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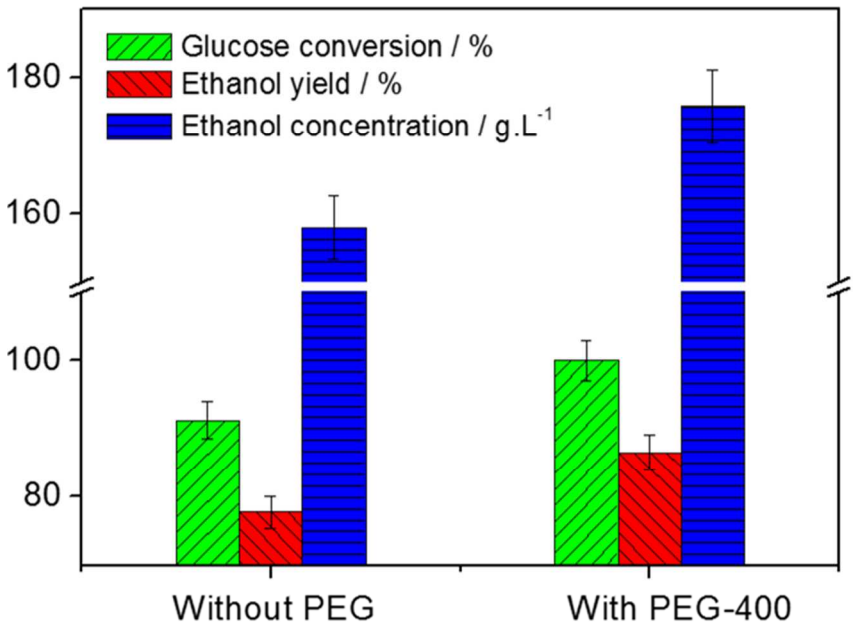

Yeast cells exo-protected by water soluble PEGs are tolerant to higher ethanol concentrations, and offer high ethanol productivity.

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Fuel ethanol is an attractive alternative to fossil-based fuels or fuel additives. Very-high-gravity(VHG) ethanol fermentation is a promising technology by reducing energy consumption in distillation. However, yeast cells subjected to a high concentration of ethanol and osmotic stress readily loses cell viability, resulting in reduced ethanol productivity. Here we report the beneficial effect of fully water-soluble polyethylene glycol (PEG) in chemically exo-protecting yeast cells during fermentation, resulting in largely boosted cell vitality and tolerance to high ethanol concentration. The final ethanol concentration and the yeast cell viability were substantially increased as compared to PEG-free fermentation. The recovered exo-protected yeast was further demonstrated to continue to deliver superior bio-catalytic performance in subsequent fermentations over that recovered from PEG-free broth. Furthermore, the water-soluble PEG was readily recycled for reuse after distillation of ethanol.

Introduction

Fuel ethanol represents the largest volume of renewable fuels world wide as an environmentally friendly alternative to fossil fuels. The production of bio-ethanol has been considerably increased over the last few years.[1-3] Feedstock and energy consumption contribute to the major cost of bio-ethanol production. The U.S. Department of Energy's research roadmap,[4] highlighted a number of challenging targets, including high yield with complete sugar utilization, higher final ethanol concentration(titer), higher overall volumetric productivity, and high tolerance to inhibitors.

High ethanol concentration has always been pursued in the fermentation industry, because significant energy savings can be achieved for downstream distillation and waste distillage treatment.[5,6] Bio-ethanol fermentation can be improved by fermentation in media containing high concentrations of dissolved solids (above 300g per liter), optimization of the ethanol fermentation process, and selection of robust microorganisms.[7,8] One of the process strategies currently applied in the fermentation industry is very high gravity (VHG) technology. Compared to conventional fermentation process, industrial application of VHG ethanol fermentation technology has exhibited obvious advantage,[9-12] as it proceeds in significantly higher final ethanol titer (usually above 15%, v/v) in the fermentation broth.[13] This fermentation technology not only saves energy consumption for ethanol distillation and decreases process water requirements, but also reduces waste distillage discharged from the distillation system.[14,15] Thus VHG fermentation has garnered great attention. However, VHG fermentation suffers from incomplete glucose utilization and decreased fermentation rate by subjecting yeast cells to high osmotic stress and severe ethanol inhibition in yeast cells.[16-21]

Research has revealed that improved cell viability and yeast growth rate needed to achieve high ethanol concentration strongly depend on the supplementation with osmoprotectant. Thomas et al. reported that glycine can promote yeast cell growth and improve cell viability in VHG medium.[22] Thomas and Ingledew showed that the fermentation time was shortened from 8 days to 3 days when 0.9% yeast extract was added in wheat mash fermentation that produced 17.1% (v/v).[23] Considering that fresh yeast extract was too costly for the industrial use, Kawa-Rygielska and Pietrzak studied the spent brewer's yeast supplements and found that ethanol concentration can be enhanced from 120g.L^{-1} to 140g.L^{-1}.[24] In that study, it was also recognized that spent brewer's yeast cell lysis released various hydrocolloids (β-glucan, polypeptides), which caused an increase in mash viscosity. Overall, the use of the most of these supplements is not economically feasible because of their relatively high prices.

Surfactants have lower vapor pressure and their physical properties can be fine-tuned with appropriate choice of molecular structure and weight. Surfactant Triton X-100 was found to improve the cell viability during post-logarithmic cell growth phase,[25] but its effect in ethanol fermentation was not assessed. PEG-6000/dextran aqueous two-phase system (ATPS) was used as a means for extractive ethanol fermentation, which encouraged growth of microorganism and improved fermentation efficiency.[26,27] The ATPS fermentation took place in the bottom H_2O/dextran phase with ethanol extracted to the upper H_2O/PEG-6000 phase. However, the ethanol concentration was very low (3.3 g/L) as compared to that in VHG fermentation. An industrial application of the ATPS extractive fermentation may also be limited due to the high cost of dextran.

The objective of this work was to identify and to study potential supplements that may provide excellent biocompatibility to microorganisms and may also enhance the vitality of the microorganisms at higher ethanol concentrations. By using such supplments, the yeasts may be reused and the distillation cost in separating ethanol from the fermentation broth may be reduced.

Up to now, there was no report in the literature on the effect of fully water soluble PEGs directly in ethanol fermentations. In this work, the addition of the PEGs to a fermentation broth was found to induce a highly favourable effect in vitalizing yeast cells, resulting in substantially enhanced cell tolerance to very high ethanol concentrations. Complete glucose conversion and substantially improved final ethanol concentration were obtained in this work. It was further revealed that the vitality of the exo-protected yeast cells was maintained and the recycled cells displayed robust activity in subsequent fermentations.

Experimental

Organisms and chemicals

A commercially available immobilized active dry yeast, Saccharomyces cerevisiae, was purchased from Angel Company (China), which was named Angel Super-Alcohol Active Dry Yeast (starch base). The yeast was kept at 4°C during storage, and was weighted and directly added to specified fermentation media as received right before each use. D-glucose ($C_6H_{12}O_6 \cdot H_2O$), glycerol (99wt%), ethanol(99wt%) were received from Sinopharm (China). Polyethylene glycol (PEG-400) was purchased from Kermel (China). Sulphuric acid (98wt%) and methylene blue were provided by a local supplier. Deionized water (DI H_2O) was produced by a Milli-Q Integral 5 system. All other chemicals were of analytical quality.

Measurement of viable cell density

The yeast viability was measured according to the Methylene-Violet Staining Procedure. A volume of 100 µl dilute sample containing cells was mixed with 100 µl of a methylene blue solution. After 20 min staining, the numbers of viable (living) cells and of total cells were counted under a microscope (Nikon Ci-L). The cell viability was calculated according to the following equation:

$$\text{Cell viability} = \frac{\text{Viable cell numbers}}{\text{Total cell numbers}} \times 100\%$$

Yeast culture: The yeast strain was grown at 33°C in YPD medium containing 10g/L yeast extract, 20g/L peptone and 20g/L glucose. The yeast cell concentration was determined by the optical density of the culture using a UV–vis spectrophotometer at 600 nm (Shimadzu UV-2600).

VHG fermentation

The ethanol fermentation was performed in the air-locked Erlenmeyer flask (50 or 100 ml). The initial yeast cell concentration was approximately 3×10^8-5×10^8 cells·mL^{-1}. The PEG concentration in water was approximately 0.25g.mL^{-1}. The pH of the media was adjusted to 3.9 with a H_2SO_4 solution. Glucose was used as a carbon source without nutrient supplement. The fermentation was performed in batch mode and the temperature was controlled at 30-36 °C. During fermentation, the flasks were placed on a rotary shaker (ZWY-240) at 160 rpm. After fermentation, the yeast cells and PEG-400 with H_2SO_4 were recycled for subsequent uses.

Recovery of yeast, PEGs and buffer H_2SO_4

A representative procedure for the recovery and reuse of yeast, PEG-400 and H_2SO_4 is shown in Figure 1. After fermentation, the yeast cells were collected by centrifugation at 8000 rpm for 5 minutes, and the yeast cells were further evaluated for their activity in subsequently fermentations. And then the separated liquid phase was subjected to vacuum distillation to separate ethanol. PEG and H_2SO_4 were reused by recycling the fermentation broth after distillation.

The sample of each fermentation broth was diluted in 2500ml solution with deionized water, and filtered through a 0.22um filter. The glucose and ethanol concentrations of fermentation samples were quantified using a high performance liquid chromatography (HPLC). An Agilent 1260 Series HPLC system equipped with a refractive index detector was used. Ion exchange columns (HPX-87H, 300 × 7.7 mm) were used in series. The column and detector temperatures were maintained at 65 °C and 50 °C, respectively, with 5 mM H_2SO_4 as the mobile phase at 0.6mL/min. The glucose conversion was calculated based on initial glucose and consumed glucose. The ethanol yield was calculated based on the theoretical ethanol yield from consumed glucose; the concentrations of ethanol and glucose were calculated based on the water volume in the fermentation broth. Data analysis were performed using the Agilent Chemstation software and Microsoft Excel. The glucose conversion, ethanol yield and ethanol concentration were calculated according to the following equations:

$$\text{Glucose conversion} = \frac{(\text{initial glucose(mol)} - \text{final glucose(mol)})}{\text{initial glucose(mol)}} \times 100\%$$

$$\text{Ethanol yield} = \frac{\text{final ethanol(g)}}{0.51 \times \text{ initial glucose(g)}} \times 100\%$$

$$\text{Ethanol concentration(g/L)} = \frac{\text{final ethanol(g)}}{\text{H2O(L)}}$$

Fermentation model and parameter estimation

The model for ethanol productivity from glucose was a modified Logistic model. The parameters in the model were evaluated by using Matlab7.5. Its parameter estimation feature seeks to minimize the residual sum of squares between the model predicted values and the experimental values. These data were fitted to Logistic model as given below.

$$v = \frac{dp}{pdt} = v_{max}(1 - \frac{p}{p_{max}})$$

where v is specific rate of ethanol formation (g. L^{-1}.h^{-1}), v_{max} is maximum specific rate of ethanol formation (g. L^{-1}. h^{-1}), P is ethanol concentration (g.L^{-1}), and P_{max}, ethanol concentration above which cells do not produce ethanol (g.L^{-1}).

Results and discussion

Effect of PEG-400 on glucose conversion at high glucose concentrations

Separation of bio-ethanol from the aqueous fermentation broth is usually by distillation. Increasing ethanol concentration in water can greatly decrease the ethanol separation cost,[28] reduce the amount of waste water, and improve the equipment utilization efficiency.

In order to get high ethanol concentration, it's prerequisite to use high glucose concentration. First, two high initial glucose concentrations (37 wt% and 40 wt% solutions) were evaluated for batch fermentation process in pure water as reference experiments. The results are shown in Figures 2a-2b. The glucose conversion of 100% and ethanol concentration of 160g.L^{-1} were obtained in 72h at the glucose concentration of 37 wt%.

To further increase the ethanol concentration, an initial glucose concentration of 40% was also evaluated. It was found that the glucose conversion was decreased to 91% at this higher glucose concentration, while the ethanol concentration (160g.L^{-1}) did not change. The results are consistent with the discussion of VHG process in the introduction; one disadvantage of the VHG process is incomplete glucose conversion at high glucose concentration. This phenomenon may be ascribed to the particular yeast cell's tolerance to an ethanol concentration ceiling due to osmotic pressure and ethanol stress. This may cause decreased yeast viability, and slow or stuck fermentations. The incomplete fermentation of glucose is undesirable as it wastes the valuable carbohydrate resource as well as brings difficulty for the subsequent treatment of waste distillage. Therefore, it can be concluded that the highest ethanol concentration of 160 g.L^{-1} which corresponds to the initial glucose concentration is 37 wt% at full glucose conversion in pure water for the yeast employed in this work. Further increase in the initial glucose concentration has no positive effect on ethanol production.

To break the ethanol concentration ceiling by the yeast in pure water, we evaluated potentially low-cost and recoverable supplements as osmoprotectant in the fermentation media. In this study, we explored the efficiency of PEG-400 as an osmoprotectant in VHG fermentation. The basis for focusing on PEG-400 among several PEGs in this study is provided later in this paper. To establish the role of PEG-400, the effect of the initial glucose concentration in the presence of PEG-400 on glucose conversion and ethanol concentration was studied. The results are shown in Figure 2c-2d. Under glucose concentration of 37 wt%, it took 48 h to convert glucose completely, and the ethanol concentration of 160 g.L^{-1} was obtained. The time to reach full conversion for 37wt% glucose was 48 h in the presence of PEG-400, as compared to 72 h for that in pure water. Full conversion of 40 wt% glucose was achieved in the presence of PEG-400 in 72 h. Moreover, ethanol concentration was increased to 175 g.L^{-1} which was substantially higher than the ethanol concentration ceiling (160g.L^{-1}) in pure water for the same yeast. The results indicate that the yeast cell's ethanol tolerance was improved when the PEG-400 was supplemented to the reaction media.

Effect of yeast cell loading and temperature

The loading of yeast cell was determined for the batch fermentation process in the presence of PEG-400 at 33℃and at 40wt% of glucose concentration. The results are shown in Figures 3a and 3b. It is clear that when the cell concentration was below approximately 5×10^8 cells mL^{-1}, glucose was not completely converted in 72h. The highest ethanol concentration of 175g.L^{-1} was obtained. Therefore, the cell concentration of approximately 5×10^8 cells mL^{-1} was chosen for VHG fermentation process in this study. It should be pointed out that this yeast loading ensures full glucose conversion in 72 h at the initial 40% glucose concentration in the presence of PEG-400. Further increase in the yeast loading may only shorten the time to reach full glucose conversion, but is not expected to increase the ethanol concentration ceiling.

The effect of fermentation temperature was investigated in the presence of PEG-400. The results are shown in Figures 3c and 3d. The glucose conversion was 90% with the ethanol concentration of 152g.L^{-1} at 36 ^oC in 72h. When the temperature was 33 ^oC, the ethanol concentration of 175 g.L^{-1} and glucose conversion of 100% were obtained in 72h. However, at 30 ^oC, glucose conversion of 91% and the ethanol concentration of 151 g.L^{-1} were obtained, indicating that the end of fermentation was not reached in 72 h.

We measured the cell viabilities, defined as the percent of survived living cells, from tests at the three temperatures to account for the different glucose conversions. The lower glucose conversion at 30 ^oC is largely due to the lower activity of the cells because the cell viability (52%, Fig. S1) at this temperature is the highest among the three. On the other hand, the lower glucose conversion at 36 ^oC can be ascribed to high cell death rate at this high fermentation temperature, as the cell viability has dropped to 3%. The cell viability of 24% for fermentation at 33 ^oC in 72 h represents a moderate cell death rate at the highest ethanol concentration, a most severe condition for the yeast cells.

Effect of PEG concentration in water

PEG-400 concentration was optimized for the batch fermentation process. It is shown in Figure 4 that when the concentration of PEG-400 added was increased, the glucose conversion, ethanol yield and concentration also increased. As the concentration of PEG-400 achieved 0.25g.mL^{-1}, the glucose conversion, ethanol yield and concentration reached maximum. When the concentration of PEG-400 was further increased, the glucose conversion, ethanol yield and concentration start to decrease. Thus, 0.25 g.mL^{-1} was chosen as the appropriate PEG-400 concentration applied in this paper.

Selection of PEGs

Effects of different PEGs on batch ethanol fermentation were investigated. Four types of PEG were used in this paper. The results are shown in Figure 5. It can be seen that when PEG-200 was added, the glucose conversion, ethanol yield and concentration were decreased. However, addition of PEG-400, PEG-600 and PEG-1000 resulted in higher glucose conversion, ethanol yield and concentration than that of the control (without adding PEGs). The three PEGs increased fermentation productivity. In this study, PEG- 400 was chosen and studied in greater detail as the appropriate additive in improving

fermentation productivity.

Effect of exo-protection by PEG-400 on yeast performance and viability

In order to understand the effect of PEG-400 on the increased ethanol productivity in VHG fermentation, fermentation performances of the yeast with and without PEG-400 were further investigated as shown in Figure 6, even though the glucose conversion rate was faster in the first 36 h in pure water than that in the presence of PEG-400, it slowed down afterward. Even after extending the fermentation to 96 h, 42.0 $g.L^{-1}$ of glucose was unconverted in the pure water system. The maximum ethanol concentration was 159 $g.L^{-1}$, which is near the ethanol concentration ceiling for the yeast in pure water. In comparison, the maximum ethanol concentration reached 175$g.L^{-1}$ at the end of fermentation (Figure 6a), with little residual sugar left when 2.0 g of PEG-400 was used. We also performed verification tests of the same experiments in 40 ml. The results obtained at smaller volumes were reproduced. Such remarkably beneficial effect of PEG-400 on the fermentation suggests that PEG-400 may have played a role of osmoprotectant for improved cell tolerance to higher ethanol concentration. This hypothesis was tested by measuring the yeast cell viability from fermentations with and without PEG-400 (Figure 6b).[29] It was found that although the yeast cell viability was the same with and without PEG-400 within 36h, it was much decreased in pure water than that in the presence of PEG-400 in 48 h. The cell viabilities in systems with and without PEG-400 started to progressively diverge after 36h. The viability results clearly indicate that exo-protection of the yeast cells by PEG-400 significantly increased ethanol concentration under VHG conditions. The mechanism of PEG additives on the improvement of cell viabilities under high ethanol concentrations is an important subject of future study. Reduced osmotic pressure to the yeast cells may be caused by employing PEG-400 as an osmoregulator.[30] Chemical interaction of PEG-400 with ethanol could play an important role in the fermentation broth.

Effect of PEG-400 on recovered yeast fermentation performance

We further studied the performance of recycled yeast cells from both fermentation systems with and without PEG-400. According to the results in Figure 6a, with fresh yeast, the ethanol concentration was near the same at 48h in both fermentation systems with and without PEG-400. It should be noted that the ethanol concentration in 48 h is already close to the ethanol concentration ceiling in the pure water system. Therefore, the yeast cells were recycled from both systems after 48h for the subsequent fermentation. As shown in Figures 7a and 7b, although in general the activity of the recycled yeast was lower than that of fresh yeast and fermentation productivity decreased with each recycle, there was a very pronounced difference between yeast recycled from prior fermentation in the presence of PEG-400 and that recycled from fermentation in the absence of PEG-400. The first recycled batch of yeast produced 62 $g.L^{-1}$ ethanol as compared to 150 $g.L^{-1}$ produced by fresh yeast in pure water system (Figure 7b). In comparison, in the system with PEG-400, the first recycled batch of yeast produced 126 $g.L^{-1}$ ethanol, which is more than doubled that produced from recycled yeast in pure water. The results of additional recycled runs further confirm the beneficial effect of PEG-400 exo-protection for the yeast cells. It was noted that the yeast cells recycled from the PEG-400-free batches ceased to produce ethanol in the 4th reuse. Comparing the total amount of ethanol produced in the five runs, the fermentation operated in the presence of PEG-400 produced 7.16 g of ethanol, equivalent to 0.90×10^{-9} g of ethanol per yeast cell, while that in the absence of PEG-400 produced 3.88 g of ethanol, equivalent to 0.48×10^{-9} g of ethanol per yeast cell. We also measured the yeast cell viability after each recycled use (Figure 7c). Overall, the yeast cell viability decreased in the first two recoveries, and then became unchanged. It is remarkable that the yeast cell viability in the presence of PEG-400 was much higher than that in the absence of PEG-400, supporting the hypothesis that PEG-400 improved cell viability. However, the yeast cell viability in cycle 3 and cycle 4 (Figure 7c) did not appear to correlate with the corresponding fermentation performances as shown in Figure 7a. This observation could be explained by decreased cell vitality with the increasing cycle times. To verify this hypothesis, the recovered cells were cultured in YPD broth, and the results are shown in Figure 7d. It became evident that cell growth in cycle 4 was slower than that in cycle 2 and cycle 3, and that cell in the cycle 3 and cycle 4 started to die rapidly after 24h. The results imply that the cell vitality decreased gradually with cell aging during the multiple cycles

Processes for PEG and H_2SO_4 recycle and reuse

Even though PEG-400 is a low cost surfactant, it is still economically meaningful to recycle and reuse this surfactant. The recovery of PEG-400 and H_2SO_4 is integrated as a natural step of ethanol separation by distillation. The PEG-400 and H_2SO_4 were readily recovered from the residual mother liquor of the distillation. By following the process as shown in Figure 1, we have verified that PEG-400 and H_2SO_4 were recoverable and remained effective without additional makeup. The results of recycled PEG-400 and H_2SO_4 in subsequent fermentation cycles are shown in Figure 8. There was little change in glucose conversion, ethanol yield and concentration from the four repeated uses of the initially loaded PEG-400 and H_2SO_4.

Integrated recycle process for yeast, PEG and H_2SO_4

We further studied an integrated recycle process for yeast, PEG-400 and H_2SO_4. In the experiments, fresh yeast cells were supplemented in late cycles to complete glucose-to-ethanol conversion. The yeast cells were recovered by centrifugation, and then PEG and H_2SO_4 were recycled by reusing the fermentation broth after distillation. The living cells and PEG-400 were reused in subsequent cycles of the fermentation process. Fresh yeast cells were supplemented in cycles 2-4 with the amount pre-determined based on the death rate of cells (40%, 60%, 65%, respectivily) from the results in Figure 9. The results obtained in recyled use of spent yeast with supplement yeast, and of PEG-400 and H_2SO_4 are shown in Figure 9. Comparing the total amount of ethanol produced in the four runs, the fermentation operated in an integrated recycle process for yeast and PEG produced 10.37g of ethanol, equivalent to 0.86×10^{-9} g of ethanol per yeast cell while that in process for reuse of recovered yeast

produced 7.16 g of ethanol, equivalent to 0.90×10^{-9} g of ethanol per yeast cell. The results not only verify the superior productivity of PEG-400 exo-protected yeast cells in cycles 2-4, but also demonstate that the spent yeast can be reused with the supplementation of a much reduced amount of fresh yeast cells. In combination with the recycled use of PEG-400 and H_2SO_4, only supplemental amount of yeast is needed to carry out the subsequent fermentations at the full efficiency due to the presnce of PEG-400.

The kinetics of ethanol production in the absence and presence of PEG-400

Figure10 shows the product ethanol profiles during VHG batch fermentation in pure water and in the presence of PEG-400. The experimental data of variation in specific ethanol productivity on glucose in the absence and presence of PEG-400 were obtained. The experimental data were also fitted to estimate the kinetics parameters $v_{max}$and P_{max}.[31] Only 162 g.L[1] of P_{max} was obtained in pure water. In comparison, the addition of PEG-400 led the P_{max} to reach 196 $g.L^{-1}$which is close to the theoretical maximum of 203 $g.L^{-1}$; the ν_{max} of 8.77 $g.L^{-1}.h^{-1}$ in pure water is higher than the ν_{max} of 5.66 $g\cdot L^{-1}\cdot h^{-1}$ with PEG-400.

Conclusions

In summary, our results demonstrate that fully water-soluble PEGs are effective for the vitalization of yeast cells in VHG fermentation for the production of ethanol in very high concentration. The final ethanol concentration was increased by over 10% with fresh yeast and no residue sugar remained in the fermentation broth in the presence of PEG-400 supplement. Very importantly, the yeast cells exhibited substantially extended viability for reuse, and the PEG and H_2SO_4 are readily recoverable for multiple subsequent recycled use. It is also deomosntrated that the spent yeast with highly maintained viability due to the presence of PEG-400 can be reused in multiple cycles only by supplementing a much reduced amount of fresh yeast cells. The combination of recycled use of spent yeast, PEG-400 and H_2SO_4, allows the subsequent fermentations at the full efficiency due to the presnce of PEG-400. The results of the present study also imply that reduced water consumption and energy consumption can be achieved by using an optimized amount of the PEG. Largely enhanced ethanol productivity can be achieved by recycling the yeast, which can be expected to help reduce the overall cost. The results of this work further imply that the method of yeast cell exo-protection may be applicable beyond ethanol production; it may be used in fermentation processes to produce other types of renewable chemicals and bio-fuels.

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‡ Footnotes should appear here. These might include comments relevant to but not central to the matter under discussion, limited experimental and spectral data, and crystallographic data.

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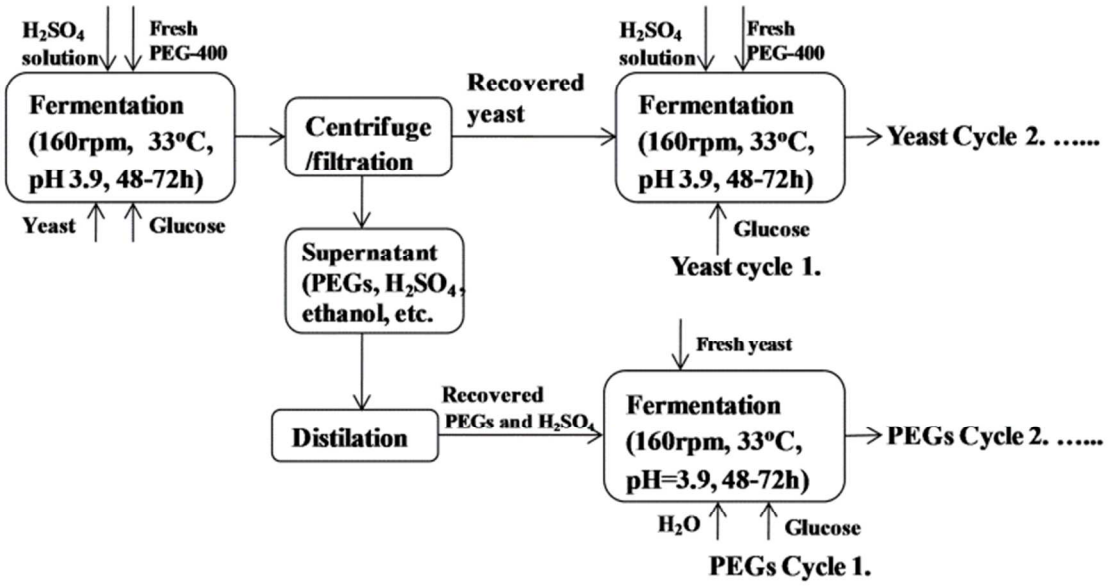

Fig. 1 Process scheme for recovery and reuse of yeast, PEGs and H_2SO_4. Experiments on combined reuse of yeast, PEGs and H_2SO_4 followed similar recovery procedure.

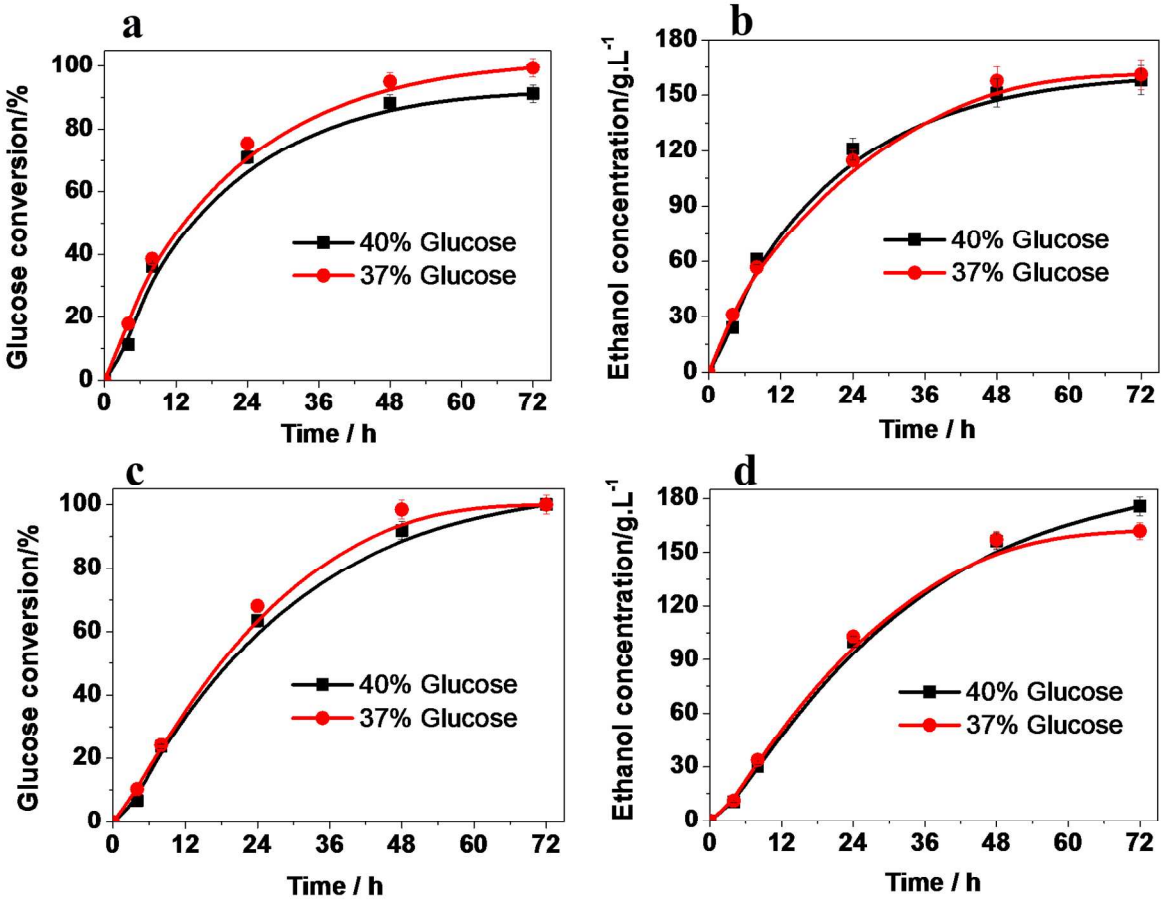

Fig. 2 Effect of initial glucose concentration on glucose conversion and ethanol concentration. **a,** Effect of initial glucose concentration on glucose conversion in pure water; **b,** Effect of initial glucose concentration on ethanol concentration in pure water; **c,** Effect of initial glucose concentration on glucose conversion in the presence of PEG-400; **d,** Effect of initial glucose concentration on ethanol concentration in the presence of PEG-400; Fermentation conditions: approximately 5×10^8 cells mL^{-1}, 8 mL water, 33 ^{o}C, 160 rpm, and pH of 3.9. 2.0 g of PEG-400 in Figures 2c and 2d. All data and error bars represent the averages and standard deviations of triplicate measurements.

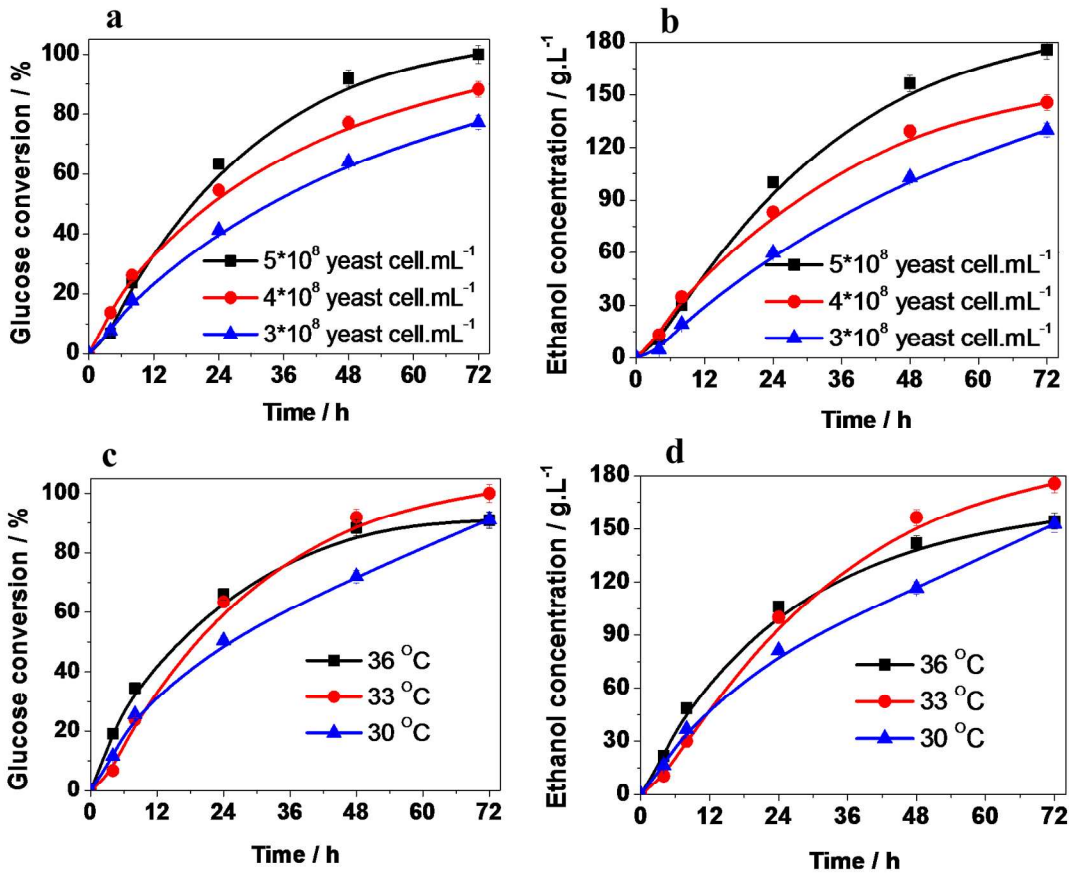

Fig. 3 Effect of yeast cell loading and temperature on glucose conversion and ethanol concentration. **a,** Effect of yeast cell concentration on glucose conversion. **b,** effect of yeast cell concentration on ethanol concentration. **c,** Effect of temperature on glucose conversion. **d,** effect of temperature on ethanol concentration. Fermentation conditions: 3.5 g glucose, 8 mL water, 2.0g of PEG-400, 160 rpm, and pH of 3.9. All data and error bars represent the averages and standard deviations of triplicate measurements.

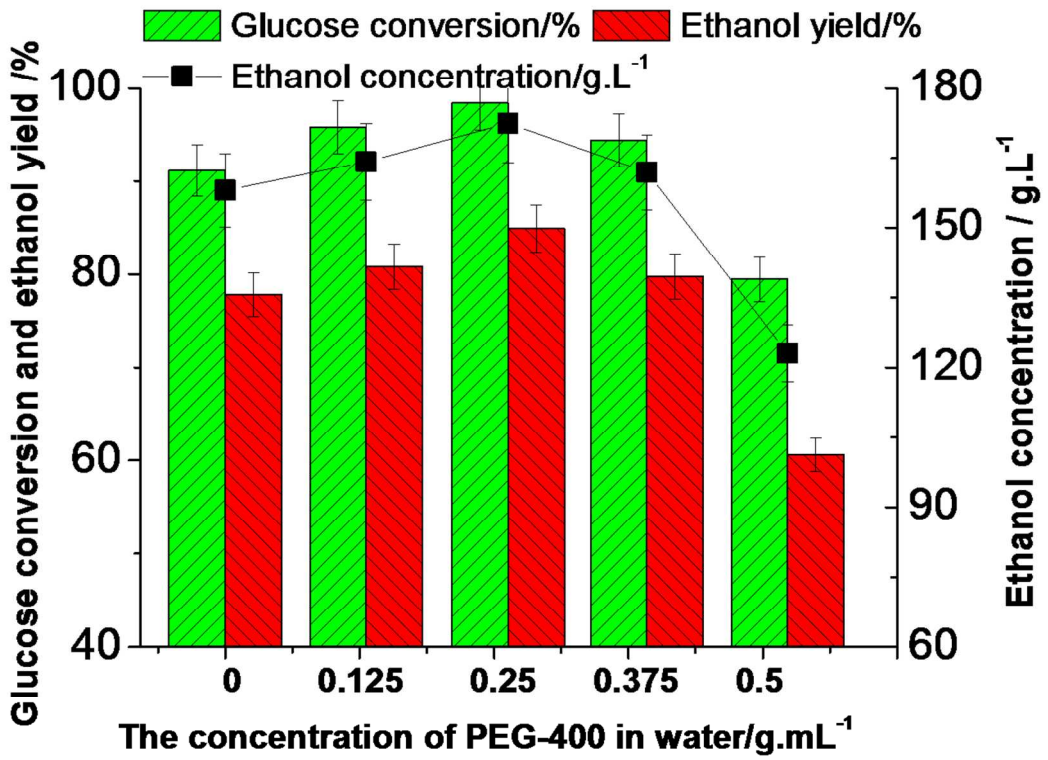

Fig. 4 Effect of PEG-400 concentration in water. Fermentation conditions: 7.0 g glucose, approximately 5×10^8 cells mL^{-1}, 16 mL water, 33 °C, 160 rpm, and pH of 3.9. All data and error bars represent the averages and standard deviations of triplicate measurements.

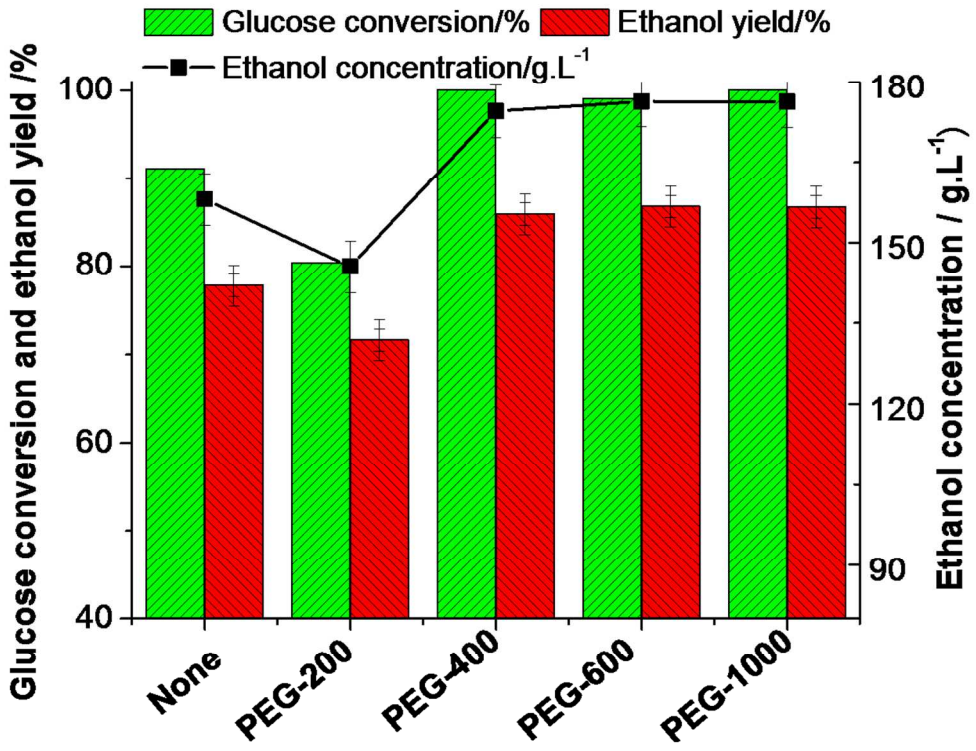

Fig.5 Effect of different PEGs on batch ethanol fermentation. Fermentation conditions: 7.0 g glucose, 4.0g of PEGs, approximately 5×10^8 cells mL^{-1}, 16 mL water, 33 ^{o}C, 160 rpm, and pH of 3.9. All data and error bars represent the averages and standard deviations of triplicate measurements.

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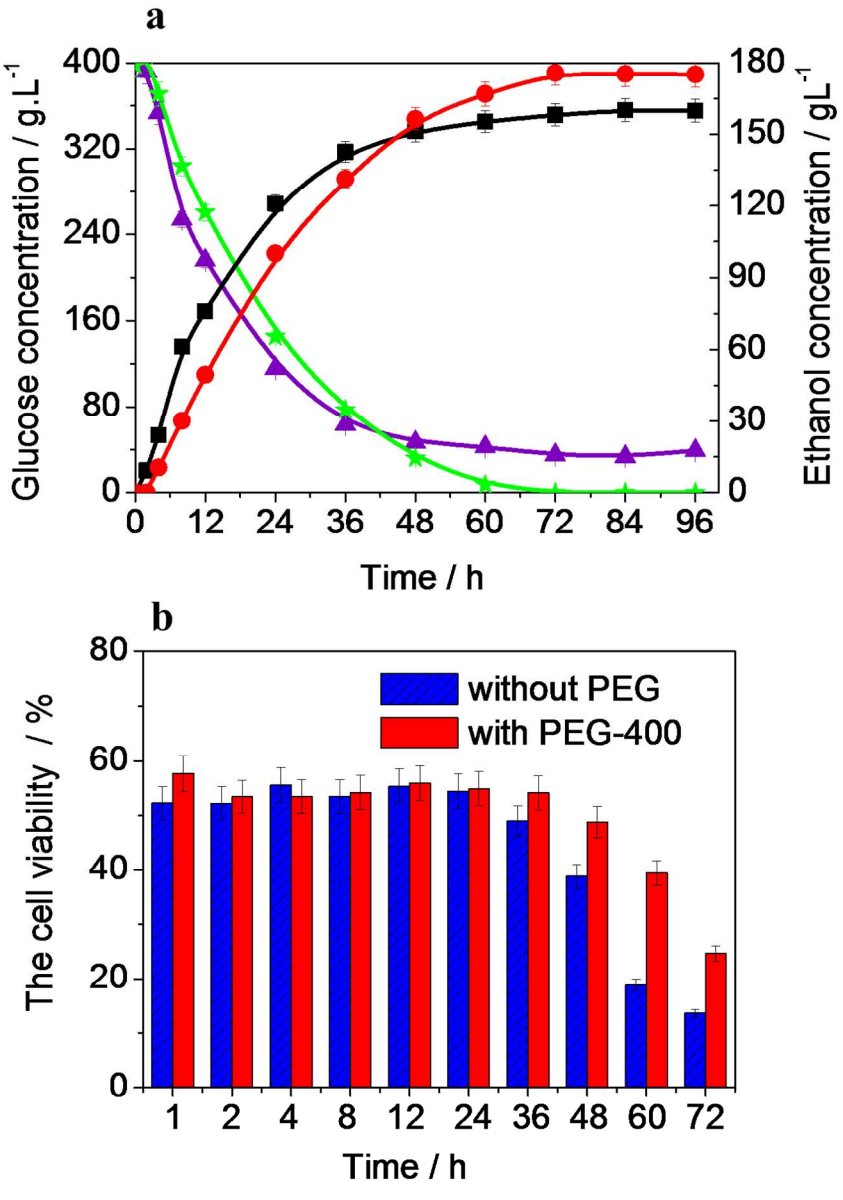

Fig. 6 Comparison of glucose fermentation at different time. a, Ethanol concentration and glucose consumption (expressed without monohydrate) with and without PEG-400; b, Yeast cell viability with and without PEG-400 from the VHG fermentation processes. Fermentation conditions: 3.5 g glucose as monohydrate, approximately 5×10^8 cells mL^{-1}, 8 mL water, 2.0 g of PEG-400, 33 °C, 160 rpm, and pH of 3.9. All data and error bars represent the averages and standard deviations of triplicate measurements.

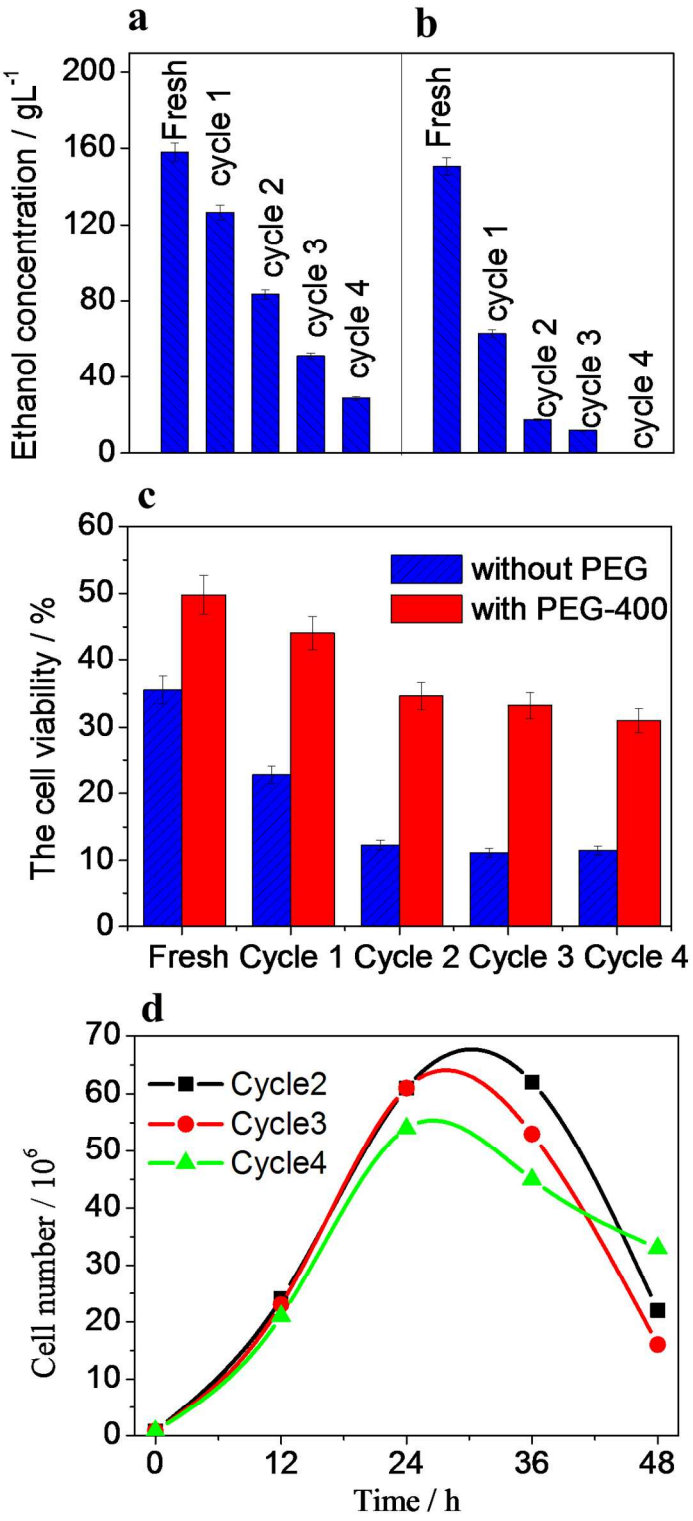

Fig. 7 The performance of recycled yeast from fermentation systems with and without PEG-400. **a,** The ethanol concentration over recovered yeast with PEG-400. **b**, The ethanol concentration over recovered yeast without PEG. **c**, Comparison of yeast cell viability with and without PEG-400. **d**, Comparison of yeast cell vitality with cycle 2, cycle 3 and cycle 4. Fermentation conditions: 6.5 g glucose, approximately 5×10^8 cells mL^{-1}, 16 mL H_2O, 4.0 g of PEG-400, 33 ^{o}C, 48 h, 160 rpm, and pH of 3.9. All data and error bars represent the averages and standard deviations of triplicate measurements.

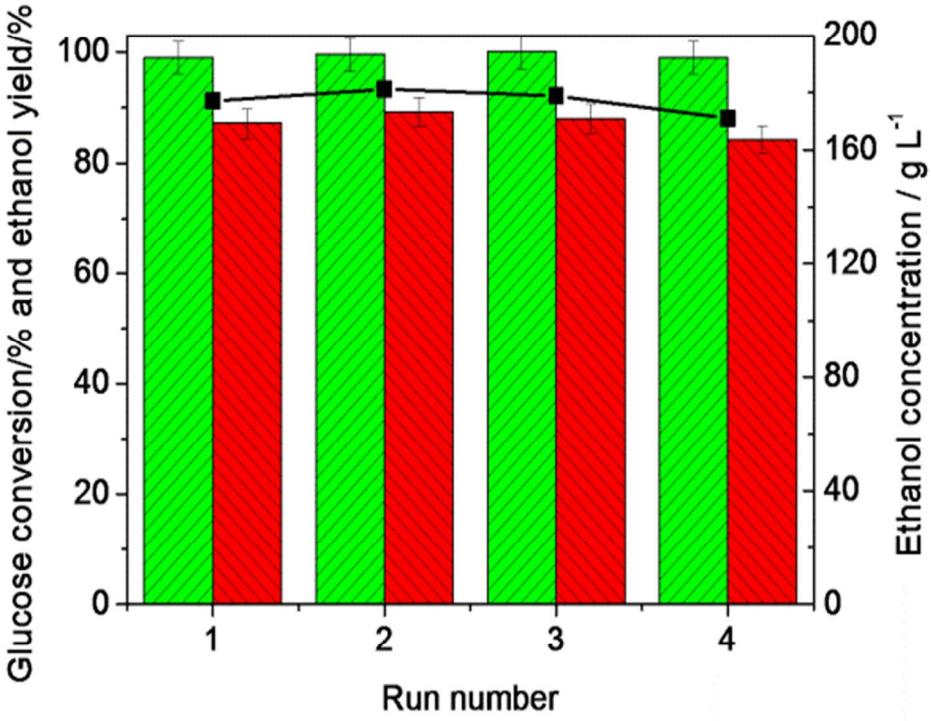

Fig. 8 Results of four successive fermentation runs by recovering PEG-400 together with H_2SO_4. Green bar, glucose conversion; red bar, ethanol yield; (■), ethanol concentration based on water; Fermentation conditions: 7.0g glucose, approximately 5×10^8 cell. mL^{-1}, 16mL water, 4.0g of PEG-400 , 72h, 33 °C, 160rpm, and pH of 3.9. All data and error bars represent the averages and standard deviations of triplicate measurements.

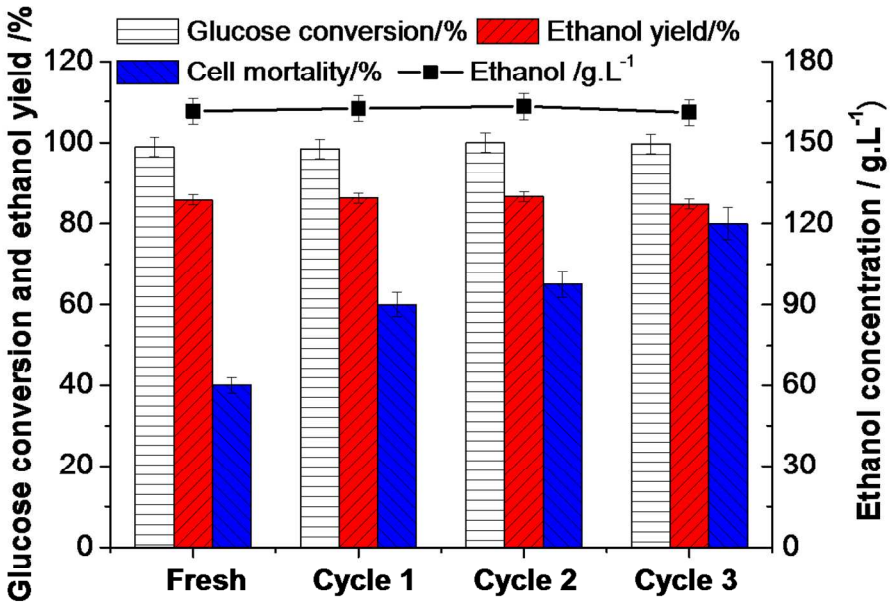

Fig. 9 Results of four successive fermentation runs by recovering yeast, PEG-400 together with H_2SO_4. Fermentation conditions: 6.5 g glucose, 16mL water, 4.0g of PEG-400, 48h, 33 °C, 160rpm, and pH of 3.9. All data and error bars represent the averages and standard deviations of triplicate measurements.

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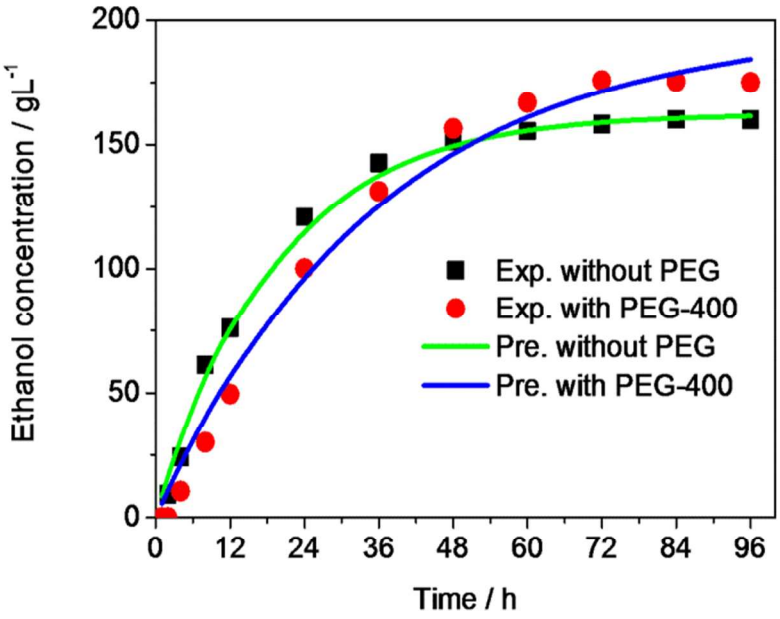

Fig. 10 Experimental and model profiles of ethanol production. Fermentation conditions: 3.5 g glucose, approximately 5×10^8 cells·mL^{-1}, 8 mL water, 2.0 g of PEG-400, 33 ^{o}C, 160 rpm, and pH of 3.9.