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ORIGINAL PAPER

Fermentation of xylose into ethanol by a new fungus strain *Pestalotiopsis* sp. XE-1

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Abstract A new fungus, *Pestalotiopsis* sp. XE-1, which produced ethanol from xylose with yield of 0.47 g ethanol/g of consumed xylose was isolated. It also produced ethanol from arabinose, glucose, fructose, mannose, galactose, cellobiose, maltose, and sucrose with yields of 0.38, 0.47, 0.45, 0.46, 0.31, 0.25, 0.31, and 0.34 g ethanol/g of sugar consumed, respectively. It produced maximum ethanol from xylose at pH 6.5, 30°C under a semi-aerobic condition. Acetic acid produced in xylose fermenting process inhibited ethanol production of XE-1. The ethanol yield in the pH-uncontrolled batch fermentation was about 27% lower than that in the pH-controlled one. The ethanol tolerance of XE-1 was higher than most xylose-fermenting, ethanol-producing microbes, but lower than *Saccharomyces cerevisiae* and *Hansenula polymorpha*. XE-1 showed tolerance to high concentration of xylose, and was able to grow and produce ethanol even when it was cultivated in 97.71 g/l xylose.

Keywords Xylose fermentation · *Pestalotiopsis* · Xylose tolerance · Ethanol production

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Abbreviations

GC	Gas chromatography
HPLC	High-performance liquid chromatography

Introduction

With the global depletion of fossil fuel within sight, there has been an increasing worldwide interest in alternative liquid fuels such as ethanol, butanol, and methanol, among the others. Over the last decades, much attention has been paid to fuel ethanol production from cheap and renewable raw materials such as lignocellulose [1, 10, 24]. Lignocellulose is the most abundant organic compounds in nature. It contains five major sugars including the hexoses D-glucose, D-mannose, D-galactose, and the pentoses D-xylose and L-arabinose. Xylose is a major constituent of lignocellulose. It accounts for about 17% of the total dry weight in woody angiosperms and up to 31% in herbaceous angiosperms [11]. The economic conversion of lignocellulose to fuel ethanol is strongly dependent on the fermentation of xylose to ethanol [2, 7]. However, *Saccharomyces cerevisiae*, the yeast most commonly used in industrial large-scale ethanol fermentation of sugar- and starch-based raw materials, cannot ferment xylose, although there were some successful instances in genetic engineering of this strain in order to confer it a capacity to utilize xylose to produce ethanol [3, 13].

Since Wang et al. found that some yeasts could convert xylulose to ethanol in the 1980s, many native and genetically engineered xylose-fermenting, ethanol-producing bacteria and yeasts were reported [4, 8, 10, 11, 20, 23]. However, only a few xylose-fermenting, ethanol-producing filamentous fungi were reported so far [8]. *Thermoanaerobacter ethanolicus* has the highest native capability for xylose fermentation of any known bacteria, and *Pichia*

stipitis, *Candida shehatae*, and *Pachysolen tannophilus* are the best native xylose fermentation yeasts known. These microbes have been studied rather intensively in the past [10, 12].

In the present study, a new fungus that produces ethanol from xylose was reported, and it was identified as *Pestalotiopsis* sp. XE-1 through the sequence alignment of the internal transcribed spacer 2 (ITS-2) in its 18S rRNA gene. This is the first report on the utilization of xylose to produce ethanol in the genus *Pestalotiopsis*. The xylose fermentation characteristics of XE-1 to produce ethanol and the factors that affect the ethanol production from xylose were also investigated. XE-1 was found to be able to grow in as high as over 97 g/l xylose and this may indicate that it can be tolerant to xylose substrate inhibition, which occurred in many of the industrial microorganisms studied so far.

Materials and methods

Strains and media

Xylose-fermenting, ethanol-producing fungi strains were isolated from soil samples at 30°C using medium plates containing the following components (g/l): xylose 20, KH_2PO_4 1, $MgSO_4$ 0.5, and $(NH4)_2SO_4$ 5, agar 15, pH 6.5. Fungi colonies were picked up and streaked on yeast extract-peptone-xylose (YEPX) agar plates consisting of (g/l): yeast extract 3, peptone 5, xylose 20, KH_2PO_4 1, and $MgSO_4$ 0.5 at pH 6.5. Each strain was tested for its capability to produce ethanol from xylose. The taxonomical identification was based on the internal transcribed spacer (ITS) DNA sequences in its 18S rRNA gene [22].

Unless otherwise stated, the fermentation medium contained (g/l): xylose or other carbohydrates 20, peptone 5, yeast extract 3, KH_2PO_4 1, $MgSO_4$ 0.5, $CaCO_3$ 20, pH 6.5. $CaCO_3$ was added as a separate powder to control the pH during cultivation.

Test the ability to ferment various carbon sources

XE-1 was cultivated in 250-ml flasks at 30°C for 72 and 120 h without shaking in 100 ml of fermentation media containing different carbon sources inoculated with 10 ml of a 48-h-old XE-1 culture in the same medium as the fermentation medium, but no $CaCO_3$ was added.

Effect of temperature and initial pH on ethanol production

To test the effect of temperature on ethanol production, XE-1 was grown in fermentation medium at 26, 28, 30, 32, and 34°C, respectively, for 96 h without shaking. To investigate the effect of pH on ethanol production, XE-1 was grown in fermentation medium at 30°C for 96 h without shaking. The pH of the medium was adjusted with H_2SO_4 and NaOH to 5.5, 6.0, 6.5, 7.0 and 7.5, respectively.

Effect of aeration on xylose fermentation

Different levels of aeration were obtained by varying the volume of fermentation media in 500-ml Erlenmeyer flasks: (1) Aerobic cultivations were conducted in 500-ml Erlenmeyer flasks containing 100 ml of the fermentation medium. (2) Semi-aerobic: in 500-ml Erlenmeyer flasks containing 250 ml of the fermentation medium. (3) Micro-aerobic: in 500-ml Erlenmeyer flasks containing 500 ml of the fermentation medium. (4) Anaerobic experiments were conducted in 500-ml serum vials filled almost completely with the fermentation medium and tightly sealed. Immediately, after inoculation and taking sample, nitrogen gas was passed through the liquid for 15 min. All flasks and serum vials were inoculated with 10% of a 48-h-old XE-1 culture in the same medium as the fermentation medium, but no $CaCO_3$ was added and shaken at 150 rpm on a rotary shaker. Fermentation was monitored for 5 days.

Test for tolerance to ethanol

Cultivations were conducted at 30°C for 72 h in 250-ml flasks containing 100 ml of YEPX broth with different concentrations of ethanol without shaking.

Test for xylose tolerance

Experiments were conducted in 250-ml Erlenmeyer flasks containing 100 ml of the fermentation medium with different xylose concentrations of 20, 40, 60, 80, and 100 g/l at 30°C for 72 and 120 h without shaking. Ethanol and xylose in the broth were examined.

Measurement of biomass

Because of the inhomogeneous distribution of the fungus mycelia, only the final biomass (dry weight) was determined in most experiments. The mycelia were washed with distilled water and the $CaCO_3$ in the mycelia ball was dissolved with 0.5 M of HCl. The mycelia were then washed with distilled water, filtered through a 0.45-μm membrane and dried at 105°C until a constant weight.

Substrate and metabolite analysis

Xylose and other carbohydrates were determined by HPLC using a Hypersil NH_2 column (4.6 × 250 mm) at 35°C and a refractive index detector. The eluent was acetonitrile/

water (85:15, v/v) at a flow rate of 1.0 ml/min. Ethanol was determined by GC as described [6].

Results

Strain

More than ten isolates of xylose-fermenting microorganisms were isolated from soil samples, and each was tested for its capability to produce ethanol from xylose. The isolate XE-1 that produced the highest level of ethanol was taxonomically identified according to the ITS sequence. The ITS sequence of strain XE-1 was shown in Fig S1 (see supplemental data) in the alignment with other most identical sequences available so far in the GenBank. It displayed the highest degree of homology with those of *Pestalotiopsis* when it was compared by BLAST analysis to the sequences in the GenBank database. The results of phylogenetic analysis suggested that strain XE-1 may represent a novel species within the genus *Pestalotiopsis* (Fig. 1).

The ability of XE-1 to ferment various carbon sources

The capability of XE-1 to ferment various carbon sources was examined. The results were presented in Table 1. XE-1 grew and accumulated ethanol from almost all substrates (xylose, arabinose, glucose, fructose, mannose, galactose, cellobiose, maltose, sucrose, and starch) tested except cellulose. Among the monosaccharides, XE-1 utilized xylose, arabinose, mannose, and galactose quickly. These monosaccharides were also the preferred carbon sources for XE-1 to produce ethanol. Among the disaccharides, cellobiose and sucrose were the preferred sugars for XE-1 to grow and produce ethanol, the biomass for cellobiose and sucrose were 5.96 and 6.37 mg/ml, respectively. Maltose was the poorest carbon source for XE-1 to grow and produce ethanol. The concentration of maltose had decreased only about 30% from the initial concentration after 120 h cultivation. The yields of ethanol and biomass from maltose were 1.72 g/l and 2.66 mg/ml, respectively. Xylose fermentation of XE-1 was further studied in more detail as below.

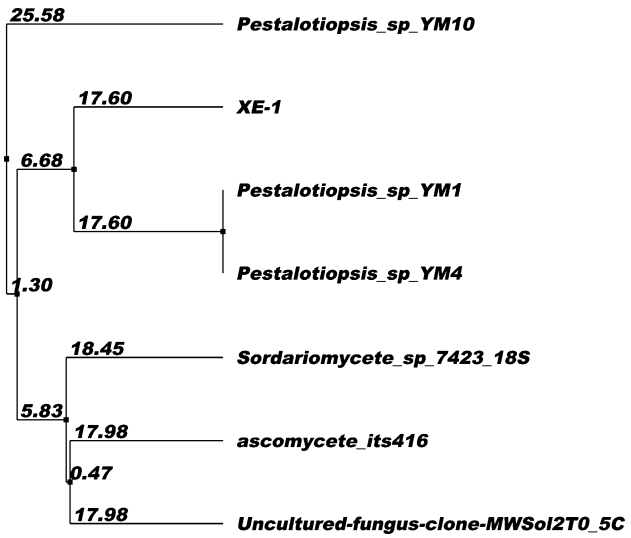

Fig. 1 Phylogenetic tree for XE-1 based on the DNA sequence of internal transcribed spacer (ITS) in its 18S rRNA gene. (see the ITS sequence alignment in Fig. S1 of the supplemental data)

Test of XE-1 for ethanol production from xylose

XE-1 was tested for conversion of 20 g/l of xylose to ethanol at pH 6.5 and 30°C under a semi-aerobic condition. The time course of batch fermentation of xylose and formation of ethanol by XE-1 is shown in Fig. 2. In pH-uncontrolled batch fermentation, the ethanol concentration was 3.56 g/l and the acetic acid concentration was 3.0 g/l (data not shown) at 96 h, and a decrease in pH from 6.1 to 4.0 was observed. Xylose was consumed only about 67% of the initial concentration after 120 h cultivation and biomass was 3.24 mg/ml (Fig. 2a). In pH-controlled batch fermentation by adding $CaCO_3$ to the medium, the xylose was consumed completely within 96 h, at which time the ethanol concentration reached its maximum (4.53 g/l). The biomass was 4.73 mg/ml (data not shown) and pH was 5.3 (Fig. 2b). The major fermentation end-products of xylose fermentations were ethanol and acetate with no xylitol accumulation (data not shown).

Effect of temperature and initial pH on ethanol production

XE-1 performed best around pH 6.5 and 30°C, and reached its maximal ethanol concentration of 4.05 g/l at this temperature (Fig. 3a). Its optimal growth temperature also seemed to be 30°C, at which the biomass was 3.63 mg/ml. When the temperature was either lowered to 26°C or increased to 34°C, the ethanol concentration, xylose consumption, and biomass decreased significantly. The ethanol concentration was almost constant between pH 6.0 and 7.0, and decreased if below and above this pH range. The best initial pH for ethanol production from xylose by XE-1 in batch fermentation was 6.5. Under these conditions, the ethanol concentration was 4.06 g/l (Fig. 3b).

Effect of aeration on xylose fermentation by XE-1

The effect of aeration on xylose fermentation by XE-1 was investigated (Fig. 4). XE-1 exhibited its highest xylose consumption rate and ethanol production capability under the semi-aerobic condition. Under the semi-aerobic and aerobic

Table 1 Ethanol production from different carbohydrates by *Pestalotiopsis* sp. XE-1

Carbon sources	Carbon sources concentration (g/l)			Biomass (mg/ml)	Ethanol (g/l)		Ethanol yield			
							g/g carbon source consumed		g/g carbon source added	
	0 h	72 h	120 h	120 h	72 h	120 h	72 h	120 h	72 h	120 h
Xylose	18.10	10.45	6.46	4.02	3.56	3.82	0.47	0.33	0.20	0.21
Arabinose	17.80	10.08	7.14	4.12	2.90	3.90	0.38	0.37	0.16	0.22
Glucose	16.81	11.78	8.74	4.27	2.35	2.75	0.47	0.34	0.14	0.16
Fructose	17.40	12.82	11.41	3.57	2.07	2.02	0.45	0.34	0.12	0.12
Mannose	17.50	11.52	7.95	4.45	2.78	3.35	0.46	0.35	0.16	0.19
Galactose	18.30	8.91	5.47	4.98	2.90	3.32	0.31	0.26	0.16	0.18
Cellobiose	17.80	11.74	5.16	5.96	1.50	2.40	0.25	0.19	0.08	0.13
Maltose	18.00	14.62	12.37	2.66	0.90	1.72	0.27	0.31	0.05	0.10
Sucrose	17.60	10.04	4.09	6.37	2.57	2.70	0.34	0.20	0.15	0.15
Starch	18.52	12.10	6.32	4.34	1.95	2.45	0.30	0.20	0.11	0.13
Cellulose	17.90	17.88	17.89	0	0	0	0	0	0	0

XE-1 was cultivated at pH 6.5 and 30°C for 72 and 120 h without shaking. Biomass was measured as the mycelia dry weight. Experiments were performed in triplicate and the results are given as mean values

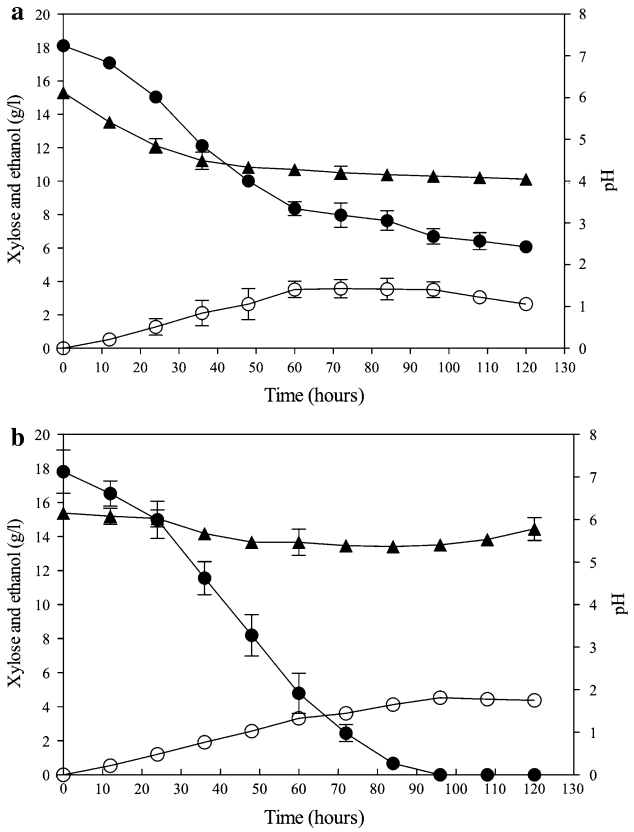

Fig. 2 Batch fermentation of xylose under pH-uncontrolled (**a**) and pH-controlled (**b**) conditions by *Pestalotiopsis* sp. XE-1. The experiments were performed in 20 g/l of xylose at 30°C and pH 6.5 under a semi-aerobic condition. *Filled circle*, residual xylose; *open circle*, ethanol; *filled triangle*, pH

conditions, XE-1 consumed xylose completely at 84 and 96 h, respectively. Under the micro-aerobic and anaerobic conditions, XE-1 utilized xylose slowly. The concentration of xylose was decreased about 75% from the initial concentration under the micro-aerobic condition, but only about 48% under the anaerobic condition after 120 h cultivation. XE-1 reached its maximal ethanol concentration of 4.1 g/l at 84 h under the semi-aerobic condition. Under the aerobic, micro-aerobic and anaerobic conditions, the maximal ethanol concentrations were 80, 78, and 41%, respectively, of that under the semi-aerobic conditions. Analysis of variance demonstrated that there were significant differences in ethanol yield between the semi-aerobic and aerobic ($p < 0.05$), micro-aerobic ($p < 0.01$), and anaerobic condition ($p < 0.01$) and in xylose consumption rates between the semi-aerobic and aerobic ($p < 0.01$), micro-aerobic ($p < 0.01$), and anaerobic condition ($p < 0.01$). The biomasses were 4.5, 4.4, 4.2, and 2.1 mg/ml under the aerobic, semi-aerobic, micro-aerobic and anaerobic conditions, respectively (data not shown).

Tolerance to ethanol

The effect of ethanol on growth of XE-1 is shown in Fig. 5. XE-1 exhibited the best growth in 0–2% (v/v) of added ethanol. Increasing concentrations of added ethanol would result in a corresponding growth decrease when the added ethanol was over 2%, and the growth was totally inhibited in the presence of 6% ethanol.

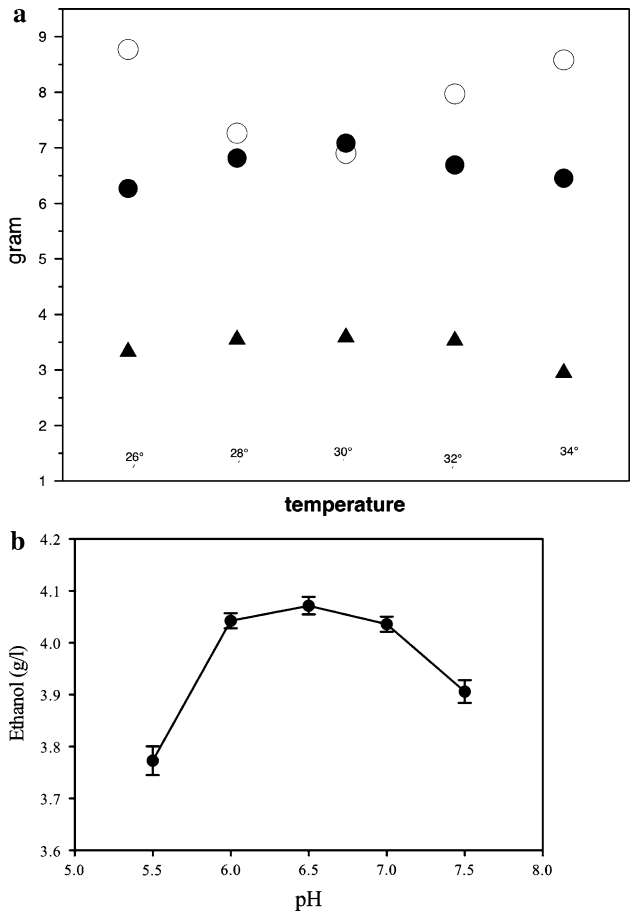

Fig. 3 Relationship between temperature and ethanol formation (**a**), pH and ethanol formation (**b**) during xylose fermentation. Cultivations were conducted at different temperature or different pH at 30°C in 20 g/l of xylose without shaking for 72 h. Biomass was measured as dry weight. *Open circle*, residual xylose; *filled circle,* ethanol; *filled triangle,* mycelia dry weight

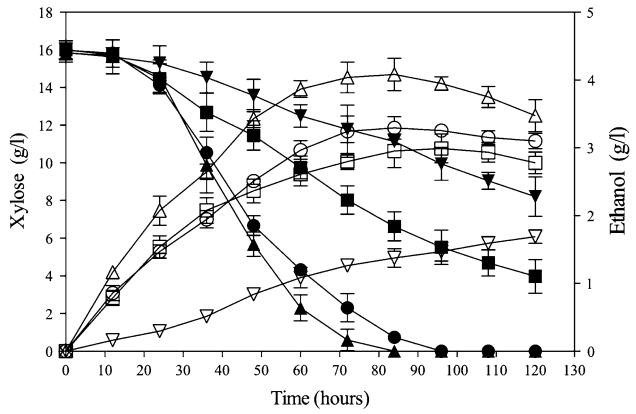

Fig. 4 Relationship between aeration and ethanol formation during xylose fermentation. Experiments were conducted at 30°C and pH 6.5 in 20 g/l of xylose under aerobic, semi-aerobic, micro-aerobic and anaerobic conditions. Xylose concentration (*closed symbols*) and ethanol concentration *(open symbols)* were shown: *filled circle*, *open circle*, aerobic; *filled triangle*, *open triangle*, semi-aerobic; *filled square*, *open square*, micro-aerobic; *filled inverted triangle*, *open inverted triangle*, anaerobic

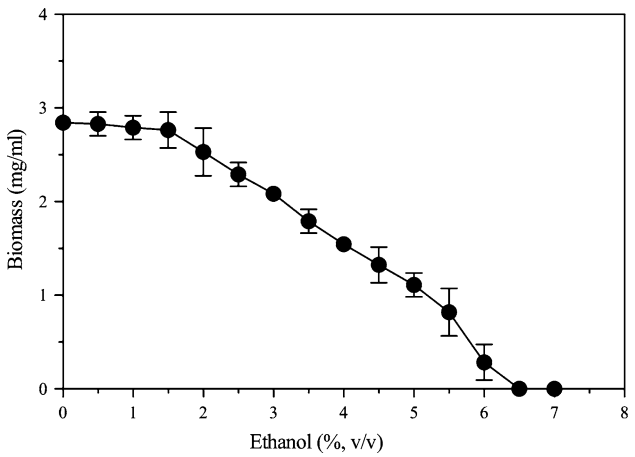

Fig. 5 Effect of ethanol on the growth of *Pestalotiopsis* sp. XE-1. Cultivations were conducted at 30°C and pH 6.5 in 250-ml flasks containing 100 ml of YEPX broth without shaking for 72 h. Biomass were measured as dry weight

Test for xylose tolerance

XE-1 was tested for ethanol production at different concentrations of xylose and the results are shown in Table 2. Xylose was almost completely utilized when the initial concentration of xylose was 20 g/l, and incompletely utilized at the higher initial concentrations over 40 g/l. At concentrations between 20 and 80 g/l of initial xylose concentration, ethanol yield increased but the ethanol conversion rate decreased. When the xylose concentration was over 80 g/l, the biomass, ethanol yield, and ethanol conversion rate decreased remarkably. When xylose concentration increased to 100 g/l, the biomass and ethanol yield at 120 h were 3.88 mg/ml and 0.11 g ethanol/g xylose consumed, respectively.

Discussion

Hemicellulosic material might be a good source for ethanol production. Economic production of ethanol from renewable biomass requires the efficient conversion to ethanol of all types of sugars in cellulose and hemicellulose [8, 24]. In this study, we reported on the isolation of a novel fungus *Pestalotiopsis* sp. XE-1 that could use xylose to produce ethanol. This is the first report on the utilization of xylose to produce ethanol in the genus *Pestalotiopsis*. The genus *Pestalotiopsis* was established by Steyaert [21]. Many species of *Pestalotiopsis* are saprobes, while others are pathogenic or endophytic on living plant leaves and twigs [9]. Some *Pestalotiopsis* species have gained much attention in recent years as they produce many important secondary metabolites with potential use in medical applications and

Table 2 Production of ethanol with different concentrations of xylose by *Pestalotiopsis* sp. XE-1

Xylose concentration (g/l)			Biomass (mg/ml)	Ethanol (g/l)		Ethanol yield			
						g/g xylose consumed		g/g xylose added	
0 h	72 h	120 h	120 h	72 h	120 h	72 h	120 h	72 h	120 h
19.60	6.65	1.37	4.14	4.61	5.10	0.36	0.28	0.24	0.26
38.90	26.37	20.81	4.03	4.55	4.32	0.36	0.24	0.12	0.11
58.92	45.35	39.77	4.22	5.05	5.00	0.37	0.26	0.09	0.08
78.12	64.45	49.68	4.22	5.48	5.08	0.40	0.18	0.07	0.07
97.71	85.18	60.77	3.88	4.30	4.12	0.34	0.11	0.04	0.04

XE-1 was cultivated at pH 6.5 and 30°C for 72 h and 120 h without shaking in different concentrations of xylose. Biomass was measured as the mycelia dry weight. Experiments were performed in triplicate and the results are given as mean values

in the control of plant diseases [14, 18, 26]. No reports showed that *Pestalotiopsis* species were pathogenic to humans. Perhaps the biggest difference between *Pestalotiopsis* sp. XE-1 and other xylose-fermenting, ethanol-producing fungal strains is that *Pestalotiopsis* sp. XE-1 did not accumulate any xylitol and could ferment xylose under anaerobic conditions. Besides, XE-1 was extremely tolerant to a high concentration of xylose (could grow in up to 100 g/l xylose). As other xylose-fermenting, ethanol-producing filamentous fungi, XE-1 fermented a wide range of carbohydrates, especially xylose, arabinose, galactose, mannose, and cellobiose, to produce ethanol (Table 1) [8]. This important feature of XE-1 would be advantageous when the simultaneous saccharification and fermentation (SSF) of the hemicellulose, along with the cellulose fraction, have to be attained in the processing.

Temperature and initial pH were the most important process parameters. They affected the rates of both microbial cell growth and product formation [15, 25]. XE-1 grew best and produced maximum ethanol from xylose at pH 6.5, 30°C and under the semi-aerobic condition. The ability of XE-1 to convert xylose to ethanol in the later stage of fermentation was poor (Fig. 2a), and the major reason may well be that it produced too much acetic acid when it fermented xylose to produce ethanol, just as the case for many other prokaryotes [10, 20]. Acetic acid is a growth inhibitor for many microorganisms [17, 25]. It is present in a protonated (undissociated) form when pH is lower than its pKa (4.75) and acts as a membrane protonophore, disrupts proton gradients by diffusing from the acidified external medium into the cells and dissociating in the more neutral environment of the cytoplasm, and then causes its inhibitory effect by bringing about the acidification of the cytoplasm [10]. In the present investigation, the acetic acid concentration in pH-uncontrolled batch fermentation with 20 g/l of xylose was 3.0 g/l at 96 h (data not shown), and the pH decreased from 6.1 at 0 h to 4.0 at 96 h. The protonated form of acetic acid in the broth inhibited the growth and xylose consumption of XE-1. Under this pH-uncontrolled condition, the biomass was 3.24 mg/ml and only 67% of xylose was utilized after 120 h cultivation (Fig. 2a). In order to avoid the inhibitory effect of acetic acid, pH was controlled by the addition of $CaCO_3$ into the fermentation media. $CaCO_3$ combined with CH_3COOH to form CH_3COOCa, CO_2, and H_2O. The acetic acid was neutralized and pH of fermentation media was kept at 5.3 and above. Under this pH-controlled condition, the biomass was 4.73 mg/ml and xylose was consumed completely at 96-h cultivation. Ethanol yield in pH-controlled batch fermentation was about 27% greater than that in the pH-uncontrolled one (Fig. 2b).

Aeration rate greatly influenced the growth rate, xylose consumption rate, ethanol production, and ethanol assimilation of xylose-fermenting, ethanol-producing microbes. It is especially one of the most important parameters in attaining maximal ethanol production with xylose-fermenting, ethanol-producing yeasts [10]. *Pachysolen tannophilus* exhibited the highest ethanol specific productivity under the semi-aerobic condition [19]. *Candida shehatae* and *Pichia stipitis* reached the maximum ethanol concentration under an oxygen-limited condition [5]. The growth and ethanol production were severely restricted under the anaerobic conditions. Most of xylose-fermenting, ethanol-producing prokaryotes reported so far were anaerobic bacteria. Aeration was harmful for these anaerobic bacteria during growth and ethanol production. In the present investigation, aeration rate greatly affected xylose consumption and ethanol production of XE-1. It exhibited the highest xylose consumption rate and ethanol yield under the semi-aerobic condition. The xylose consumption rate and ethanol production under the aerobic and microaerobic conditions were much lower than that obtained in the semi-aerobic cultures, and decreases sharply under the anaerobic condition (Fig. 4). These results illustrated that oxygen was required for xylose consumption and ethanol production of XE-1. Aeration greatly stimulated ethanol

productivity, but the ethanol yield decreased at higher aeration levels.

The ethanol tolerance of XE-1 was higher than most of the xylose-fermenting, ethanol-producing yeasts which were completely inhibited by ethanol concentrations near 3%, and xylose-fermenting, ethanol-producing bacteria which were in the range of 1–5%, but lower than *S. cerevisiae* and *H. polymorpha*, when cultivated on xylose as a sole carbon source [16]. XE-1 grew well in 0–2% (v/v) of added ethanol and was completely inhibited by ethanol concentrations of 6%. Added ethanol concentration of 4% led to a 52% decrease in growth.

Most of the xylose-fermenting, ethanol-producing microbes can not grow when the xylose concentrations are over 6% (403 mM). Some species of *Thermoanaerobacter* can tolerate higher xylose concentration. When *T. ethanolicus* was grown in pH-controlled batch experiments with 27.75 g xylose/l, a yield of 0.57 mol of ethanol/mol of xylose fermented (0.17 g ethanol/g xylose fermented) could be obtained. *T. ethanolicus* JW200 produced 0.68 mol of ethanol/mol of xylose (0.21 g ethanol/g xylose fermented) from 35 g xylose/l [20]. Compared to other xylose-fermenting, ethanol-producing microbes, XE-1 had the highest xylose tolerance ever reported. XE-1 used almost all of the 19.60 g xylose/l with a yield of 0.36 g ethanol/g xylose fermented and was capable of growing and producing ethanol in 97.71 g xylose/l with a biomass of 3.88 mg/ml and a yield of 0.34 g ethanol/g of xylose fermented (Table 2). This is especially important if one considers that it takes less energy for modern distillation processes.

The major advantage of using XE-1 for ethanol production is its ability to ferment a variety of carbohydrates derived from lignocellulosic biomass. It could not compete with *S. cerevisiae* or *Zymomonas mobilis* when it comes to the conversion of hexoses and to the ethanol tolerance. However, it will be a promising candidate for conversion of the hemicellulose fraction (xylose, arabinose, mannose, and galactose) of lignocellulosic biomass to bioethanol.

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