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The Ethanol Tolerance of *Pachysolen tannophilus* in Fermentation on Xylose

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Abstract The influence of ethanol on fermentation by *Pachysolen tannophilus* was studied. When xylose utilization rate was 80%, ethanol concentration began to decline. Fermentation of *P. tannophilus* was affected by ethanol addition in the beginning of fermentation; average xylose consumption rate was 0.065 $g{\cdot}l^{-1}{\cdot}h^{-1}$, and maximum specific growth rate was 0.07 h^{-1} at 28 $g{\cdot}l^{-1}$ ethanol, comparing with the average xylose consumption rate of 0.38 $g{\cdot}l^{-1}{\cdot}h^{-1}$ and maximum specific growth rate of 0.14 h^{-1} in fermentation with no ethanol addition; *P. tannophilus* stopped growth at 40 $g{\cdot}l^{-1}$ ethanol. When the initial ethanol concentration was 30 $g{\cdot}l^{-1}$, the addition of glucose in xylose media made the growth of *P. tannophilus* better, and the most favorable glucose concentration was 15 $g{\cdot}l^{-1}$ with the highest biomass of 1.51 $g{\cdot}l^{-1}$ as compared with that of 0.95 $g{\cdot}l^{-1}$ in pure xylose media.

Keywords Ethanol · Glucose · *Pachysolen tannophilus* · Xylose

Introduction

Fuel ethanol can be produced from lignocellulose, comprising cellulose, hemicellulose, and lignin [1]. Lignocellulosic hydrolysates contain a mixture of sugars with glucose and xylose as the major components [2]. *Saccharomyces cerevisiae* can ferment glucose to ethanol easily, but it cannot use xylose, which is often difficult to ferment [3]. The natural yeasts that can ferment xylose to ethanol include *Pichia stipitis*, *Candida shehatae*, and *Pachysolen tannophilus* [4–6]. Owing to the characteristic of lignocelluloses, mixed culture biotechnology (MCB) can be applied in ethanol production using lignocellulosic hydrolysates [7].

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P. tannophilus was the first yeast discovered capable of significant ethanol production from xylose; however, it could not tolerate high ethanol concentrations [8]. *S. cerevisiae* can produce ethanol concentrations more than 15% (*v*/*v*) using media containing more than 25% (*w*/*v*) sugar in very high-gravity ethanol production technologies [9]. So, the ethanol tolerance of *P. tannophilus* will be a difficulty in MCB. Glucose is preferred over xylose in fermentation by *P. tannophilus*, and *P. tannophilus* grows better in glucose media than in xylose media [10]. In this paper, fermentation on xylose by *P. tannophilus* with different initial ethanol concentrations and glucose additions were studied.

Materials and Methods

Microorganism

P. tannophilus 1771 was provided by China National Research Institute of Food and Fermentation Industries.

Fermentations

The inoculum was prepared using medium containing ($g \cdot l^{-1}$): xylose, 20; yeast extract, 20; peptone, 10 at 30 °C and 140 rpm until the midexponential growth phase. The composition of the fermentation medium ($g \cdot l^{-1}$) was: D-xylose, 20; yeast extract, 2; $(NH_4)_2SO_4$, 10; $MgSO_4$, 2; KH_2PO_4, 2.

Fermentations were performed in sterile 500-ml Erlenmeyer flasks in a shaker incubator at 30 °C and 100 rpm. Each Erlenmeyer flask contained 50 ml of sugar medium and 5 ml inocula, covered with two layers of plastic wrap. All the media were autoclaved at 116 °C for 25 min before use.

Analytical Methods

Glucose concentration was determined using a biosensor (SBA-40C; Biology Institution of Shandong Academy of Science, Jinan, China) [11]. Residual sugars were determined using the 3,5-dinitrosalicylic acid method [12, 13]. Dry cell weight was determined by the absorbance of the suspension at a wavelength of 620 nm measured against a previously obtained absorbance versus dry-weight calibration line [14].

The ethanol concentration was measured by Shimadzu GC-2050 gas chromatography with cbp-20 capillary column and a flame ionization detector. The chromatogram was run at 180 °C oven temperature and 90 °C injection temperature using N_2 as a carrier gas and H_2 as a flaming gas [15].

Results and Discussion

Relationship Between Ethanol Concentration and Xylose Consumption

In fermentation on xylose by *P. tannophilus*, ethanol produced could be consumed for cell productivity in spite of the presence of xylose [8]. For example, in the fermentation of 30 $g \cdot l^{-1}$ xylose, *P. tannophilus* consumed 85% xylose in 48 h (Fig. 1). The average xylose consumption rate during this period was 0.55 $g \cdot l^{-1} \cdot h^{-1}$, and a maximum ethanol

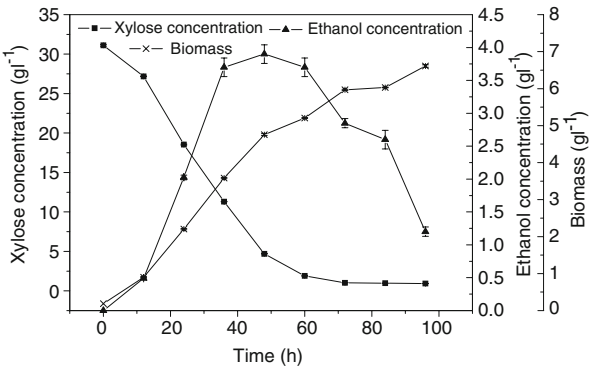

Fig. 1 Fermentation of 30 g·l^{-1} xylose by *P. tannophilus*

concentration of 3.9 g·l^{-1} was obtained. Then, xylose concentration declined at an average xylose consumption rate of 0.23 g·l^{-1}·h^{-1}, and ethanol concentration began to decline at the rate of 0.017 g·l^{-1}·h^{-1}. After 72 h, the xylose concentration was nearly zero, while ethanol concentration decreased faster at a rate of 0.069 g·l^{-1}·h^{-1}. Biomass increased during the whole fermentation, so *P. tannophilus* used ethanol for cell productivity in the fermentation anaphase. Fermentations in other xylose concentrations (10, 20, 40, 50, 60 g·l^{-1}) were carried out, and similar conclusions were got as in 30 g xylose l^{-1} that ethanol concentration began to decline when xylose utilization was around 80%.

According to the experiment, formerly, although there was still a small quantity of xylose in media, ethanol was consumed (Fig. 2). During the fermentation, ethanol concentration increased, and xylose concentration decreased**.** When xylose utilization was around 80%, ethanol concentration was higher, while xylose concentration was lower in the media, and ethanol began to be consumed. It might be because ethanol produced in fermentation could inhibit xylose consumption by *P. tannophilus***.** After xylose utilization reached 90%, ethanol concentration decreased fast and xylose could not be used anymore; *P. tannophilus* just used ethanol for cell productivity in the fermentation anaphase.

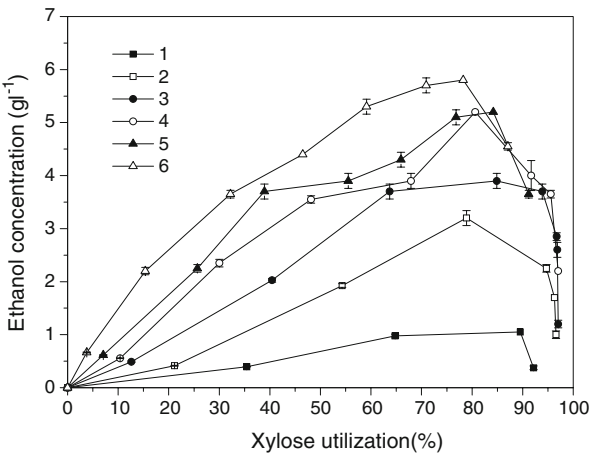

Fig. 2 Relationship between ethanol concentration and xylose utilization. *1*, 10 g xylose l^{-1}; *2*, 20 g xylose l^{-1}; *3*, 30 g xylose l^{-1}; *4*, 40 g xylose l^{-1}; *5*, 50 g xylose l^{-1}; *6*, 60 g xylose l^{-1}

Influence of Various Ethanol Concentrations on *P. tannophilus*

Different ethanol concentrations were added at the beginning of fermentation in order to observe the influence on fermentation by *P. tannophilus*. As indicated by Fig. 3a, with increasing ethanol concentration, residual xylose concentration became higher in the end; in other words, xylose used by *P. tannophilus* became less. When initial ethanol concentration was lower (14, 20 $g \cdot l^{-1}$), xylose consumption was inhibited slightly, and xylose concentrations at the end of fermentation were 1.49 and 6.02 g xylose l^{-1}, respectively, compared with 0.6 g xylose l^{-1} in fermentation without ethanol added. When ethanol concentrations were 28 and 33 g ethanol l^{-1}, xylose consumption was inhibited seriously, for residual xylose concentrations at the end were 19.27 and 21.43 $g \cdot l^{-1}$. Xylose was not consumed at 40 g ethanol l^{-1} during the whole fermentation. The average xylose consumption rate decreased as initial ethanol concentration increased (Fig. 3b). The average xylose consumption rate with no addition of ethanol was 0.38 $g \cdot l^{-1} \cdot h^{-1}$; it reduced to 0.29 $g \cdot l^{-1} \cdot h^{-1}$

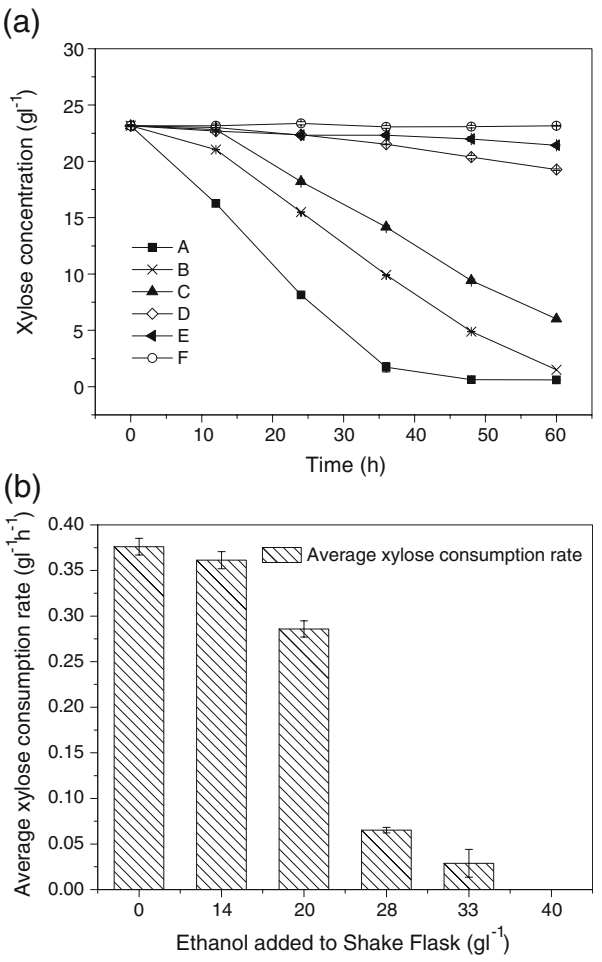

Fig. 3 Change of xylose concentration and average xylose consumption rate when different concentrations of ethanol was added. **a** Change of xylose concentration when different concentrations of ethanol were added. **b** Average xylose consumption rate when different concentrations of ethanol were added. *A*, 0 g ethanol l^{-1}; *B*, 14 g ethanol l^{-1}; *C*, 20 g ethanol l^{-1}; *D*, 28 g ethanol l^{-1}; *E*, 33 g ethanol l^{-1}; *F*, 40 g ethanol l^{-1}

in fermentation with 20 g ethanol l^{-1} and to 0.065 $g \cdot l^{-1} \cdot h^{-1}$ at 28 g ethanol l^{-1}; the one at 40 g ethanol l^{-1} was 0 $g \cdot l^{-1} \cdot h^{-1}$.

Biomass increased more slowly, and the final value became lower as ethanol concentration got higher (Fig. 4a). Biomass of 5.68 $g \cdot l^{-1}$ was obtained in fermentation with no ethanol added, and biomass had no increase at 40 g ethanol l^{-1}. Maximum specific growth rate fell concomitantly with an increasing ethanol concentration (Fig. 4b). With no ethanol addition, the maximum specific growth rate was 0.14 h^{-1}, compared to 0 h^{-1} at 40 g ethanol l^{-1}.

High concentration of ethanol inhibits yeast growth and xylose consumption. For these reasons, ethanol modifies the fluidity of the plasma membrane [16], stimulates the ATPase activity [17], and affects various transport systems. Also, it inhibits the activity of crucial glycolytic enzymes and has been reported to damage mitochondrial deoxyribonucleic acid in yeast cells [18].

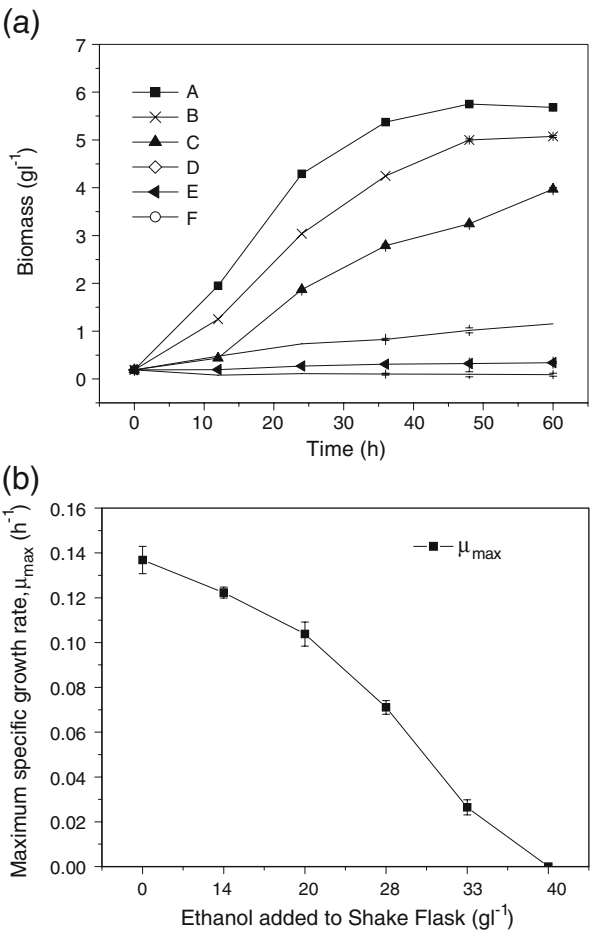

Fig. 4 Change of biomass and maximum specific growth rate when different concentrations of ethanol was added. **a** Change of biomass when different concentrations of ethanol were added. **b** Maximum specific growth rate when different concentrations were ethanol added. *A*, 0 g ethanol l^{-1}; *B*, 14 g ethanol l^{-1}; *C*, 20 g ethanol l^{-1}; *D*, 28 g ethanol l^{-1}; *E*, 33 g ethanol l^{-1}; *F*, 40 g ethanol l^{-1}

Influence of Various Additions of Glucose on *P. tannophilus* at 30 g Ethanol l^{-1}

Glucose was added to improve the growth of *P. tannophilus* at 30 g ethanol l^{-1}, and *P. tannophilus* grew better in media containing glucose (Fig. 5). Maximum biomass was 0.95 $g \cdot l^{-1}$, and the average cell growth rate was 0.0136 $g \cdot l^{-1} \cdot h^{-1}$ in the pure xylose fermentation medium. After adding glucose, both maximum biomass and average cell growth rate increased. At 30 g ethanol l^{-1}, both the highest biomass of 1.51 $g \cdot l^{-1}$ and the highest average cell growth rate of 0.023 $g \cdot l^{-1} \cdot h^{-1}$ were obtained at 15 g glucose l^{-1}, which was the most favorable glucose concentration. Biomass and average cell growth rate decreased at 25 g glucose l^{-1} for the reason that total sugar concentration was high here and sugar tolerance might become weak at high ethanol concentration.

Time courses of ethanol concentration, glucose concentration, and xylose concentration were shown in Fig. 6. In the first 12 h of fermentation, sugar in media was used slowly by *P. tannophilus*, because there might be a period for *P. tannophilus* to adapt to the condition of high ethanol concentration of 30 $g \cdot l^{-1}$. Sugar was used to produce ethanol besides for biomass. In fermentation at 15 g glucose l^{-1}, ethanol concentration had an increment of

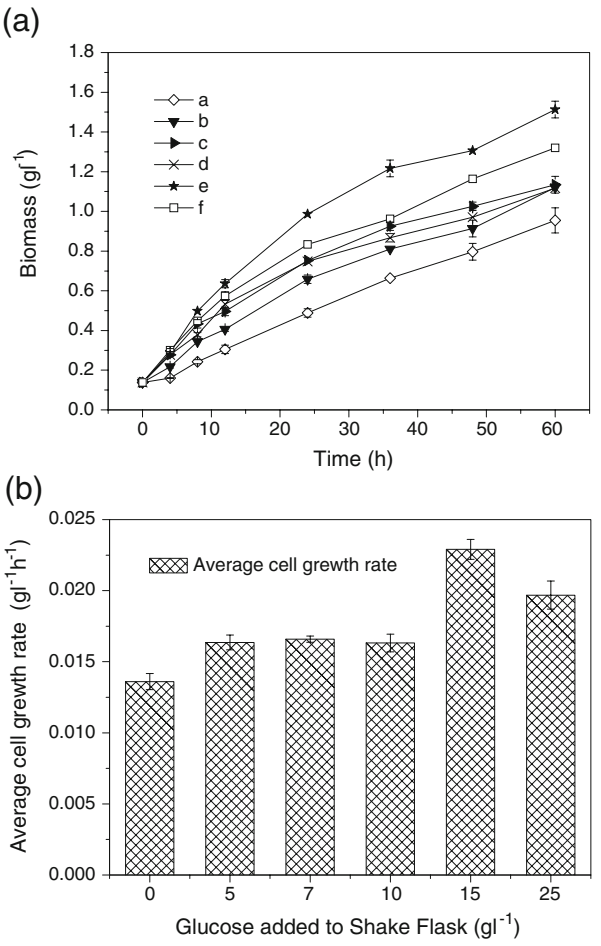

Fig. 5 Biomass and average cell growth rate at 30 g ethanol l^{-1} when different concentrations of glucose were added. **a** Biomass at 30 g ethanol l^{-1} when different concentrations of glucose were added. **b** Average cell growth rate at 30 g ethanol l^{-1} when different concentrations of glucose were added. *a*, 0 g glucose l^{-1}; *b*, 5 g glucose l^{-1}; *c*, 7 g glucose l^{-1}; *d*, 10 g glucose l^{-1}; *e*, 15 g glucose l^{-1}; *f*, 25 g glucose l^{-1}

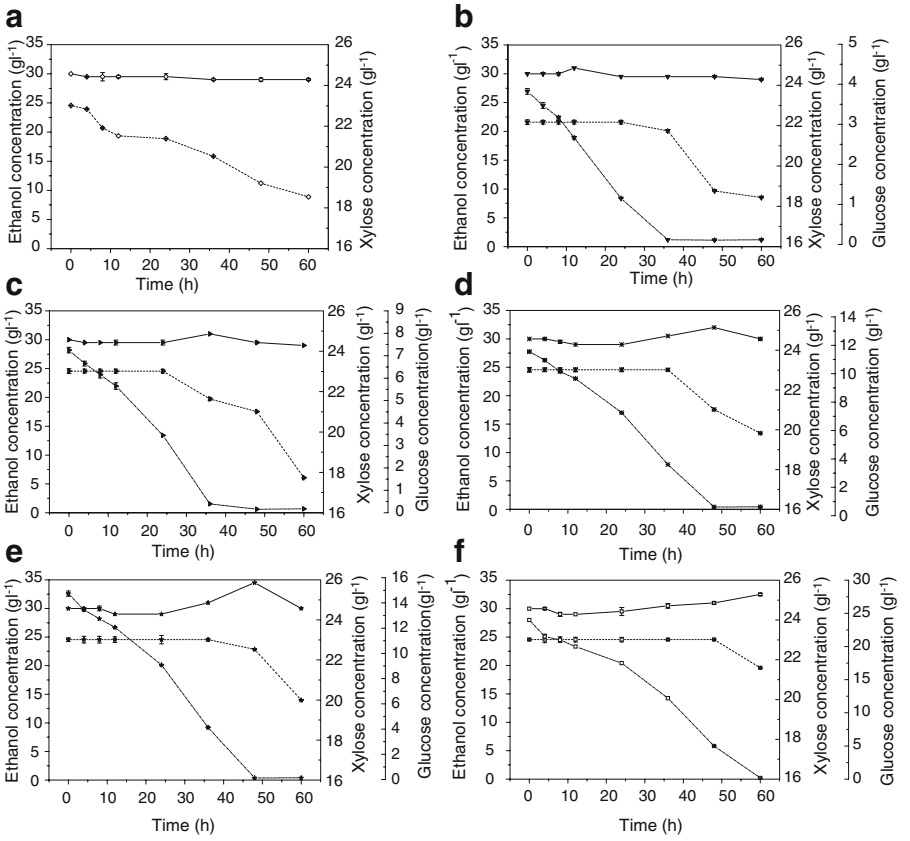

Fig. 6 Ethanol concentration and sugar concentration of fermentation in 30 g ethanol l^{-1} with different added glucose concentrations. *Solid line*, ethanol concentration; *dashed line*, xylose concentration; *dash-dot line*, glucose concentration. **a** 0 g glucose l^{-1}; **b** 5 g glucose l^{-1}; **c** 7 g glucose l^{-1}; **d** 10 g glucose l^{-1}; **e** 15 g glucose l^{-1}; **f** 25 g glucose l^{-1}

5.5 g·l^{-1}, and at 5 g glucose l^{-1}, the increment was 0.5 g·l^{-1}. When glucose and xylose existed in media simultaneously, glucose was the preferred substrate to xylose for *P. tannophilus*.

Conclusion

P. tannophilus has obtained attention for its ability to use xylose to produce ethanol; however, various problems existed such as low ethanol productivity and weak ethanol tolerance. In this paper, we studied the ethanol tolerance of *P. tannophilus* and offered some advice for ethanol production by mixed culture. In fermentation on xylose to produce ethanol by *P. tannophilus*, ethanol was utilized in spite of the presence of xylose. When xylose utilization was around 80%, ethanol concentration declined; when xylose utilization exceeded 90%, xylose was not almost consumed, and ethanol produced in the fermentation prophase was used for cell productivity. The reason might be that ethanol could inhibit the xylose consumption in the fermentation anaphase.

The high ethanol concentration affected fermentation efficiency. The inhibiting effect of ethanol on fermentation by *P. tannophilus* got more serious as the ethanol concentration increased. Xylose consumption rate and maximum specific growth rate were affected when ethanol was above 20 $g \cdot l^{-1}$, and *P. tannophilus* stopped growth at 40 g ethanol l^{-1}. This conclusion was in agreement with studies by Slininger et al. [8]. Glucose addition was attempted to improve the growth of *P. tannophilus* in high ethanol concentration. When the glucose concentration added was 15 $g \cdot l^{-1}$, biomass and average cell growth rate had the highest increase.

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