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Direct ethanol production from dextran industrial waste water by *Zymomonas mobilis*

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Abstract−The direct production of ethanol from dextran industrial waste water was investigated by using *Zymomonas mobilis* via batch and semi-continuous fermentation mode. In batch fermentation, pretreated waste water (unsterilized and sterilized), pH value (3.8 and 6.0), and Mg^{2+} (with and without) was compared with OD_{600}, sugar and ethanol concentration. After 24 h fermentation, sugar in the dextran waste water was almost exhausted, and the amount of ethanol accumulated reached 24.33-29.92 g/*l*, which is nearly 99% of the theoretical yield of ethanol. Kinetic parameters of *Z. mobilis* in batch fermentation were also investigated. The raw dextran waste water was also used in semi-continuous fermentation. After 48 h fermentation, the production of ethanol was 28.65 g/*l*. These results indicated that dextran waste water may be used as a candidate substrate and *Z. mobilis* could convert the raw material into ethanol directly.

Keywords: Ethanol, Dextran Industrial Waste Water, *Zymomonas mobilis*, Batch Fermentation, Kinetic Parameters, Semi-continuous Fermentation

INTRODUCTION

Although lignocellulosic biomass provides an abundant and renewable source for bioethanol production, the process on a commercial scale is also limited in biomass pretreatnent, enzymatic hydrolysis and robust microorganisms, etc. Actually, most process concepts for bioethanol from lignocellulose start with thermo-chemical hydrolysis of the hemicellulose part (pretreatment), followed by enzymatic hydrolysis of the cellulose part and engineered strain-based fermentation of the resulting sugars [1].

First, biomass pretreatment is necessary to make carbohydrates available for enzymatic hydrolysis and further fermentation [2]. However, the optimal conditions on an industrial-scale are also not constructed; the various pretreatment methods need to be reassessed at more industrial-like conditions, considering the whole integrated process and taking into consideration the influence on all process steps [3]. Second, the cost of enzymes is another major barrier for bioethanol production from lignocellulosic biomass [4]. For example, the cost contribution of enzyme to ethanol produced by the conversion of corn stover was found to be $0.68/gallon if the sugars in the biomass could be converted at maximum theoretical yields, and $1.47/gallon if the yields were based on saccharification and fermentation yields that have been previously reported in the scientific literature [5]. Third, absence of robust, engineered ethanologenic strains is also a key bottleneck in cellulosic ethanol. Especially, xylose fermentation and inhibitor tolerance remain challenging due to the complexity of lignocellulosic biomass hydrolyzate. Many efforts have been made to construct recombinant strains to enhance xylose fermentation over the past few decades [6-18]. Engineered ethanologenic strains tolerance to hydrolyzate by-products is another attractive method for improving lignocellulosic bioethanol production based on the mechanism of stress response [19]. Alper et al. [20] constructed an engineered yeast by global transcription machinery engineering (gTME) method to improve ethanol tolerance and production [20]. Recently, extensive reviews concentrated on inhibitors formed by pretreatment of lignocellulosic materials and their inhibition of ethanol production in yeast and bacteria [21-23].

So, other candidate materials may be searched for these bottleneck problems. For example, macroalgae showed faster growth compared to terrestrial crops, does not compete with agricultural land area for mass cultivation, high carbohydrate content, and does not contain lignin, which may be used as feedstock for bioethanol production [24,25]. However, the utilization of macroalgae as bioethanol feedstock is still scattered in literature [26].

Recently, dextran industrial waste water containing an amount of fructose (50-150 g/*l*) aroused our interest. Dextran $(C_6H_{10}O_5)n$ is a polysaccharide consisting of glucose monomers linked mainly (95%) by α(1-6) bonds [27]. It has many industrial applications, such as blood volume expander (in the pharmaceutical industry) and food industry [27]. Currently, commercial dextran production is mainly accomplished by *Leuconostoc mesenteroides* from sucrose. During this process, the equal mol fructose is also produced as by-product, as showed in the following:

$$\underset{\text{Sucrose}}{n C_{12}H_{12}O_{11}} \xrightarrow{\text{Enzyme}} \underset{\text{Dextran}}{(C_6H_{10}O_5)n} + \underset{\text{Fructose}}{n C_6H_{12}O_6}$$

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Then, separation was performed after sucrose fermentation. Traditionally, dextran is harvested from the fermentation medium by alcohol precipitation and purified by further precipitation after redissolution in water. Finally, the theoretical concentration of residual fructose was 53% of sucrose concentration. Actually, industrial production of dextran uses 300 g/*l* sucrose as substrate. So, the concentration of fructose may reach to about 160 g/*l* in dextran industrial waste water. Currently, this waste water is treated by different methods, which lead to increasing the costs of dextran industry. As *Z. mobilis* could utilize fructose as substrate, in this study, the dextran waste water used for ethanol production was investigated. The aim was to find a new biomass resource for ethanol production.

MATERIALS AND METHODS

1. Substrate

Dextran industrial waste water was obtained from Jianyang Pharm, Co., Ltd. (Sichun Province). The composition of waste water was quantified according to our previous studies [29,30], and also summarized in Table 1.

2. Bacterial Strains

The strain used was *Z. mobilis* ZM4 (ATCC 31281). The strain was cultured in rich media (RM) [28] at 30 ºC without shaking. Cultures were maintained on glucose agar (20.0 g/*l* glucose, 10.0 g/*l* yeast extract and 15.0 g/*l* agar). Organism was subcultured to fresh inoculum media for 24 h at 30 ºC before being inoculated into the fermentation medium. Inoculum medium (g/*l*) consisted of 10.0 g yeast extract, 1.0 g $MgCl_2$, 1.0 g $(NH_4)_2SO_4$, 1.0 g KH_2PO_4, 20.0 g glucose.

3. Fermentation of Dextran Industrial Waste Water

The batch fermentations were carried out in duplicate; 250 ml shaken flasks containing 100 ml dextran waste water and 10.0% (v/v) inoculum were inoculated for 96 h at 30 ºC without shaking according to the experimental design (Table 2). Different pretreated methods including sterilize, pH adjusted and Mg^{2+} addition were performed to detect its effect on ethanol yield. Semi-continuous fermentation was carried out as follows according to the results of batch fermentation (based on ethanol yield and base medium). Actually, a 2.5 *l* bioreactor containing 500 ml dextran waste water and 10.0% (v/v) inoculum were inoculated for 12 h at 30 ºC without shaking, and 500 ml dextran waste water was added at 12 h-intervals to a total amount of 2.0 *l* in bioreactor for 96 h fermentation.

Table 1. Composition of dextran industrial wasted water

Component	Concentration (g/*l*)
Total carbohydrates	58.89
Glucose	5.44
Fructose	50.61
Sucrose	3.84

4. Cell Growth, Sugar and Ethanol Analysis

Cell growth was determined by monitoring the optical density at 600 nm (with an initial OD_{600} of 0.15 when the inoculum was added to each flask) by using a multiscanner spectrometer (Thermo Inc.) at 6 or 12-h intervals. Fermentation supernatant was prepared by passing through 0.2 μm membrane (Millipore) and used to determine the concentrations of sugar and ethanol. Ions Chromatography (Switzerland, Metrohm Bio-Scan 871) was applied to measure the concentration of sugar (glucose, fructose and sucrose) with sodium hydroxide (0.1 M) as mobile phase at a flow rate of 1 ml/min. Sugars were quantified by comparing their peak areas with standard sugar of known concentrations [29,30]. Ethanol was assayed using GC122 gas chromatography with a glass column (0.26×200 cm) filled with Porapak Type QS (80-100 mesh, Waters, Milford, MA) at 150 ºC and a FID detector at 80 ºC. N_2 was used as the carrier gas (30 ml/min), and butylacetate was added as inner reference [29,30].

5. Kinetic Parameters of *Z. mobilis* ZM4 in Batch Fermentation

The maximum specific fructose uptake rates ($q_{max,s}$) and maximum specific ethanol production rates ($q_{max,p}$) were calculated over the exponential phase of growth and based on the following formulae: $q_{max,s}=(1/x)\ (ds/dt)$ and $q_{max,p}=(1/x)\ (dp/dt)$, where x, s, and p are the concentrations of biomass, sugars, and ethanol, respectively [31]. Biomass concentration (g/*l*) could be determined from OD_{600} 1.0=0.28 g/*l* dry cell weight [32]. The overall yields for biomass ($Y_{x/s}$) and ethanol ($Y_{p/s}$) production on sugar mixture media were based on the initial and final concentrations of biomass, sugar, and ethanol [31].

6. Scanning Electron Microscopy

Cells from fermentation culture were spread onto RM agar plate for determination of contamination. Contamination was also investigated by scanning electron microscopy. Biomass collected by centrifugation at 10,000 ×g for 1 min in an Eppendorff microcentrifuge was washed twice with distilled water, dehydrated in 30.0, 50.0, 70.0, 80.0, 85.0, 90.0, 95.0, 100.0% ethanol gradient, and then air-dried, directed observation of the morphology by using a Hitachi TM-1000 scanning electron microanalyzer.

Table 2. Different culture conditions used in this study

Dextran industrial wasted water	Pretreated methods		
	Sterilize or unstrilize	pH	Mg^{2+} (1.0 g/*l*)
Batch fermentation	Unsterilize	Start (pH 3.8)	No addition
	Unsterilize	Adjusted pH to 6.0	No addition
	Sterilize	Start (pH 3.8)	No addition
	Sterilize	Adjusted pH to 6.0	No addition
	Unsterilize	Start (pH 3.8)	Added Mg^{2+}
	Sterilize	Start (pH 3.8)	Added Mg^{2+}
Semi-continuous fermentation	Unsterilize	Start (pH 3.8)	No addition

RESULTS AND DISCUSSION

1. Dextran Industrial Waste Water is a Candidate Substrate for Bioethanol Production

Although many starchy or lignocellulosic materials could be used as candidate substrate for bioethanol production, pretreatment and enzymatic hydrolysis are necessary steps for releasing fermentable sugars for ethanol production [1]. We first investigated the feasibility of using dextran industrial waste water for bioethanol production. As shown in Table 1, the selected dextran industrial waste water contained of 50.61 g/*l* fructose, 5.44 g/*l* glucose and 3.84 g/*l* sucrose. Currently, this waste water is treated by different methods, which leads to increasing cost for the dextran industry. On the other hand, as a candidate ethanologenic microorganism for converting sugar into ethanol or other valuable chemicals, *Z. mobilis* showed many desirable industrial characteristics for its special Entner-Doudoroff pathway [33]. It could convert glucose and fructose rapidly to ethanol [34]. Based on this consideration, dextran industrial waste water contained nearly 58.89 g/*l* fermentable sugar, which may be suitable substrate for ethanol production by *Z. mobilis*.

2. Comparative Batch Fermentation Performance of *Z. mobilis* ZM4 on Dextran Industrial Waste Water

Although dextran industrial waste water contained nearly 58.89 g/*l* fermentable sugar and may be utilized directly by wild type of *Z. mobilis*, the effect of different conditions on ethanol yield should also be performed. In this study, different pretreated methods including sterilize, pH adjusted and Mg^{2+} addition were investigated to detect its effect on ethanol yield by *Z. mobilis* via batch fermentation mode (Table 2). On the other hand, 96 h for incubation time was selected for determination of sugar utilization and microorganism contamination during the process of fermentation. Fig. 1 shows the time course of batch fermentation of ZM4 with dextran industrial waste water under different environmental conditions (Table 2). As shown in Fig. 1(a), different pretreated methods including adjusted pH and added Mg^{2+} will help to get higher cell growth during fermentation. However, there are minor differences in cell growth under different conditions. Actually, after 24 h fermentation, the optical density (OD_{600}) reached to 1.62, 1.79, 1.53, 1.86, 1.58, and 1.88 under different conditions. However, in our study, we also found OD_{600} showed a sharp increase in unsterilized (pH 6) condition after 72 h fermentation (Fig. 1(a)). Actually, when pH is adjusted to 6.0, it may be suitable for other unknown microorganism growing. Furthermore, the same phenomenon was not found in other conditions, even in unsterilized conditions (lower pH value). Based on this analysis, we determined that the contamination may be happening during fermentation.

Importantly, after 24 h fermentation, sugars including fructose, glucose, and sucrose in the dextran waste water were almost exhausted, and the amount of ethanol accumulated reached 24.33-29.92 g/*l*, which is nearly 99% of the theoretical yield of ethanol from the dextran waste water (as shown in Fig. 1(b) and (c)). The kinetic parameters of ZM4 for different conditions are given in Table 3. The higher maximum specific rates of fructose uptake and ethanol production were found under the condition of unsterilized treated, pH 3.8, no addition of Mg^{2+}. However, the amount of ethanol was nearly of the theoretical yield of ethanol from the dextran waste water in all conditions. Based on these results and the simple process, the dextran waste water may be used as a candidate substrate for ethanol production without any pretreatment.

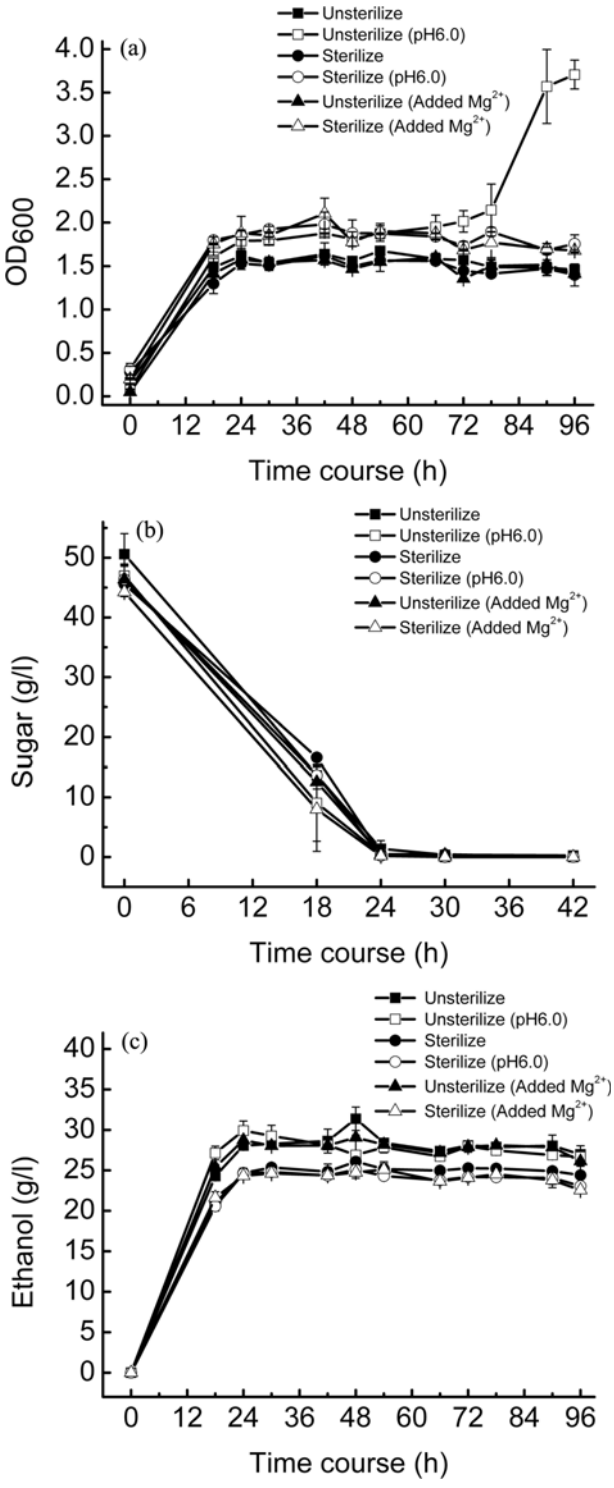

Fig. 1. Ethanol production from dextran industrial waste water by *Z. mobilis* via batch fermentation mode. (a) Cell growth; (b) Fructose concentration; (c) Ethanol. Data comes from two independent experiments.

3. Ethanol Production from Dextran Industrial Waste Water by *Z. mobilis* via Semi-continuous Fermentation Mode

As shown in Fig. 1, there are minor differences in cell growth

Table 3. Kinetic parameters of *Z. mobilis* ZM4 in batch fermentation mode in dextran industrial wasted water[a]

Conditions	$q_{max,s}$ (g/g/h)	$q_{max,p}$ (g/g/h)	[b]$Y_{p/s}$ (g/g)	[c]$Y_{p/s}$ (g/g)	$Y_{x/s}$ (g/g)
Unsterilize, pH 3.8, No addition Mg^{2+}	4.52	2.58	0.56	0.50	0.009
Unsterilize, pH 6.0, No addition of Mg^{2+}	3.86	2.49	0.64	0.50	0.011
Sterilize, pH 3.8, No addition of Mg^{2+}	4.39	2.40	0.54	0.49	0.0094
Sterilize, pH 6.0, No addition of Mg^{2+}	3.69	1.97	0.53	0.49	0.011
Unsterilize, pH 3.8, added Mg^{2+}	4.33	2.71	0.62	0.50	0.0095
Sterilize, pH 3.8, added Mg^{2+}	3.49	1.93	0.55	0.50	0.012

$q_{max,s}$, maximum specific fructose upstake rate
$q_{max,p}$, maximum specific ethanol production rate (based on fructose)
[a]Data comes from two independent experiments
[b]$Y_{p/s}$, ethanol yield on fructose sugar
[c]$Y_{p/s}$, ethanol yield on total sugar (fructose, glucose and sucrose)
$Y_{x/s}$, yield for biomass

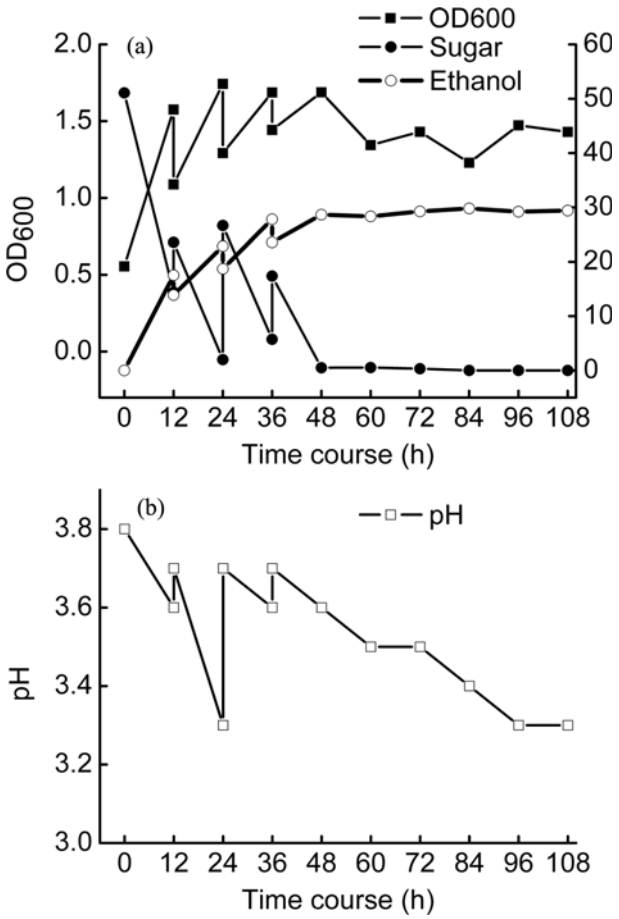

Fig. 2. Ethanol production from dextran industrial waste water by *Z. mobilis* via semi continuous fermentation mode. (a) Profiling of cell growth, fructose and ethanol concentration; (b) pH value during fermentation. Filled square symbols represent OD_{600}, filled cycle symbols represent sugar concentration, open cycle symbols represent ethanol concentration, and open square symbols represent pH value during fermentation.

and sugar utilization under different conditions after 24 h fermentation via batch mode. On the other hand, contamination may have happened during fermentation in unsterilized (pH 6.0) condition.

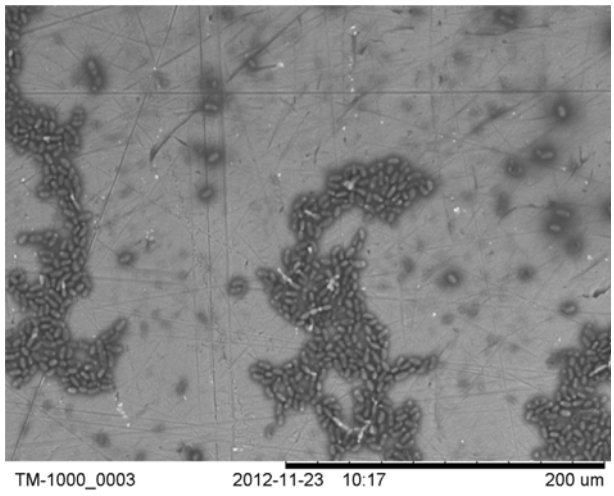

Fig. 3. Scanning electron micrograph of the dextran waste water culture in semi-continuous fermentation.

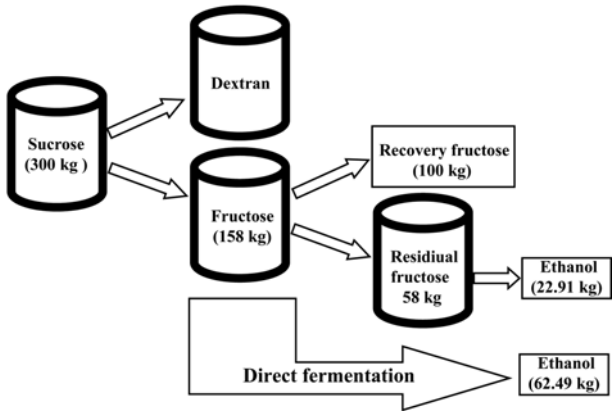

Fig. 4. Ethanol production from dextran industrial waste water by *Z. mobilis* with or without recovery of fructose.

Based on these results, the unsterilized dextran waste water (pH 3.8) was used directly in semi-continuous fermentation. As shown in Fig. 2, after 108 h fermentation, the production of ethanol was 29.41 g/*l*. Furthermore, all sugars were almost exhausted after 48 h fermentation, and 28.65 g/*l* ethanol produced in this time point.

The amount of ethanol accumulated reached nearly 30 g/*l*, which is nearly 99% of the theoretical yield of ethanol from the dextran waste water; the commercial scale ethanol production will be limited for its low concentration of ethanol. However, the concentration of ethanol may have reached 70 g/*l* if the concentration of total sugar increased to 150 g/*l* without recovery of fructose during the process of dextran fermentation and separation, which would lead to higher ethanol yield (Fig. 4), indicating that *Z. mobilis* could convert the dextran waste water into ethanol rapidly.

Cells from semi-continuous fermentation culture were also spread onto RM agar plate for determination of contamination by scanning electron microscopy. As shown in Fig. 3, no other microorganism was found, which indicated that no contamination will take place during the process of direct fermentation of the raw material. It may be depend on the lower pH value. As shown in Fig. 2(b), the pH value varied from 3.3-3.8.

Furthermore, *Z. mobilis* could directly ferment the dextran waste water without any pretreatment and contamination. So, the advantages of the proposed process over the conventional process are related to cost reduction due to the elimination of ethanol usage and recovery of fructose during dextran production. The dextran waste water could be used as other chemical product production by engineered *Z. mobilis* strains. Taken together, the raw dextran waste water may be suitable for ethanol or other chemical product production by using *Z. mobilis*.

CONCLUSION

We conclude that dextran waste water could be a potential feedstock for bioethanol production. And *Z. mobilis* could convert dextran waste water into ethanol directly. This simple but effective method could facilitate an energy-efficient and cost-effective conversion of the waste water into bioethanol.

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