Effect of Different Biological Surfactants on Engineering *Saccharomyces cerevisiae* in Simultaneous Saccharification and Fermentation of Corncob

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Lignocellulose is considered to be a good resource for producing renewable energy. This paper reports on the effect of three surfactants [polyoxyethylene (80) sorbitan monooleate (POE80), rhamnolipid, and tea saponin] on cellulase (CBH/EG/BG) expression of *Saccharomyces cerevisiae* in simultaneous saccharification and fermentation (SSF) of corncob. In this work, the optimal surfactant concentrations for yeast growth were 0.1% POE80, 0.05% rhamnolipid, and 0.002% tea saponin. In the process of SSF, the reducing sugar content with 0.1% POE80 was 13.5% higher than the control at 24 h. The reducing sugar content with 0.05% rhamnolipid was higher than the control at 120 h, and reached the maximum difference of 18.2% in 120 h. The addition of 0.002% tea saponin exhibited the lowest promotion effect on the reducing sugar content in SSF compared with POE80 and rhamnolipid. However it reached the maximum difference of 8% in 120 h. Compared with the control, 0.1% POE80, 0.05% rhamnolipid, and 0.002% tea saponin presented different degrees of increase in reducing sugar content and viable count in the SSF. The results showed that the addition of the surfactants in SSF increased the growth rate of strains and promoted the saccharification efficiency of the substrate. This study lays a foundation for the application of surfactants in bio-energy research.

Keywords: Surfactant; Saccharomyces cerevisiae; Lignocellulose; Cellulase

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INTRODUCTION

Lignocellulose is composed of three main components: cellulose, hemicellulose, and lignin. It is a renewable resource with abundant reserves on Earth (Hahn-Hagerdal *et al.* 1994; Cherubini 2010). Therefore, the production of bio-ethanol using lignocellulose as the raw material has great significance for rational utilization of straw resources and the alleviation of the environmental energy crisis (Camargo *et al.* 2014). Lignocellulose has a highly complex and recalcitrant structure that limits the hydrolysis of stable carbohydrate polymers into reducing sugar (Chen *et al.* 2015). The energy loss and pollution emission caused by pretreatment of the substrate, which improves the saccharification efficiency, and the high enzyme cost, are the constraints of the industrialization of lignocellulosic bio-ethanol (Liu *et al.* 2015). Covalent bonding between hemicellulose and lignin constitutes a barrier in the enzymatic hydrolysis process and reduces the enzymatic conversion

efficiency of biomass feedstock (Meng *et al.* 2012). To make lignocellulosic conversion more efficient, the common methods are to optimize pretreatment, improve the hydrolysis process, and promote the fermentation process (Hammel 1996; Romaní *et al.* 2010; Soccol *et al.* 2010; Wang and Hu 2011). The optimized pretreatment method mainly treats the lignocellulose by physical or chemical methods. The removal of lignin components after pretreatment leads to accessibility of cellulose and thus an easier process of hydrolysis of cellulose to glucose during saccharification (Ming *et al.* 2009). However, due to environmental pollution and energy waste caused by pretreatment, many researchers hope to improve the utilization efficiency of lignocellulose in other ways, such as the use of multiple effective strains or additives.

The surfactant is a type of common additive in fermentation, which has hydrophilic and lipophilic groups. In terms of the hydrolysis of cellulose, some studies have shown that surfactants, especially nonionic surfactants, can promote the hydrolysis of cellulose by cellulose (Eriksson *et al.* 2002; Jeya *et al.* 2012; Cao and Aita 2013; Eckard *et al.* 2013; Zhou *et al.* 2013). For example, the addition of 1.80 mg/mL tea saponin increases the glucose production of lignocellulose, which produces the glucose from 34.3 g to 46.3 g per 100 g lignocellulose (Feng *et al.* 2013). During the process of wheat-straw saccharification, 0.016% and 0.048% polyoxyethylene (80) sorbitan monooleate (POE80) addition can promote cellulose degradation (Mo *et al.* 2008). However, the effect of rhamnolipid in cellulose degradation have some controversy. Some studies have shown that the rhamnolipids are conducive to the hydrolysis of cellulose (Jia *et al.* 2006; Mo *et al.* 2008). Others reported that the rhamnolipids are disadvantageous to cellulose degradation, because the rhamnolipids inhibit the contact of substrate and enzymes (Zhang *et al.* 2009; Wang *et al.* 2011). Nonetheless, the addition of surfactants during the hydrolysis of the substrate promotes the saccharification efficiency of the substrate and improves the substrate utilization and the conversion rate of ethanol. The addition of 0.5% (w/w) of surfactant (EOPO5) improved saccharification yields 31% and 55% with dilute acid pretreated wheat straw (DAWS) and steam exploded wheat straw (SEWS), respectively (Agrawal *et al.* 2017). Some studies have focused on the role of surfactants in cellulosic saccharification, such as the use of non-ionic surfactants (Triton X-100) in assisting β-glucosidase to convert bagasse and microcrystalline cellulose into glucose (Qu *et al.* 2017), and how Tween-20 (POE20) enhances the enzymatic saccharification of highly crystalline cellulose (Mizutani *et al.* 2002). Moreover, surfactants have certain applications in reducing the enzyme cost in the saccharification process. For example, some studies indicated that the role of some surfactants (POE80, Triton X-100, *etc*.) involved reducing non-productive binding of cellulase (Okino *et al.* 2013). The addition of the surfactant can make the enzyme rapidly desorb from the substrate and avoid enzyme inactivation (Helle *et al.* 2010). The presence of the surfactant can also increase the stability of the enzyme in the enzymatic hydrolysis and prevent the cellulase from deactivating (Kim *et al.* 1982; Kaar and Holtzapple 2015).

To solve the problems of low cell concentration and inhibition of high-concentration matrix during separate hydrolysis and fermentation (SHF), SSF is more selected (Öhgren *et al.* 2007). SSF is considered to be the most appropriate process for bio-ethanol production from cellulose (Alfani *et al.* 2000). Because the optimum temperature for enzymatic hydrolysis and fermentation differs greatly, the intermediate temperature in SSF is generally used. However, the intermediate temperature has a certain influence on the strain growth and the utilization efficiency of the substrate. Biomass instability and low substrate utilization will further lead to the decline of bio-ethanol production. Therefore,

how to ensure the stable growth of biomass and the saccharification efficiency of substrates under conditions of intermediate temperature becomes the key point of SSF condition optimization. To improve the utilization of the substrate, sometimes cellulase is continuously added during the saccharification, which greatly increases the cost. To solve this dilemma, many different approaches are used. One such approach is the use of an ultrafiltration membrane reactor, which retains the larger cellulase component and allows smaller molecules, such as reducing sugars and water molecules, to pass (Tian *et al.* 2015). Moreover, immobilizing cellulase on nanomaterials (Lee *et al.* 2010) or polymers can also reduce cellulase addition, such as recycling β-glucosidase by immobilization on a methacrylamide polymer carrier Eupergit C (Tu *et al.* 2006). In addition, studies have constructed genetically modified strains that enable production of the enzymes needed for cellulose degradation autonomously, thus reducing the addition of enzymes (Hong *et al.* 2015). Many studies have focused on the role of the surfactant in cellulase hydrolysis with a particular emphasis on fermentation process optimization, constructing enzyme recycling strategies, and promoting saccharification, *etc.* However, the role of the surfactant in cellulase hydrolysis by recombinant strain in SSF needs more related research.

In a previous study, the *S. cerevisiae* strains expressing three different kinds of cellulase (CBH/EG/BG) were constructed respectively, and these recombinant strains were used to hydrolyze the corncob during SSF (Song *et al.* 2016). Based on previous research in the authors' lab, surfactants with potential for development in SSF by using genetically modified *S. cerevisiae* to produce bio-ethanol from corncob were investigated. Three different surfactants (POE80, rhamnolipid, and tea saponin) are explored for their role in the SSF of lignocellulose based on mixed fermentation of a genetically modified strain.

EXPERIMENTAL

Materials

Corncob powder (Wuhan, Hubei, China) was washed with deionized water at room temperature to remove soluble components. The insoluble components were obtained by vacuum suction filtration and dried by an oven. POE80, rhamnolipid, tea saponin, and other reagents were purchased from Wuhan Times Bochi Technology Co., Ltd. (Hubei, China).

Strains and media

The *S. cerevisiae* INVSc1 used in this study were constructed in the authors' lab. The yeast cells were grown in yeast extract peptone dextrose medium (1% yeast extract, 2% peptone, and 2% glucose) at 28 ℃. The process of SSF was fermented in YPC medium (1% yeast extract, 2% peptone, and 2% corncobs). Table 1 summarizes the genetic characteristics of the recombinant strains (INVSc1 –SB, INVSc1 –SC, and INVSc1 – SE).

Optimized concentration of surfactants for engineering S. cerevisiae growth

To choose the suitable surfactants concentration for the *S. cerevisiae* growth, INVSc1 was cultured in 100 mL YPD medium with a concentration gradient of surfactants (POE80 and rhamnolipid 0.05% to 0.8%, tea saponin 0.002% to 0.01%). Sampling occurred every 2 h and the biomass was analyzed by a spectrophotometer (UV-1000; Hanyi Technology Co., Ltd., Shanghai, China) in 12 h culture.

Table 1. Genetic Characteristics of the Engineering Strains (INVSc1-SB, INVSc1-SC, and INVSc1-SE)

Strains	Relevant Features
S. cerevisiae INVSc1	*His⁻leu⁻trp⁻ura⁻*
S. cerevisiae INVSc1-SB	*His⁻leu⁻trp⁻*, integration of β-glucosidase gene
S. cerevisiae INVSc1-SC	*His⁻leu⁻trp⁻*, integration of exoglucanase gene
S. cerevisiae INVSc1-SE	*His⁻leu⁻trp⁻*, integration of endoglucanase gene

Methods

Simultaneous saccharification and fermentation

Corncobs were used as the sole carbon source for fermentation with the addition of surfactants to confirm the role of surfactants in the fermentation of natural lignocellulose. The recombinant strains (INVSc1–SB, INVSc1–SC, and INVSc1–SE) were cultured in 50 mL YPD medium for 16 h at 28 ℃ at 200 rpm. The yeast cells were used as the inoculation seed when the OD_{600} reached 2.0. Because the hydrolysis of cellulose requires the joint action of three kinds of cellulases (CBH/BG/EG), a mix of three recombinant strains to treat the lignocellulosic materials in this study was used. 5 mL aliquots of each recombinant strains were mixed together and then added into 1 L YPC medium, which, respectively, had optimized concentration of three different types of surfactants. Fermentation was performed at 28 ℃ for 120 h and sampling occurred every 24 h in the clean bench for parameter measurement.

Reducing sugar content in SSF of corncob

Concentration of the reducing sugars was determined by 3,5–dinitrosalicylic acid (DNS) colorimetry with glucose as the standard (Miller 1959). The samples (0.5 mL) were mixed with 1.5 mL of DNS in a 5 mL tube. The mixed solutions (2 mL) were heated at 100 ℃ for 5 min and then cooling to ambient temperature. The heated mixed solution was then diluted five times with deionized water. The absorbance of the solutions was measured at 540 nm with a spectrophotometer. The concentration of reducing sugars was calculated based on Eq. 1:

$$Y = 0.822x - 0.059, \ R^2 = 0.99 \quad (1)$$

Determination of biomass by plate colony counting and turbidimetry

Using deionized water as a blank control, the OD_{600} value of the YPD shake flask fermentation sample was measured using a spectrophotometer, 100 μL each of the YPDC and YPC fermentation sample was diluted 10^2, 10^3, 10^4 times with sterile water. After dilution, 100 μL of the sample was uniformly spread on the YPD solid medium and cultured at 28 ℃ for 48 h. The plate colony counting experiments were performed in triplicate. The number of colonies in the plate between 50 and 300 was selected to be the most appropriate concentration for dilution. The average of the growth colonies of the three plates at this concentration were calculated, and the colony status on each optimal plate was recorded.

RESULTS AND DISCUSSION

POE80 Effect on Engineering *S. cerevisiae* in SSF of Corncob

POE80 is a non-ionic surfactant. The engineering of *S. cerevisiae* cultured with 0.05% to 0.8% POE80 and its growth curve was measured to study the effect of surfactant concentration on strain growth. The growth curves involving different concentrations of POE80 (Fig. 1a) showed no noticeable difference in OD_{600} absorbance at 42 h.

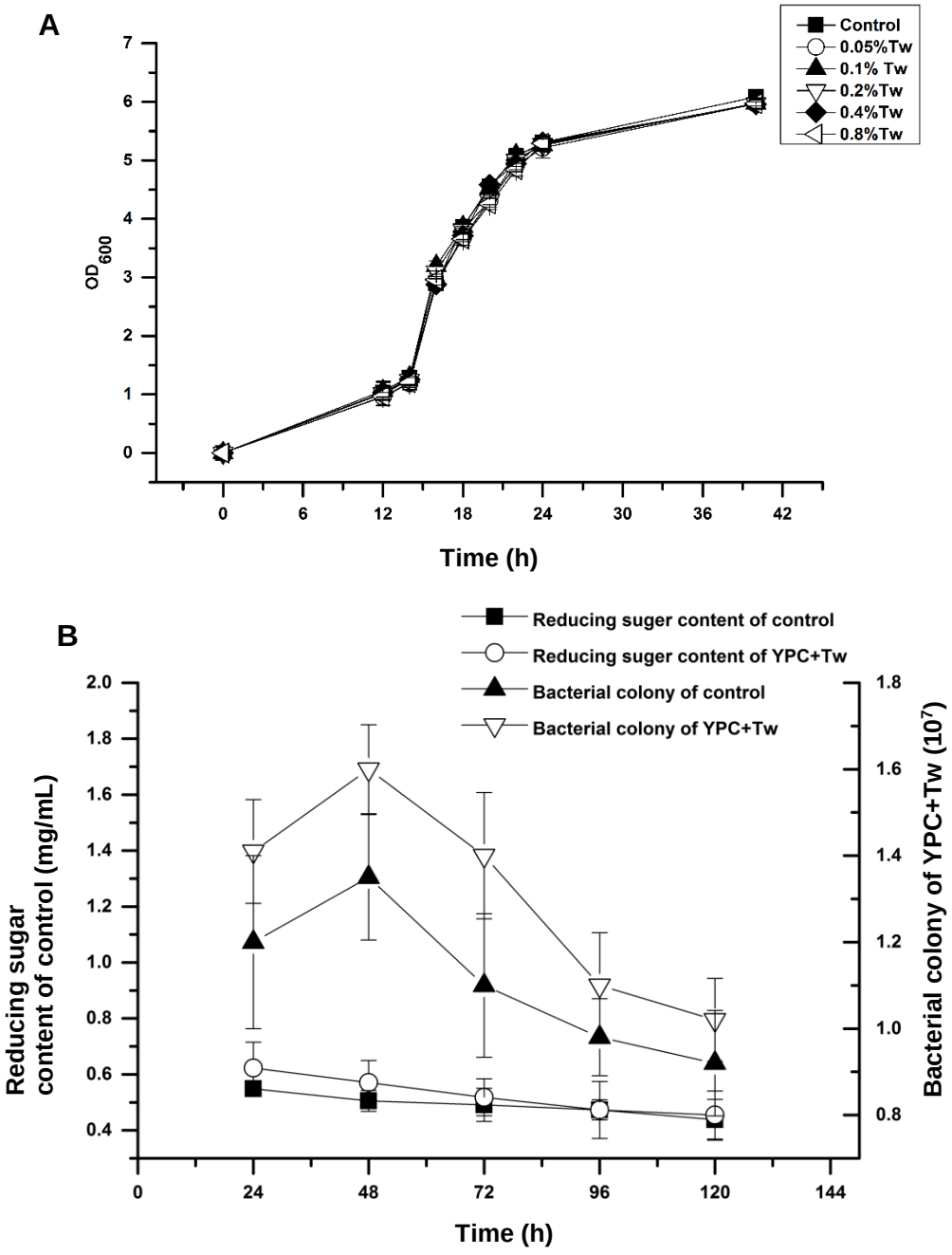

Fig. 1. The growth curve of fermentation in YPD (A) and the curve of reducing sugar content and biomass (B) with POE80

The 0.4% POE80 had no remarkable effect on the growth of *S. cerevisiae*, but it had a slight inhibitory effect on the cell growth after 20 h. Compared to other concentrations, 0.1% and 0.2% POE80 were more suitable for the growth of *S. cerevisiae*. The principle of choosing the lower concentration based on the same effect is preferred, 0.1% POE80 was selected as the most suitable concentration for subsequent research.

From the reducing sugar content and colonies number, the addition of 0.1% POE80 made the lignocellulose produce more reducing sugars within 96 h compared to the control (Fig. 1b). At 24 h, the reducing sugar content of 0.1% POE80 added was 13.5% higher than the control. With the increase of time, the difference of reducing sugar content gradually decreased. At 120 h, the reducing sugar content of 0.1% POE80 was still 3.88% higher than the control. The addition of POE80 promoted the production of reducing sugars in the system at the early stage of fermentation. From the comparison of colony numbers in Fig. 1b, the growth of strains increased after the addition of 0.1% POE80 within 48 h, and the expression of cellulase increased consequently, which resulted in higher reducing sugars content in SSF during first 48 h. The results showed that the growth of the yeast was promoted by the addition of 0.1% POE80. Within 48 to 120 h of fermentation, the glucose content and the colonies number with or without POE80 both decreased. However, the decrease of the reducing sugar with POE80 added was faster than the control. The reducing sugar content with 0.1% POE80 added and the control exhibited no difference in 96 h, which was probably due to POE80 being more conducive to the growth of the *S. cerevisiae* and its ability to more effectively decrease the reducing sugars. The product inhibition during the enzymatic hydrolysis of cellulose is an important factor that limits the efficiency of hydrolysis and reduces the utilization of the substrate (Miao *et al.* 2012). The promotion effect of POE80 on the growth of *S. cerevisiae* increased the substrate utilization during SSF, which increased the saccharification rate of the substrate. In summary, the engineering *S. cerevisiae* under the promotion effect of POE80 can use the reducing sugar in SSF more efficiently, and reduce the product inhibition in the cellulose substrate.

Table 2. Reducing Sugar Content (mg/mL) in the Saccharification of Corncob

	24 h	48 h	72 h	96 h	120 h
Control	0.549	0.505	0.491	0.473	0.438
YPC+Tw	0.523	0.571	0.518	0.473	0.455
YPC+Rh	0.633	0.558	0.546	0.498	0.518
YPC+Ts	0.583	0.522	0.515	0.487	0.474

Rhamnolipid Effect on Engineering of *S. cerevisiae* in SSF of Corncob

Rhamnolipid is a kind of biosurfactant produced by microorganisms. It has many advantages such as non-toxic nature, biodegradability, *etc*. The growth of *S. cerevisiae* with added rhamnolipid was inhibited compared to the culture without surfactant (Fig. 2a). The 0.05% rhamnolipid had no obvious effect on the growth of *S. cerevisiae*. With the increased concentration of rhamnolipid, the inhibitory effect on the growth of *S. cerevisiae* was gradually increased. The OD_{600} value was 5.47 when cultured with 0.8% rhamnolipid for 40 h, which was 10% less than the control without surfactant. Compared to other concentrations, 0.05% rhamnolipid was more suitable for the growth of *S. cerevisiae*. Therefore, 0.05% rhamnolipid was selected as the most suitable concentration for subsequent research.

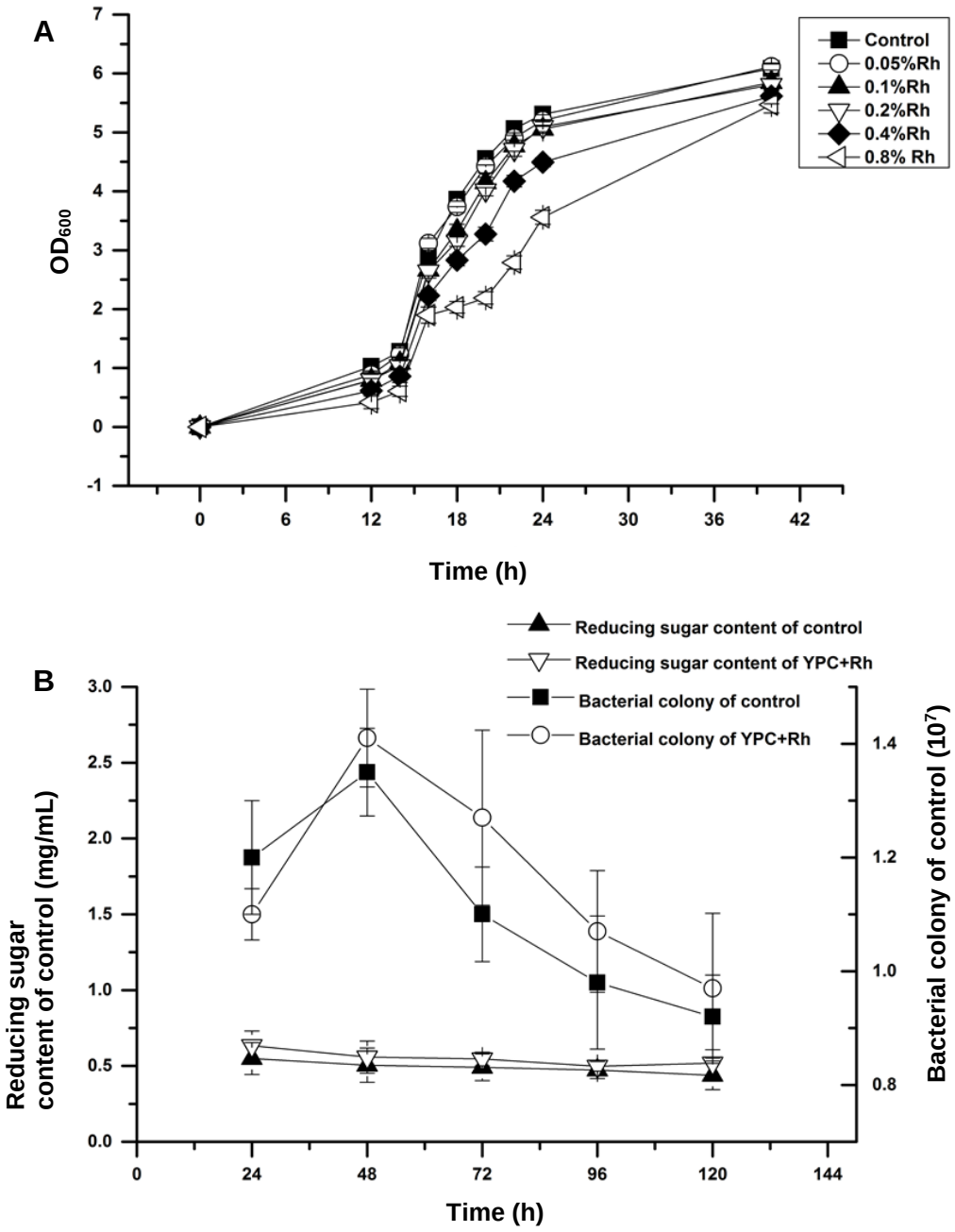

Fig. 2. The growth curve of fermentation in YPD (A) and the curve of reducing sugar content and biomass (B) with rhamnolipid

With the addition of 0.05% rhamnolipid, the reducing sugar content increased compared to the control within 120 h. The reducing sugar content of the control exhibited a tendency to decline within 120 h, while the rhamnolipid added showed an increase in reducing sugar content at 96 to 120 h (Fig. 2b). The number of colonies of *S. cerevisiae* with 0.05% rhamnolipid added was lower than the control at 24 h, but it was more than the control at 48 h, which clearly demonstrated the promotion of the strain growth by rhamnolipid. In the trend of strain growth and the change trend of reducing sugar content, the addition of 0.05% rhamnolipid was similar to 0.1% POE80, which all showed the improvement of the substrate utilization. In contrast to POE80, the reducing sugar content

with the 0.05% rhamnolipid added showed an increase trend at 96 to 120 h. This appearance was probably due to the inhibition of the product decreasing in the system and the sugar produced by hydrolysis was greater than consumed. It can be concluded that the addition of rhamnolipid can improve the utilization of the substrate, and it is more conducive to the simultaneous saccharification and fermentation.

Effect of Tea Saponin on Engineering *S. cerevisiae* in SSF of Corncob

Tea saponin (TS) is a type of tea seed-derived natural non-ionic biosurfactant. It mainly comes from the residue after producing tea seed oil (Feng *et al*. 2013). *S. cerevisiae* added with the tea saponin led to noticeable inhibition compared to without surfactant addition (Fig. 3a).

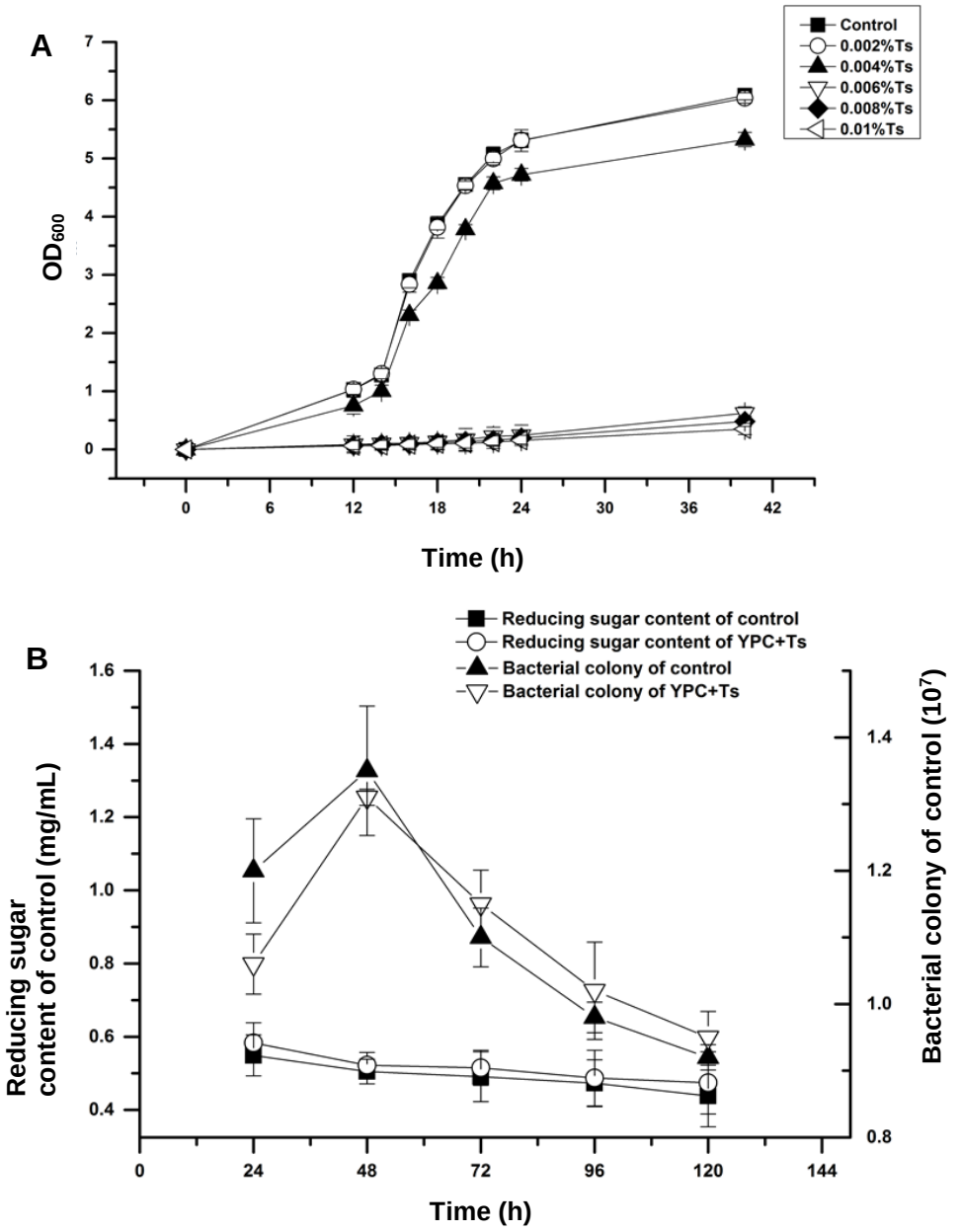

Fig. 3. The growth curve of fermentation in YPD (A) and the curve of reducing sugar content and biomass (B) with tea saponin

The 0.002% tea saponin had no inhibitory effect on the growth of *S. cerevisiae*. However, with the increase of the concentration of tea saponin, the *S. cerevisiae* growth was considerably inhibited. From 0.006% of tea saponin, the inhibition of tea saponin on *S. cerevisiae* growth was remarkable. The OD_{600} value was only 0.35 at 40 h when the culture was treated with 0.01% tea saponin, 0.002% tea saponin was more suitable for the growth of *S. cerevisiae*, and 0.002% tea saponin was selected for subsequent studies.

Compared to the addition of 0.1% POE80 and 0.05% rhamnolipid, the addition of 0.002% tea saponin resulted in a lower promotional effect on the content of reducing sugar in SSF. Nevertheless, it still presented a certain degree of improvement compared with the control. Moreover, the effect of 0.002% tea saponin on reducing sugar content and colony number was similar to 0.05% rhamnolipid, but the decline rate of colony number in the addition of 0.002% tea saponin within 48 to 120 h was lower than the control (Fig. 3b). Therefore, the tea saponin was probably not as effective as POE80 and rhamnolipid, which promoted the growth of *S. cerevisiae* but delayed the aging and death of strains.

The effects of chemical/biological surfactants on substrate utilization efficiency and biomass in SSF were focused in this work. Through the above research, although rhamnolipid and tea saponin are both non-ionic surfactants of natural origin, the promoting effect of tea saponin was not as effective as rhamnolipid in the saccharification of lignocellulose. In addition, rhamnolipid was able to achieve similar effects as POE80, which is synthetic chemical surfactant. However, rhamnolipid is biological surfactant, has lower toxicity and environmental friendliness than chemical surfactant, and has greater advantages in industrial production (Cooper *et al.* 1986; Mulligan 2005). In this study, both POE80 and rhamnolipid can improve the content of reducing sugar in SSF. Some studies have shown that the effect of adding non-ionic surfactants was caused by reduced contact of enzyme with the air–liquid interface due to the surface activity of the surfactant, and avoid cellulase deactivation by shear forces (Kim *et al.* 1982). It has also been reported that non-ionic surfactants can reduce the adsorption capacity of cellulase to residual substrates, avoid enzyme inactivation due to irreversible adsorption (Tengerdy and Szakacs 2003). In addition, the nonproductive adsorption of enzymes on lignin has a negative effect on the enzymatic hydrolysis saccharification of cellulose. Some studies shown that POE80 can reduce nonproductive adsorption by occupied the hydrophobic surface of lignin (Li, Y. *et al.* 2016). Because of complex reaction system and many influential factors, more researches are needed to investigate the mechanism of the surfactants. In addition, the effects of surfactants on fermentation products in SSF (*i.e.* ethanol) will be focused in our future work.

CONCLUSIONS

1. This study analyzed the effects of POE80, rhamnolipid, and tea saponin on the SSF of cellulose.
2. Surfactants impacted the growth of engineered *S. cerevisiae* and further promoted the saccharification of the substrate, thereby increasing the efficiency of synchronous saccharification and fermentation of lignocellulose.
3. The results show that the addition of different surfactants in SSF of lignocellulose by engineered *S. cerevisiae* has potential industrial application.

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