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Influence of furfural concentration on growth and ethanol yield of *Saccharomyces kluyveri*

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Abstract

Furfural is an important inhibitor in ethanol fermentation process using lignocellulosic hydrolysates as raw materials. In order to find out the furfural concentration range in which furfural inhibits the fermentation process, we used one strain *Saccharomyces kluyveri* selected from soil and cultured in several different furfural content media under low glucose concentration condition. Experiment results showed that microorganism growth was stimulated and dry cell weight increased when furfural concentration in the medium was 0.25 mg/ml. Furfural had negative effect on cell growth when its concentration was above 1.00 mg/ml. At the same time, the strain growed better and had a higher glucose consumption rate in 5% original glucose concentration condition than in 3% original glucose concentration condition. The results showed that appropriate exaltation of original glucose concentration in stalk hydrolysates will increase the strain resistance to furfural.

Key words: furfural concentration; *Saccharomyces kluyveri*; fermentation; growth; ethanol yield

Introduction

It is nearly 7×10^8 t of crop stalks produced each year in China (Dong *et al*., 2006). Crop stalks were replaced by coal, natural gas or other energy materials for changes of energy consumption structure in rural areas. Farmers spend a lot of money in grinding stalks and plowing them into soil after crop harvesting, and composting occupies more land and needs more labor force. Stalks incineration is common, and the total amount reaches 1.30×10^8 t/a (Cao *et al*., 2006). Such phenomenon increases air pollution and resource waste. Using crop stalks as raw materials for ethanol fermentation can produce renewable energy. And it also can contribute to the reuse of solid waste and environmental protection. This technology has good economic and social performances.

When using crop stalks as raw materials for ethanol fermentation, the hydrolysates acquired in the pretreatment process (diluted acid treatment) are different from traditional ethanol fermentation using starch-based original materials.

Firstly, the saccharide in the hydrolysates is complicated and total sugar content is low, generally not exceeding 5% (w/v). Secondly, fermentation inhibitors such as fatty acids, furfural and phenolic compounds etc. always generate during hydrolyzing pretreatment process (Carlos and Leif, 2003; Nurdan and Yesim, 2000). Hemicellulose can be hydrolyzed to xylose and glucose at low temperature (below 200°C) and diluted acid concentration (less than 3% v/v). Then xylose and glucose can be hydrolyzed to furfural at such temperature and acid conditions. During traditional alcoholic fermentation process, furfural comes from various raw materials and accessories, such as grain, rice chaff, wheat bran and rice bran etc. (Lu, 1994). The analysis of furfural content in various distilled spirit show that: furfural in it generally does not exceed 100 mg/L and almost all are below 30 mg/L (Zhang *et al*., 2002; Xu and Zhao, 1997). China distilled spirit Mao Tai has the highest furfural concentration, about 209 mg/L (Xu and Zhao, 1997). It has no prominent inhibiting influence to ethanol fermentation when furfural concentration is below 0.2 g/ml (209 mg/L).

The previous research results also show that furfural inhibiting effects are different in ethanol fermentation by using different yeast (Nurdan Eken-Saracoglu *et al*., 2000; Mohammad *et al*., 1999). It has prominent inhibiting effects to ethanol fermentation when furfural concentration increases. The yeast growth would be restrained and obvious second growth curve would appear (Steve *et al*., 2003; Eva and Barbel, 2000). Fed-batch fermentation (25 mg/h) treatment can reluctantly complete ethanol fermentation (Mohammad *et al*., 1999), but it is difficult to apply this technology to commercial ethanol production. Therefore,

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furfural concentration in hydrolysates is one of the most important factors during the fermentation.

A Chinese researcher (Fang *et al*., 2005) found that when furfural concentration in culture medium was above 1 mg/ml, it had prominent inhibiting effects during xylitol fermentation. It restrained fermentation severely when furfural concentration in culture medium was above 2.0 mg/ml. They have less related reports about the furfural inhibiting effect during ethanol fermentation. *Saccharomyces kluyveri* is one of microorganisms that have high ability to convert raw materials to ethanol. In this experiment, we explored the growth and fermentation characteristics of *S. kluyveri* under different furfural concentrations.

1 Materials and methods

1.1 Microorganisms

The source of strain *S. kluyveri* was collected from orchard soil in Haidian District, Beijing, and screened by enrichment and restrictive culture. The pure culture was identified by Microorganism Research Institute of Chinese Academy of Sciences. *Saccharomyces cerevisiae* Meyen ex Hansen, was selected as CK strain (bought from China Agricultural Microbial Culture Collection Center, serial number is 20251).

1.2 Culture medium preparation and culture condition

1.2.1 Substrate medium constitute

The original sugar (3%) treatment culture medium (100 ml) was prepared with peptone 1 g, powder yeast extraction 0.5 g, and dextrose 3 g.

The original sugar (5%) treatment culture medium (100 ml) was prepared with peptone 1 g, powder yeast extraction 0.5 g, and dextrose 5 g.

1.2.2 Culture and treatment conditions

Furfural stock solution was prepared by filtration through a 0.22-μm microporous film, put the sterilized stock solution into culture medium by sterile operation, prepared culture medium which contain different furfural concentration gradient: 0.25, 0.50, 0.75, 1.00, 1.25 and 1.50 mg/ml. Experiments were carried out thrice.

The microbial strain was activated in slant medium, then inoculated active strain to 50 ml triangular flasks, put the flasks in the shaker and cultivated them at rotational speed of 140 r/min, and set cultural temperature at 30°C. Strain suspension fluid cultivated for 24 h was used as experimental strain and 2.5 ml was inoculated in 50 ml triangular flask.

1.3 Fermentation parameter testing

1.3.1 Determination of cell dry weight

Strain suspension 10 ml was cultivated for 24 h, centrifuged for 10 min (4000 r/min), collected the cells, then put it into bake oven, and dried at 80°C until constant weight. Finally, cells were weighed by ten thousandth scale and calculated the cells dry weight in 1 ml fermentation fluid.

1.3.2 Determination of strain density

Fermentation broth was diluted 10 times using 0.9% NaCl solution. The turbidity of solution was detected at 600 nm with a 752N spectrophotometer (Shanghai Precision & Scientific Instrument Corp., China).

1.3.3 Determination of residual dextrose

The fermentation solution cultivated 24 h was centrifuged, and the supernatant fluid was extracted after appropriate diluting. The residual dextrose concentration was analyzed using 3–5 dinitrosalicylic acid (DNS) method following the literature (Miler, 1959).

1.3.4 Determination of ethanol yield

One part of the supernatant fluid was filtrated by 0.22-μm microporous film. Then put quantitative *n*-propyl alcohol (0.64% w/v) into 0.5 ml colature as internal standard material. Gas chromatography with MS detector (GCMS-QP2010, Shimadzu Corp., Japan) was used to analyze ethanol concentration (internal standard method).

Chromatographic setting: peg capillary column and FID detector; column temperature: 110°C; injector temperature: 200°C; detector temperature: 219°C; carrier gas: N_2 (60 kPa); flow rate: 31 ml/min; spilt ratio: 1/20; voltage of detector: 75 kV; velocity of H_2 flow: 30 ml/min; sample quantity: 1 μl.

All of the experiments repeated thrice, and the data used in the article were the average of repeats.

2 Results and analysis

2.1 Influence of furfural concentration gradient on strain cell dry weight

With the furfural concentration increasing in the culture medium during fermentation process, strain cell dry weight increased at low concentration and decreased at high concentration (Fig.1). The maximum cell dry weight occurred at 70.3 mg/ml when furfural concentration was 0.25 mg/ml. When furfural concentration was higher than 0.25 mg/ml, cell dry weight decreased. Apparently, the increase of furfural concentration inhibited the increase of cell dry weight. As furfural concentration was lower than 0.25 mg/ml, furfural promoted the increase of cell dry weight in all treatments.

Furfural can promote the increase of cell dry weight apparently under the condition of 3% original sugar. The cell dry weight began to decrease gradually while furfural concentration exceeded 0.25 mg/ml.

In 5% original sugar culture medium, higher cell dry growth weight could be obtained comparing with 3% original sugar treatment. The minimum difference between the two groups of treatment of the cell dry weight was nearly 11 mg/ml. The results of variance analysis show that (Table 1) different sugar concentration in culture medium had prominent influence on dry cell weight. Furfural concentration gradient also had different inhibiting effects on two groups of treatment.

Table 1 Variance analysis of cell dry weight of *S. kluyveri* culture of different original sugar content treatment

Difference source	SS	d*f*	MS	*F*	*P*-value	$F_{0.05}$
Between groups	476.1	1	476.1	20.6	0.001892	5.3
In group	184.55	8	23.07			
Total	660.65	9				

$F>F_{0.05}$, there is marked difference between two treatment. SS: sum of squares; MS: mean square.

2.2 Influence of furfural concentration on strain turbidity in fermentation broth

The strain density of fermentation solution can be expressed by turbidity. At different furfural concentrations, the changing tendency of turbidity is similar to that of cell dry weight. There is a peak when the furfural concentration increased to 0.25 mg/ml in all sugar content treatments (Fig.2). With the furfural concentration rising continuously, turbidity began to decrease. Strain turbidity decreased at a uniform speed (the rates of slope of ever point are similar) when furfural concentration changed from 0.25 to 1.25 mg/ml under 5% original sugar treatment. In 3% original sugar treatment, the absolute value of the rate of slope acceleratively decreased while furfural concentration increased in the same concentration changing scope. Furfural inhibiting effects on strain density enhanced under the low original sugar concentration circumstance as it concentration increased.

The reaction of *S. kluyveri* and that of CK strain are different at the same furfural concentration. The turbidity of CK treatment decreased when furfural concentration increased (Fig.2). And it decreased rapidly when furfural concentration increased to 0.5 mg/ml. It is inferred that the number of cells of CK strain was more easily inhibited by the furfural compared with *S. kluyveri* undered the same condition.

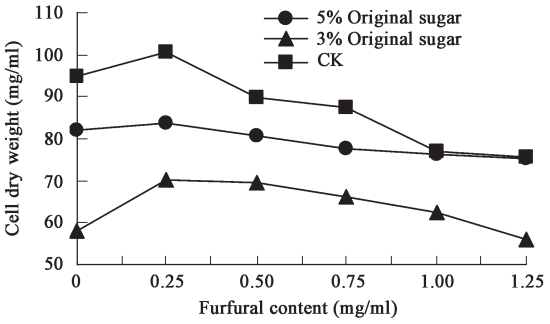

Fig. 1 Influence of furfural concentration on cell dry weight of yeast microbes.

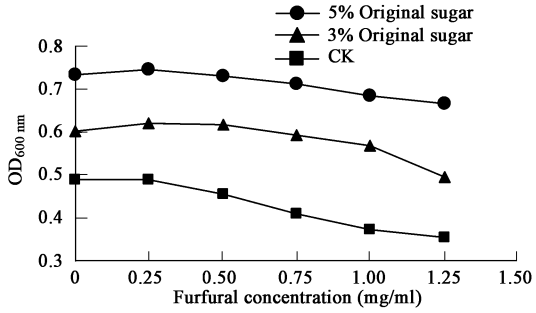

Fig. 2 Influence of furfural concentration on turbidity of *S. kluyveri*.

2.3 Influence of furfural concentration on glucose consumption during fermentation process

Glucose can be converted into other materials rapidly in normal fermentation environment. The consumption of glucose will decrease when fermentation function of strain is inhibited. The degree of inhibiting can be determined by measuring the residual glucose content in the fermentation solution under different furfural concentration (Carlos and Leif, 2003; Liu, 2001).

By analyzing the residual glucose concentration after fermenting 24 h (Fig.3), we found that furfural had no obvious inhibiting effects on glucose consumption if furfural concentration was below 1 mg/ml. But when furfural concentration was above 1 mg/ml, the glucose consumption decreased quickly (Fig.3). Therefore, the point of furfural concentration at 1 mg/ml is very important for the strain fermentation.

Comparing the residual glucose concentration in fermentation broth after 24 h, the changing trend of residual sugar in the same changing scope of furfural concentration of different sugar treatments was similar. The reducing sugar concentration in 5% original sugar treatment was less than that in 3% sugar treatment (Fig.3). With increasing the concentration of the original sugar, utilization ratio of glucose increased. The difference of residual glucose concentration between the two treatments became obviously when furfural concentration was above 1 mg/ml from the furfural concentration increasing direction (Fig.3). This phenomenon showed that the change of furfural concentration had more effects on 3% original sugar treatment than on 5%. Appropriate increase of original sugar can promote the conversion of glucose. And it also contributed to the accomplishment of fermentation as furfural concentration is higher than 1 mg/ml.

The residual glucose after 24 h fermentation of 3% sugar treatment accumulated obviously when furfural concentration reached 1.0 mg/ml (Fig.3). However, in CK treatment (5% original sugar content), the residual glucose increased rapidly when furfural concentration reached 0.75 mg/ml (Fig.3). The furfural tolerance ability of *S. kluyveri* was prominently higher than that of CK strains under the same

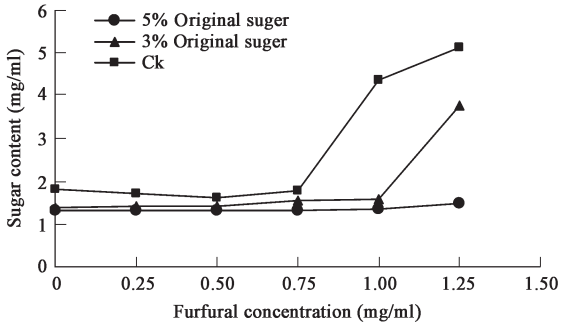

Fig. 3 Influence of furfural concentration on glucose consumption of S kluyvri strain.

original sugar content conditions. The substrate utilization rate of *S. kluyveri* was higher than that of CK strains.

The furfural concentration had no prominent influence on ethanol output of *S. kluyveri* train in the experiment scope, but original sugar content had prominent influence on ethanol output (Table 2). Ethanol yield of *S. kluyveri* under 5% original glucose treatment was similar to that of CK treatment.

3 Discussion

3.1 Influence of sugar content in the hydrolysates on ethanol fermentation

During the pretreatment process, the stalk hydrolysates are a mixture which contains glucose, glucan, xylose, xylan and many other low molecular weight materials. The total sugar concentration of this mixture is generally less than 5% (w/v). The original sugar concentration is above 10% (w/v) while using starch as raw materials during ethanol fermentation process. Glucose concentration in ethanol fermentation is from 10% to 12% (w/v). In this scope, yeast can make use of glucose sufficiently to produce the highest ethanol productivity and keep the lowest sugar remains (Liu, 2001). Therefore, the hydrolysates must be distilled to increase the sugar concentration before using yeast in ethanol fermentation process. The sugar concentration should be lower than the above mentioned while using bacteria as fermentation microbe at 35°C. If glucose concentration is above 5%, the ethanol yields decrease as the sugar content rises. The ethanol conversion rate is lower than 50% when original sugar concentration approaches 10% (Tian *et al*., 2005). Concentrating hydrolysates need to be added in the producing procedures, increase the ethanol production cost by using cellulosic materials as raw materials. When using xylose as substrate, the ethanol conversion rate is less than using glucose (Steve *et al*., 2003).

Because sugar content is always less than 5% and companies with a lot of non-fermentation materials in stalk hydrolysates after pretreatment process, the sugar consumption rate in fermentation of the experiment may be higher than that of practical production. In addition, 5% original sugar content can be easily reached by adjusting solid-to-liquid ratio appropriately. Therefore, how hydrolysates can be converted to ethanol at low sugar content directly is the priority research area. *S. kluyveri* strain has a good practical potential in this area.

3.2 Furfural influence on strain growth

Some research results show that the furfural inhibiting influence on strain growth is greater than that on ethanol fermentation (Eva and Barbel, 2000), especially when the concentration of the inhibitor is low (Carlos and Leif, 2003). The results of the experiment show that the ethanol productivity of the strain was not influenced obviously. Besides, the inhibiting effects of the mixture of various inhibitors were much greater than that of single inhibitor (Carlos and Feif, 2003; Nurdan and Yesim, 2000; Steve *et al*., 2003; Lohmeier-Vogel *et al*., 1998). The research results of Steve *et al*. (2003) show that the co-inhibition effects of two mixed inhibitors are greater than each single inhibitors. In addition, the furfural and hydroxymethylfurfural has different influence on microbe stains (Carlos and Leif, 2003) and needs more research in outcomes during hydrolytic process.

There are less related reports on the scope of furfural concentration that inhibits ethanol fermentation. Because stalk hydrolysates are a mixture which contains various saccharide and inhibitors, the constituents will change when the conditions in the pretreatment process change. It is very difficult to study all inhibitors in the hydrolysates directly because different saccharide and inhibitors can interact with each other. And we use similar system to imitate the reaction in stalk hydrolyzing solution. It is an innovative point that the strain inhibited concentration can be studied by setting furfural concentration gradient.

The lower limit value for *S. kluyveri* strain growth and glucose fermentation is near 1.00 mg/ml and higher than that of CK treatment, about 0.75 mg/ml. The furfural endurancing ability of *S. kluyveri* is higher than that of reference strain. In the same time, low concentration of furfural (0.25 mg/ml) can promote the growth of the strain (both *S. kluyveri* and CK), and the mechanics needs to be further studied.

3.3 Influence of furfural concentration on ethanol fermentation

Massive ethanol begins to yield after the furfural in the substrate has already been metabolized (Steve *et al*., 2003). In the designed scope of furfural concentration, the furfural had no obviously inhibiting effects on ethanol yields. On the contrary, furfural can promote ethanol production and can not anastomose with the growth curve of microbes. This phenomenon may be caused by the low furfural concentration in the experiment; or the disturbance of volatilization of ethanol. Therefore, using fermentation tank to further study is necessary.

4 Conclusions

The growth of *S. kluyveri* can tolerate low furfural concentration. Furfural concentration at 0.25 mg/ml can promote the growth of the strain. The inhibiting effects on the strain growth appear when furfural concentration

Table 2 Ethanol content after 24 h fermentation (% w/v)

Furfural concentration (mg/ml)	0	0.25	0.50	0.75	1.00	1.25
S. kluyveri (3% original sugar)	1.57	1.63	1.61	1.62	1.58	1.65
S. kluyveri (5% original sugar)	2.45	2.56	2.47	2.53	2.44	2.45
CK	2.35	2.54	2.55	2.61	2.52	2.59

is above 1.00 mg/ml.

The ethanol yields are not influenced apparently with the increasing of furfural concentration in the experimental. And it has no prominent differences in different furfural concentration treatment. The ethanol yields of *S. kluyveri* and CK strain, *S. cerevisiae*, are similar.

The strain can be more easily inhibited by furfural cultured in 3% original sugar medium compared with cultured in higher sugar condition (5%). During the pretreatment process, setting a proper stalk-to-water ratio will increase the original sugar content in hydrolysates, which can increase the strain tolerance to furfural and promote ethanol fermentation.

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