease in ethanol concentration, ethanol yield and volumetric ethanol productivity. The highest ethanol concentration (95.9 g/L) with the ethanol yield of 0.50 g/g and volumetric ethanol productivity of 1.93 g/L.h was attained when the medium was supplemented with 0.50 g/L ammonium nitrate, followed by the medium supplemented with 0.50 g/L diammonium phosphate, whereas the lowest values were obtained from the medium supplemented with 1.0 g/L urea. These results indicate that ammonium nitrate and diammonium phosphate favor the production of ethanol from Jerusalem artichoke juice by *Z. mobilis*. The maximum ethanol yield (expressed as percentage theoretical yield) obtained in the medium supplemented with 0.50 g/L ammonium nitrate was 98%, which was 1.28-fold higher than the control treatment (Jerusalem artichoke juice medium without nitrogen supplementation). This value was also higher than those reported by Szambelan et al. (2004) and Bekers et al. (2008).

Conclusion

The production of ethanol from Jerusalem artichoke juice was depended on many factors such as microorganism and fermentation conditions. In this study, the *Z. mobilis* TISTR 548 was the most effective strain capable of producing highest quantity of ethanol at 30 to 35°C, as compared to other TISTR strains and the type strain, *Z. mobilis* ZM4. Its ability to produce ethanol from Jerusalem artichoke juice was investigated and the maximum ethanol concentration produced by this strain under the optimal conditions was 95.9 g/L, with 98% of the theoretical ethanol yield. Based on these results, *Z. mobilis* TISTR 548 could be recommended as an alternative candidate bacterium for ethanol production when Jerusalem artichoke juice was used as a raw material.

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