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SHORT COMMUNICATION

The effect of cyclodextrins on the ethanol tolerance of microorganisms suggests potential application

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Abstract Cyclodextrins (CDs) are used in food, pharmaceutical, and chemical industries, as well as agriculture and environmental engineering. Cyclodextrin glucanotransferase (CGTase) is an important industrial extracellular enzyme which is used to produce CDs and oligosaccharides. We previously developed a novel yeast-surface CGTase expression system which was used for the production of CDs from starch. In the present study, we showed that the presence of CDs may increase the ethanol tolerance of microorganisms. The cell numbers of *Saccharomyces cerevisiae* and *Escherichia coli* in the presence of β-cyclodextrin and ethanol were 1,000-fold and 10-fold higher than that without CDs. The yeast strain with the immobilized CGTase produced 13 g CDs/l and 1.8 g ethanol/l when it was incubated in yeast medium supplemented with 4% starch. The effect of CDs on microorganisms suggests a potential application for the co-production of CDs and ethanol.

Keywords Cyclodextrins · Ethanol · Yeast · Cyclodextrin glucanotransferase (CGTase)

Introduction

Cyclodextrins (CDs) are hydrophilic on the outside and have a hydrophobic cavity on the inside and are able to form inclusion complexes with a wide variety of hydrophobic compounds by partially encapsulating them in their apolar cavities. Cyclodextrins are therefore used in food, pharmaceutical, chemical, cosmetic, and agricultural industries [13, 14]. CDs are cyclic α-(1,4)-linked D-glucose oligosaccharides typically consisting of 6, 7, or 8 glycosyl units known as α-, β-, and γ-CD (CD6, CD7, CD8) [6, 8]. The glycosyl units are produced as a mixture by the action of cyclodextrin glucanotransferase (CGTase, EC 2.4.1.19) [2, 13, 16] on starch [2]. The type of enzyme that is employed to generate oligosaccharide units determines the composition of CDs obtained [3, 8, 16].

To improve the starch conversion rate and CDs yield, Lima et al. [9] proposed the simultaneous application of CGTase and fermentation by *Saccharomyces cerevisiae*. In our previous experiments, CGTase was immobilized on the surface of *S. cerevisiae* which was endowed with direct starch-utilizing capability [15]. This surface engineered yeast is able to utilize starch as the sole carbon source directly and produce the expected CD products as well as glucose and maltose. This application was later investigated in another system [7].

Ethanol is a versatile oxygen-containing organic chemical because of a combination of properties. For example, it is used as a solvent, germicide, beverage, antifreeze, fuel, depressant, and intermediate for the synthesis of other organic chemicals. According to Gunasekaran [4], the yeast *S. cerevisiae* is a good candidate for industrial alcohol production. However, the yeast is highly susceptible to inhibition by ethanol. Ethanol concentrations of between 1 and 2% (w/v) are sufficient to retard the growth of *S. cerevisiae*, whereas at 10% (w/v) its growth almost stops. Overproduction of ethanol during the fermentation process may inhibit cell growth, cell viability, solute accumulation, and proton fluxes [17]. Ethanol mainly affects the plasma

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membrane and this changes the membrane organization and permeability [11]. In addition, all alcohol produced for consumption and industrial alcohol are currently made from grains. The fermentation of starch from grain is more time consuming than the fermentation of sugars because starch requires conversion to sugar and then to ethanol [10].

The objective of this study was to investigate the influence of CDs on the ethanol tolerance of microorganisms and ethanol production. This could signal the potential application for the co-production of CDs and ethanol from starch with an engineered yeast.

Materials and methods

Strains and media

Saccharomyces cerevisiae EBY-100 was purchased from Invitrogen Life Technology. *S. cerevisiae* (pYD/cgt) was previously constructed in our lab [15]. *Escherichia coli* DH5α was obtained from Gibco-BRL Life Technology. *E. coli* was grown in LB medium (1% tryptone, 0.5% yeast extract, 1% sodium chloride), and *S. cerevisiae* was grown in YPD medium (1% yeast extract, 2% polypeptone, 2% glucose) or YNB-CAA medium (0.67% yeast nitrogen base without amino acids, 0.5% casamino acids, and 2% glucose or galactose).

Ethanol tolerance experiments

The *S. cerevisiae* EBY-100 was incubated in 50 ml YNB-CAA medium at 30°C and grown to an optical density (OD600 nm) between 0.5 and 1. Ten percent (v/v) ethanol was added to the flasks supplemented with or without 1 g β-CD/l. Samples were taken every 12 h and viability was determined by plating appropriate dilutions of cells onto YNB-CAA medium containing 2% glucose. The plates were then incubated at 30°C for 2 days. All experiments were performed in duplicate and repeated at least three times. The ethanol tolerance experiment of *E. coli* DH5α was performed in the same way as for *S. cerevisiae* EBY-100 except a 6% (v/v) ethanol concentration was used.

Production of CDs and ethanol

The yeast that harbors the pYD/cgt plasmid was inoculated in 50 ml YNB-CAA medium and incubated for about 48 h at 30°C until the OD was between 2 and 5. Cells were harvested and washed with phosphate-buffered saline (PBS) and were then resuspended in YNB-CAA medium containing 2% galactose and grown up to an OD of 1.0. Following that, the resuspended cells were incubated on a shaker for 36 h at 20°C. Engineered *S. cerevisiae* cells were resuspended in YNB-CAA medium containing 4% glucose or 4% α-amylase pretreated soluble starch (80°C, 20 min). Ten percent (v/v) ethanol was added to flasks supplemented with or without 1 g β-CD/l. Yeast viability was measured by plating cells onto YNB-CAA medium containing 2% glucose followed by incubation at 30°C for 2 days.

Determination of CDs and ethanol by HPLC

Substrates and fermentation products were quantitatively analyzed by high-performance liquid chromatography (HPLC). The instrument was supplied with a refractive index detector using a 15-cm Shim-pack CLC-NH_2 6-μm column (Shimadzu Scientific Instruments Inc, Maryland, USA). Samples (1 ml) were taken at 12-h intervals and boiled for 5 min. Samples were centrifuged and filtered prior to HPLC analysis. CDs were eluted with an acetonitrile/water (60:40 v/v) mobile phase at 0.5 ml/min. Standard CDs (concentration, 10 mol/l) were dissolved in the same buffer and analyzed under identical conditions.

Results and discussion

Ethanol tolerance of yeast and *E. coli* in the presence of β-CD

Several reports suggest that the presence of ethanol could enhance the enzymatic production of CDs [3, 9]. The effect of CDs on ethanol tolerance and production of ethanol by microorganisms, however, is still unclear. To investigate the effect of CDs on ethanol tolerance, we performed fermentation experiments with *S. cerevisiae* EBY-100 by supplementing glucose with 2% (w/v) β-CD and 10% ethanol (v/v). We found that cell numbers were higher in the culture with β-CD than the culture without β-CD (Fig. 1). Cell numbers were approximately 1,000-fold higher at 84 h. Almost all yeast cells died after a 96-h incubation period (10% ethanol (v/v), without β-CD), whereas many cells were still alive in the culture where β-CD was added. CD quantities were constant during the incubation period and this indicates zero β-CD degradation. The reduction of the toxicity of ethanol to other microorganisms was shown in the experiments with *E. coli* (Fig. 2). Cell numbers of the culture in the presence of β-CD and 6% (v/v) ethanol were 10-fold higher than that without β-CD at 48 h. This indicates that the presence of CDs increased ethanol tolerance probably by formation of ethanol–CDs complexes.

Cyclodextrins can enhance the microbial conversion of either toxic [1] or water-soluble/insoluble organic substrates [5]. CDs were also successfully employed to reduce the toxicity of sludges by forming complexes with pesticides

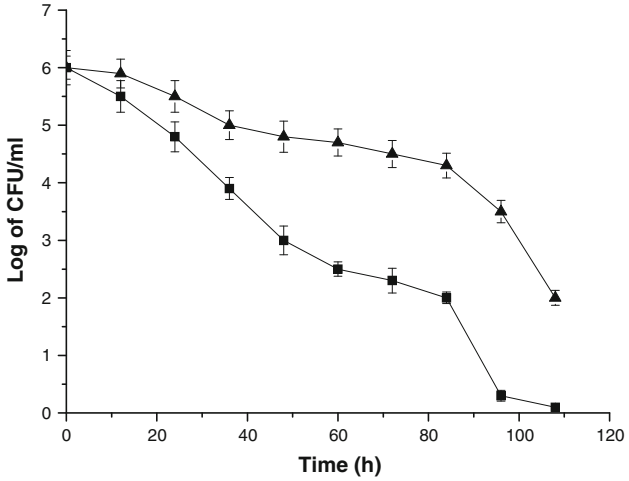

Fig. 1 Effect of β-CD on ethanol tolerance of *S. cerevisiae*. *Filled squares* cell number for cultivation without β-CD; *filled triangles* cell number for cultivation with β-CD

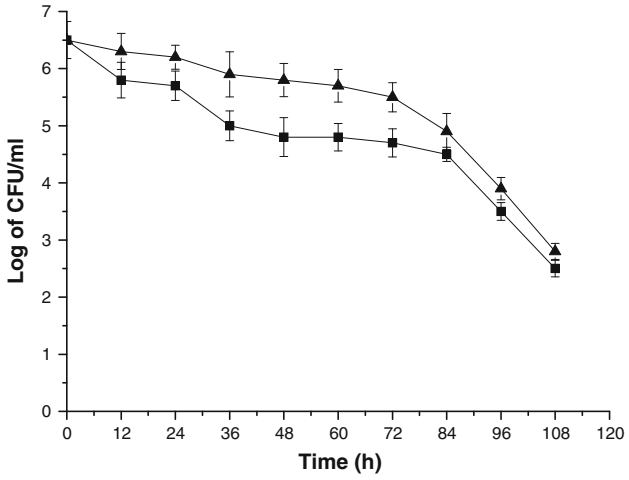

Fig. 2 Effect of β-CD on ethanol tolerance of *E. coli*. *Filled squares* cell number for cultivation without β-CD; *filled triangles* cell number for cultivation with β-CD

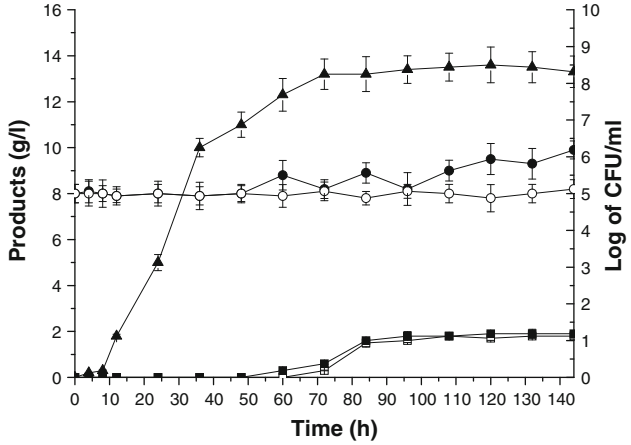

Fig. 3 Fermentation of surface engineered yeast. *Filled triangles* β-CD produced from soluble starch; *filled circles* cell number for cultivation in soluble starch; *open circles* cell number for cultivation in glucose; *filled squares* ethanol produced from soluble starch; *open squares* ethanol produced from glucose

[12]. CDs could also significantly alleviate the inhibitory effects of aromatic aldehydes and aid in the microbial transformation of aromatic aldehydes by *S. cerevisiae* [1]. Here we showed that the presence of CDs may attenuate ethanol toxicity to microorganisms by forming an inclusion complex with ethanol. This indicates that the presence of CDs increases the ethanol tolerance of microorganisms which may benefit ethanol production by the yeast.

Production of ethanol and CDs with surface engineered yeast

In our previous study, we developed a novel system in which CGTase was anchored on the surface of *S. cerevisiae*. The engineered yeast could utilize starch and produce CDs. The removal of glucose and maltose by the yeast facilitated an increase in the CD conversion ratio [15]. To investigate the capability of the engineered yeast to produce more ethanol than the wild-type *S. cerevisiae* EBY-100, fermentation experiments with the surface engineered yeast were conducted by adding 4% pretreated starch as the carbon source. The wild-type strain EBY-100 produced 1.9 g ethanol/l when grown on starch and 1.8 g ethanol/l when grown on glucose, which is a more convenient substrate, after 120 h (Fig. 3). The engineered yeast produced 13 g CDs/l from pretreated soluble starch. Co-production of ethanol and CDs may increase the substrate conversion rate and facilitate subsequent product recovery. The production of CDs did not affect the fermentation efficiency of the yeast. The yeast grew slightly better in YNB-CAA medium containing pretreated soluble starch than it did in YNB-CAA medium containing glucose. The ethanol yield was low because we employed a laboratory yeast. The possibility exists that an industrial strain could produce more ethanol.

In conclusion, this study to our knowledge is the first report that β-CD could enhance the tolerance of microorganisms to ethanol. By employing the surface engineered yeast, co-production of ethanol and CDs from starch can be obtained. The formation of an ethanol–CD complex has been shown to increase the production of CDs [3]. Our previous experiment confirmed that surface immobilization of CGTase may increase the CD conversion ratio from starch because of the removal of glucose, which is an efficient acceptor in CGTase coupling reactions and an inhibitor of CGTase [15]. In the present study we found that the formation of an ethanol–CD complex can not only increase starch conversion but also improve the ethanol

tolerance of the yeast. The effect of CDs on microorganisms suggests a potential application for the co-production of CDs and ethanol.

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