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An integrated bioprocess for fermentative production of protopanaxadiol by recycling ethanol waste during down-stream extraction process

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ABSTRACT:

Solvent recovery and reutilization in bioprocess have great potential to reduce production cost and environmental harm. However, solvent recovery process is often energy-intensive and costly. In this study, we developed an integrated bioprocess for fermentative production of protopanaxadiol (PPD) from ethanol waste recycled in down-stream extraction process, achieving solvent recovery and reutilization in a cost-effective manner. In this integrated process, PPD isolation and purification were achieved by foam separation and resin chromatography, respectively; ethanol solution used as PPD extractant and chromatography eluent was recycled to be reused directly as yeast carbon source for PPD biosynthesis. Notably, in 3 batches of 5 L-fermentation productions, the recycled ethanol could compensate 81.3% of the ethanol used in fermentation. Since the lost PPD during chromatography elution was returned into the next-batch fermentation together with the recycled ethanol, the overall yield of 3-batch production (85.78%) was higher than the yield of single batch production (~75%). This study demonstrates a promising integrated bioprocess for triterpene compounds production from ethanol waste.

1. Introduction

Protopanaxadiol (PPD), a major bioactive component of *Panax ginseng*, is regarded as promising high-value pharmaceutical compound for tumor inhibition and depression treatment (Li et al., 2006; Popovich and Kitts, 2004; Hui et al., 2012b). Traditional PPD production relies on phytoextraction that is followed by hydrolysis or enzymolysis, and is a labor-intensive and highly polluting process. Synthetic biology technology provides an alternative method for producing PPD in engineered *Saccharomyces cerevisiae* (*S. cerevisiae*) (Xu et al., 2013). In recent years, considerable progress has been achieved for engineering yeast for high production of PPD (Dai et al., 2013; Zhao et al., 2016, 2017). However, the down-stream bioprocess of PPD isolation and purification has not yet been studied.

The down-stream bioprocess is known to be relatively solvent intensive. It has been reported that for the production of 1 kg of commercially available active pharmaceutical ingredients a median value of 45 kg of material needs to be used, of which about 50% has been organic solvents (Henderson et al., 2007). Recovery of waste solvent through crystallization or membrane separation can offer significant benefits with regards to reduced storage and waste costs as well as increased compliance with environmental legislation. However, these recovery processes are highly energy-intensive and costly (Constable et al., 2007). Therefore, it is of great interest to find a new way to reutilize the wasted organic solvent to be recycled during down-stream bioprocess. PPD is a triterpene saponin which can be dissolved in many organic solvents such as methanol, ethanol, ethyl acetate, acetone et al. Among these solvents, ethanol is a widely used solvent for saponins or sapogenins extraction and chromatography eluent (Kwon et al., 2003). In addition, ethanol can also be used as carbon source for *S. cerevisiae* growth (Keulers et al., 1996). In a previous report, ethanol feeding strategy improved amorpha-4,11-diene (sesquiterpene) production to 41 g/L, which showed huge potential for terpene production by *S. cerevisiae* from

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ethanol (Westfall et al., 2012). The multiple application of ethanol inspirits us to recycle the solvent in downstream process and reutilize it as feeding carbon source, thereby decreasing the production cost.

Another problem in PPD production arises due to hard control of foam formation during fermentation. Almost all of the PPD produced by *S. cerevisiae* secreted to outside space of cells and they caused the formation of abundant foam. The foam cannot be eliminated even after adding high dose of antifoaming agent. In addition, PPD mainly exists in foam, if foam is not removed timely, PPD attends to adhere to the surface of stainless pipe and the inner tank wall (Zhao et al., 2017), which complicates the extraction process. Two phase extraction fermentation was an effective method for in situ PPD removal (Dai et al., 2013), however, extraction fermentation also facilitated dammarenediol II (DMD, precursor of PPD) secretion, which markedly decreased PPD yield (Zhao et al., 2016). Fortunately, foam is not always a headache in fermentation process, it is also low-cost medium for separation of biological molecules such as cells (Parthasarathy et al., 1988), proteins (Aksay and Mazza, 2007; Jiang et al., 2011), DNA (Lalchev et al., 1982), and small molecular compounds (Yan et al., 2012; Zhang et al., 2011). The continuous foam generated along with PPD production could be harnessed as PPD transferring medium to facilitate its isolation.

In this study, to develop an efficient and cost-effective method for organic solvent reutilization and to simplify the product isolation, an integrated bioprocess for PPD production was designed. This integrated bioprocess includes ethanol feeding fermentation, foam separation, ethanol extraction and macroporous resin purification (Fig. 1). In overall, PPD was isolated from fermenter by foam separation. Then PPD was extracted by ethanol solution and it was enriched on macroporous resin. The enriched PPD on resin was washed with ethanol solution, and the solvent was recycled and used directly as yeast carbon source in next batch fermentation. Because the lost PPD during chromatography elution gets returned into the fermenter together with recycled ethanol, this process is expected to improve the overall yield of PPD. This study demonstrates an attractive integrated bioprocess for PPD production, and this novel process can also be used for other terpene compounds production.

2. Materials and methods

2.1. Overall design of the integrated bioprocess for PPD production

The integrated bioprocess for PPD production comprises fed-batch fermentation, foam separation, ethanol solution extraction and macroporous resin chromatography technology. As shown in Fig. 1, tank A serves as the bioreactor for yeast fermentation and PPD production. PPD isolation from tank A to tank B was achieved by foam separation. Besides PPD, some yeast cells and broth also get transferred along with the foam. In tank B, the PPD attend to adhere on inner wall, and the yeast cells and broth accumulate in bottom. These cells and broth will be pumped back into tank A by pump 1. PPD in tank B was extracted using ethanol solution. Undissolved substance in extract solution was filtrated and discarded. PPD extract solution flows past the macroporous resin in chromatographic column for PPD enrichment. Afterwards, the PPD on resin will be washed by 90% ethanol (v/v). We can get PPD product by distillation, and ethanol solution will be condensed by a rotary evaporator. The elution effluent (containing ethanol) and condensed ethanol will be recycled and reutilized as fermentation carbon source in next batch production.

2.2. PPD fermentation in 5 L fermenter using ethanol as carbon source

Strain W3a-ssPy (Zhao et al., 2017) was used for PPD biosynthesis in this study. Yeast uses glucose and ethanol as carbon source. The growth rate of yeast under ethanol is lower than under

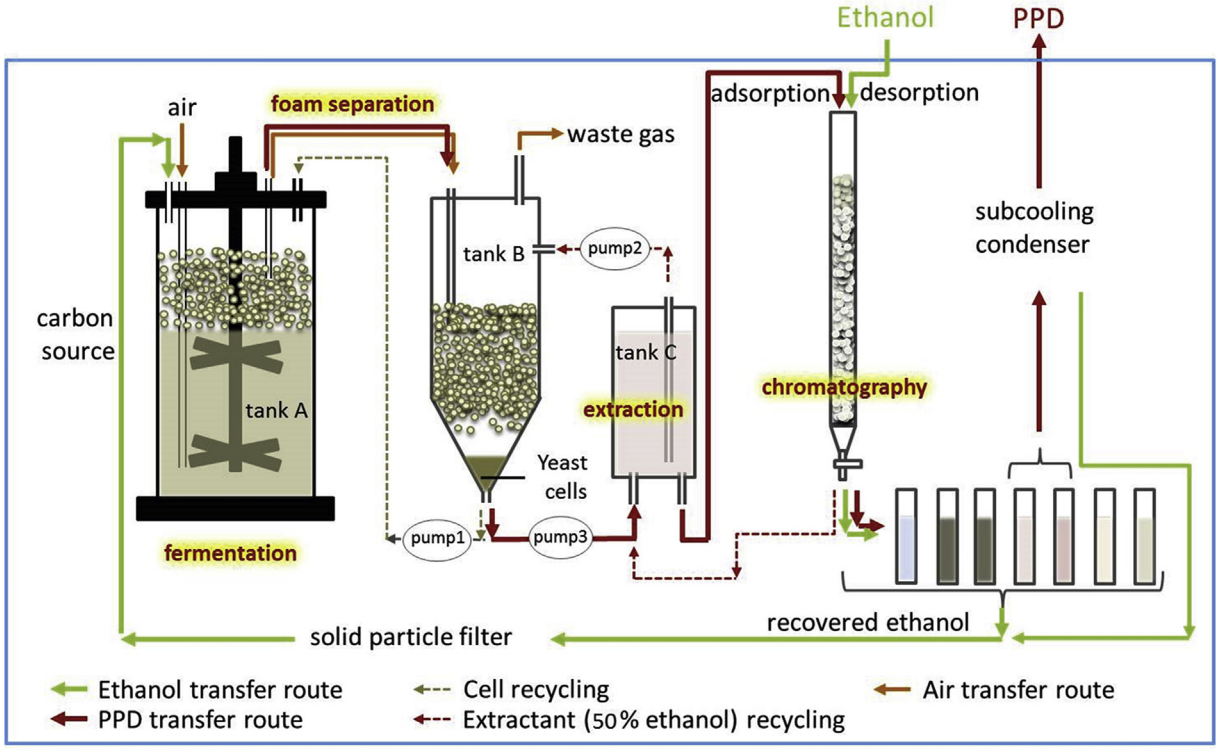

Fig. 1. Overall design of the integrated bioprocess for PPD production.

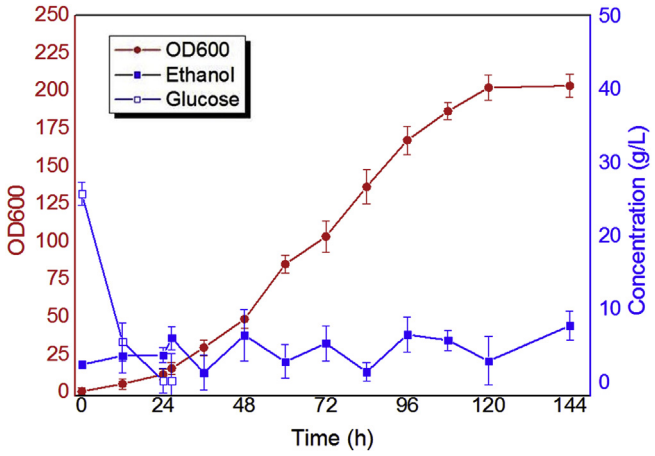

Fig. 2. Fermentation curve of ethanol fed fermentation.

glucose (Zampar et al., 2013). To shorten lag-phase time, YPD medium (glucose 25 g/L, yeast extract 10 g/L and peptone 20 g/L) was used as seed medium and initial medium for fermentation. 2 L YPD medium (initial working volume) was added into 5 L fermenter (BLBIO-5GL, ShangHai Bailun Bio-Technology CO., LTD, China). After ethanol feeding fermentation, the final volume of broth was 3 L.

200 mL seed solution (incubated at 30 °C for 18 h) was added into the fermenter. Fermentation was performed at 30 °C. PPD biosynthesis requires oxygen as substrate, so the dissolved oxygen during fermentation process was controlled higher than 40%. The pH was controlled at 5.5 by automatic addition of 5 M ammonia hydroxide. At 26 h, glucose and ethanol was depleted, 500 mL solution (pH 5.5) containing 9 g/L KH_2PO_4, 5.12 g/L $MgSO_4 \bullet 7H_2O$, 3.5 g/L K_2SO_4, 0.28 g/L Na_2SO_4, 0.5 g/L adenine, 0.6 g/L uracil, 1.2 g/L lysine monohydrochloride, 10 mL/L trace elements solution and 12 mL/L vitamin solution were added to the fermenter. After 26 h, ethanol solution was added repeatedly to the tank A and ethanol concentration was controlled at a range of 1–6 g/L. After fermentation, a total of 540.0 ± 25.6 mL ethanol (100%, v/v) was consumed by yeast.

2.3. Optimization of fermentation ventilation rate

The fermentation ventilation rate was optimized to ensure high-efficiency transfer of PPD from tank A to tank B. The initial ventilation rate was set at 2–3 L/min·L to meet the demand of strain growth. From 12 h, PPD began to produce and the foam was formed largely. From then on, the ventilation rates were regulated, and four different ventilation modes were tested (Fig. 3).

The foam separation efficiency (FEE) can be calculated as follow:

$$FEE = PPD_{tankB}/(PPD_{tankB} + PPD_{tankA}) \times 100\% \quad (1)$$

Where PPD_{tankA} and PPD_{tankB} were the PPD collected in tank A and B respectively after fermentation. PPD_{tankA} comprises intracellular and extracellular PPD in tank A. The method for extraction of intracellular and extracellular PPD was presented as Zhao (Zhao et al., 2017) has described.

2.4. Macroporous resin adsorption and elution

PPD is a hydrophobic compound. Several nonpolar or weak-polar resins were tested, and Diaion HP20 was selected based on its excellent PPD adsorption and desorption performance (Table SI).

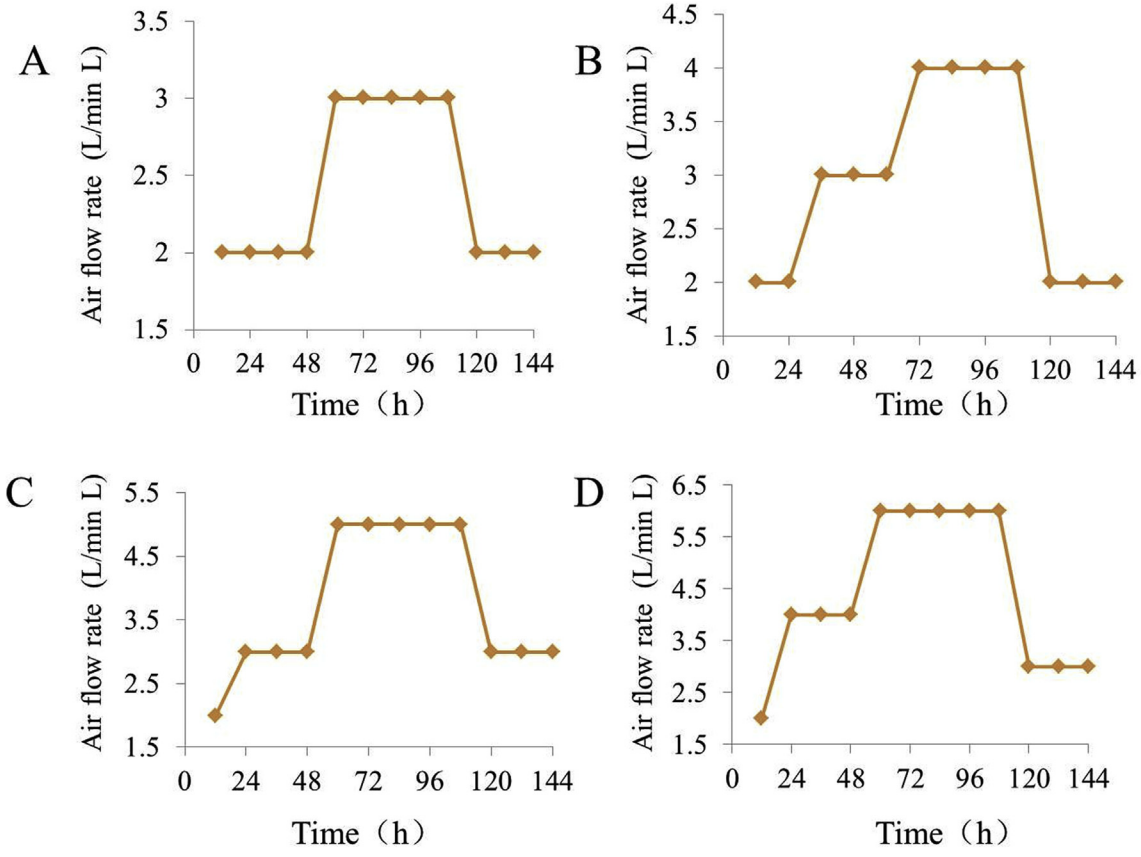

Fig. 3. Four ventilation models for optimization of fermentation ventilation rate.

Before adsorption experiment, the Diaion HP20 was equilibrated with 50% ethanol. 50% ethanol solution (PPD concentration: ~500 mg/L) was filtered by 0.45 μm nylon membrane and was pumped into the 1.5 × 30 cm chromatographic column filled with 20 mL preprocessed resin. The flow velocity was set constantly at 2, 3, 4 and 6 BV/h, respectively. And the effluent was pumped back to tank C as PPD extract solution. The resin was reactivated after 3-round adsorption and elution.

For chromatography elution, we used 2 BV deionized water (1 BV/h) to wash the pigment and other impurities. Then 90% ethanol (v/v) was added at a velocity of 1, 2 and 3 BV/h, respectively to wash the PPD down. PPD solution was collected every 5 mL and the PPD concentration was measured by HPLC.

PPD product was obtained by crystallization. The eluent containing PPD was joined together and condensed by a rotary evaporator (RE-2000A, Yarong, Shanghai, China) at 50 °C. Distilled ethanol was collected, and the overall yield of ethanol during chromatographic purification can be calculated as follow:

$$Y_{ethanol} = \frac{Ethanol_{recovered}}{Ethanol_{used}} \times 100\% \quad (2)$$

2.5. Recovery yield calculation

In fermentation, PPD product yield (SPY) can be calculated as:

$$SPY = \frac{PPD_{pro}}{PPD_{tot}} \times 100\% \quad (3)$$

where PPD_{pro} means that the PPD product obtained after crystallization and PPD_{tot} is the total PPD production by yeast cells during fermentation.

2.6. OD600, ethanol and glucose measurement

Quantification of OD600 was performed using a UV–Vis spectrophotometer (Oppler, 752 N, China). For measurement of glucose and ethanol, 2 mL broth was collected during fermentation process at different time points. Yeast cells in broth were precipitated by centrifugation and the supernatant was used for glucose and ethanol measurement with a semi-automatic bioanalyzer (SBA-40C, Shandong academy of sciences, China). If concentrations were above the maximum assay range, samples were diluted with PBS buffer.

2.7. PPD extraction by different ethanol solutions

After fermentation, the faint yellow solid was collected and it was freeze-dried in a vacuum freeze drier at −40 °C. Then 2.5 g dried powder (Fig. S1A) was added into 25 mL 40%, 50%, 60%, 70%, 80%, 90% and 100% ethanol solutions, respectively. Then the mixtures were subjected to ultrasonic treatment for 1 h and the insoluble substance was discarded after centrifugation. After that, 1 mL supernatant was used for PPD quantitative analysis by HPLC. And 20 mL supernatant was transferred into a clean centrifuge tube and dried at 80 °C for 24 h. These extracts were dried and weighted. The PPD purity in these extracts can be calculated as:

$$PPD\ \ purity = \frac{20\ (\mathrm{mL}) \times PPD_{\mathrm{concentration}}(\mathrm{mg/mL})}{\text{extract dry weight}\ (\mathrm{mg})} \times 100\% \quad (4)$$

2.8. PPD product purity analysis

PPD standards were purchased from Sigma Aldrich. It was used to make standard curve (PPD concentration versus HPLC peak area). To determine PPD product purity, 1000 mg PPD product was completely dissolved in 1 L ethanol. Then the PPD concentration was calculated by measuring its peak area. The PPD product purity can be calculated as:

$$PPD\ \ product\ \ purity = \frac{1000\ (\mathrm{mL}) \times PPD_{\mathrm{concentration}}(\mathrm{mg/mL})}{1000\ (\mathrm{mg})} \times 100\% \quad (5)$$

2.9. HPLC and LC-MS analysis

The intracellular and extracellular PPD and DMD in tank A were extracted as Zhao (Zhao et al., 2017) has described. Before HPLC analysis, the extract solutions were filtrated by 0.22 μm membrane. For quantitative analysis, 20 μL extract were injected on an Elite P230II high-pressure pump system equipped with UV detection at 203 nm. Chromatographic separation was realized on SinoChrom ODS-BP column (4.6 mm × 250 mm, 5 μm; Elite Analytical Instruments Co., Ltd., Dalian, China). Methanol-acetonitrile (4:6, v/v) was used as mobile phase and the flow velocity was 1 mL/min.

LC-MS analysis was performed as Zhao has described (Zhao et al., 2016).

3. Results and discussion

3.1. Fermentation process using ethanol as feeding carbon source

Previously, we used glucose as carbon source for PPD production (Zhao et al., 2017). In this study, to construct an integrated bioprocess using ethanol as PPD extractant, chromatography eluent and yeast carbon source, the 5 L scale fermentation (3 L working volume in 5 L bioreactor) was tried using ethanol as the single feeding carbon source. YPD was used as initial medium, and ethanol was fed into the fermenter from 26 h when glucose was depleted. Since high level of ethanol in broth will be toxic to yeast cells, ethanol concentration was controlled within range of 1–6 g/L. After fermentation, a total of 540.0 ± 25.6 mL ethanol (100%, v/v) was consumed by yeast. OD600 increased from 12.8 at 26 h to 204.5 ± 8.9 at 144 h (Fig. 2). During fermentation, most of the intracellular PPD secreted from yeast cells, and large amount of foam was generated. The PPD attached to the side of fermenter as yellowish solid. After fermentation, 12.35 ± 0.87 g PPD was collected from 3 L broth and the inner tank wall. The PPD yield on ethanol was ~2.9 mmol/mol. The production (~4 g/L) was about the same with the glucose-feed fermentation (Zhao et al., 2017). This result indicated that ethanol could be used as single carbon source for PPD production.

3.2. Optimization of PPD foam separation

To construct this integrated bioprocess, one of the key points is to remove the produced PPD from the broth in a timely manner. Here, foam separation was optimized to improve the high-efficiency of PPD isolation. Foaming ability plays a key role in foam separation. Several factors such as pH, temperature, ionic strength and ventilation rate may affect the foaming ability (Akintayo et al., 1999; Bera and Mukherjee, 1989; Temelli, 1997).

Table 1
Results of the different ventilation rate tests.

	Model 1	Model 2	Model 3	Model 4
OD600	216.3 ± 6.4	224.5 ± 5.3	209.6 ± 8.9	218.3 ± 6.5
PPDtotal	12.43 ± 0.8 g	11.56 ± 1.2 g	11.89 ± 1.3 g	12.31 ± 0.6 g
PPDtransfer	9.34 ± 0.31 g	9.74 ± 0.22 g	11.48 ± 0.58 g	11.97 ± 0.74 g
FEE[a]	75.14 ± 0.31%	84.2 ± 0.32%	96.5 ± 0.24%	97.2 ± 0.33%
Ethanol volume[b] (mL, 100% v/v)	545.4 ± 6.5	580.4 ± 8.3	682.3 ± 5.6	745.5 ± 4.5

[a] Foam separation efficiency (FEE) can be calculated as ***Function 1***.
[b] Ethanol consummation in four models test.

Table 2
Results of PPD extraction experiment.

Ethanol solution	PPD solubility (30 °C, mg/L)	PPD purity[a] (%)	Resin adsorption capacity (mg/mL)	PPD desorption capacity (%)
40%	235.6 ± 21.6	80.4 ± 1.2	78.6 ± 4.5	1.2 ± 0.6
50%	725.9 ± 20.2	80.2 ± 1.4	66.3 ± 2.2	2.3 ± 2.5
60%	2759.8 ± 103.2	80.6 ± 1.5	32.3 ± 2.5	15.2 ± 3.4
70%	7550.3 ± 201.6	78.3 ± 1.3	11.6 ± 1.2	63.5 ± 3.5
80%	_	72.2 ± 1.8	_	88.3 ± 1.2
90%	_	70.9 ± 1.5	_	92.6 ± 3.7
100%	_	65.6 ± 2.7	_	92.2 ± 2.5

PPD solubility in 30% ethanol solution is only 1–2 mg/L, the detail date on lower ethanol concentration is not shown.
[a] PPD purity can be calculated as **Function 4**.

However, changes of the pH, temperature, and ionic strength conditions will affect the normal growth of the yeast cells. Since ventilation rate is easy to control, it was selected as the key option for optimization. Under the same conditions for strain culture and ethanol feeding, 4 different ventilation modes were tested (Fig. 3). OD600 and total PPD productions of these 4 batches were almost not affected (Table 1). The results also indicated that, when air flow rate was set at 5 and 6 L/min·L (from 48 h to 120 h), transfer efficiency was 96.5 ± 0.24% and 97.2 ± 0.33% respectively (Table 1). Nevertheless, ethanol can also be released along with the exhaust gas. Under the 4 ventilation modes, ethanol consumption used as feeding carbon source were 545.4 ± 6.5 mL, 580.4 ± 8.3 mL, 682.3 ± 5.6 mL and 745.5 ± 4.5 mL, respectively (Table 1). This indicated that with the increase of air flow rate, more ethanol was volatilized and wasted. Since transfer efficiency of mode 3 is comparable with mode 4 and ethanol consumption of mode 3 is less than mode 4, therefore, ventilation mode 3 (Fig. 3C) was adopted for further experiment.

3.3. PPD extraction in tank B

Ethanol is commonly used solvent in saponins and sapogenins extraction and chromatographic separation. It can also be used for PPD extraction. The solubility of PPD in different ethanol aqueous solution was measured in this study and the results can be found in Table 2. After foam separation, PPD was existed in faint yellow solid (contained 56.8% of PPD) on the inner wall of tank B. The PPD in faint yellow solid can be completely dissolved in ethanol, while some impurities cannot be dissolved in ethanol. We also used 40%–100% (v/v) ethanol solutions as extraction for PPD extraction. The insoluble substance can be precipitate by centrifugation to improve PPD purity. Table 2 showed that, improving water ratio in extraction reagent decreased proportion of the impurities. While improving water ratio also decreased PPD solubility, which leaded to reduce conveyor loading of extraction reagent. To choose an appropriate ethanol concentration, another factor need to be considered was the adsorption efficiency of PPD on macroporous resin. So we also compared the PPD adsorption efficiency using different ethanol solutions as loading solutions. Results in Table 2 showed that the adsorption efficiency decreased as the ethanol concentration rose from 40% (v/v) to 100% (v/v). PPD purities in 40%–60% ethanol solutions are the same, while the PPD solubility in 40% ethanol solution and resin absorption capacity in 60% ethanol solution are lower than those of 50% ethanol solutions

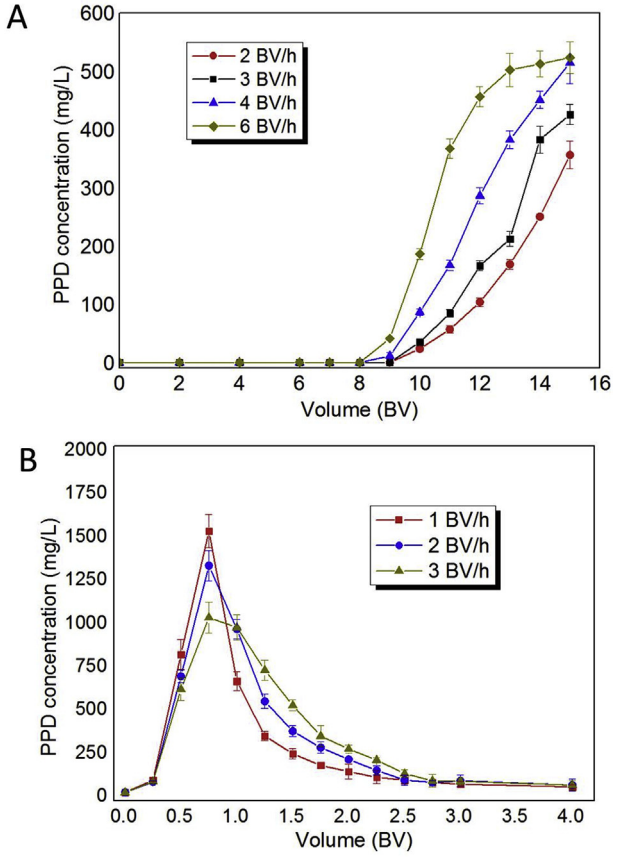

Fig. 4. Results of the PPD dynamic adsorption and desorption experiment. (A) PPD adsorption at various eluent flow velocity. (B) The eluting curve of PPD at different eluent flow velocity.

Table 3
Volume of ethanol consumption in fermentations and recovered during 3 batch productions. During fermentation, the PPD adhered on tank B was extracted by ethanol solution at 60 h, 96 h, and 144 h, respectively. In actual operating stage, ethanol solution was recovered and reused as carbon source. To be convenient for analysis, the volume presented here was standardized as absolute ethanol volume (unit, mL).

	Batch 1			Batch 2			Batch 3		
	60 h	90 h	144 h	60 h	90 h	144 h	60 h	90 h	144 h
Ethanol used as resin eluent	150.3 ± 8.9	162.8 ± 11.2	206.9 ± 7.9	164.2 ± 14.3	176.5 ± 10.5	193.6 ± 13.2	143.1 ± 7.7	155.8 ± 10.2	189.6 ± 16.9
Recovered ethanol from resin elution	114.6 ± 12.3	123.5 ± 9.3	152.3 ± 8.1	125.5 ± 18.9	137.6 ± 12.4	156.8 ± 14.7	125.3 ± 21.9	139.3 ± 15.8	163.5 ± 18.1
Ethanol used for resin regeneration	160			160			160		
Recovered ethanol from resin regeneration	142.8 ± 12.9			137.7 ± 8.9			138.2 ± 16.4		
Total recovered ethanol	543.2 ± 22.1			557.6 ± 26.9			566.3 ± 12.8		
Total ethanol used in fermentation	686.6 ± 32.2			668.0 ± 24.7			696.3 ± 28.3		

(Table 2), therefore 50% ethanol solution was selected as PPD extraction reagent. In addition, it was reported that, PPD can change molecular configuration in acid condition (Chen et al., 2003), so the pH of extraction reagent and loading solutions was adjusted to 8.0 by ammonium hydroxide before used.

3.4. Resin-based column chromatography

Macroporous resin is an excellent adsorption material for saponins or sapogenin separation, and the resin chromatography technology is also easy for industrial scaling-up. So macroporous resin was selected as the PPD adsorption material. However, as far as we know, no study has been reported using macroporous resin for PPD isolation. Therefore, PPD adsorption and desorption processes were optimized in this study.

The pH value of sample solution is said to be very important for resin adsorption properties (Jia and Lu, 2008). However, pH change did not show obvious influence on PPD adsorption capacity on HP20 resin (Table SII), so the pH was kept at 8 to prevent formation of PPD isomer. For dynamic adsorption, the liquid flow rate affected the interaction between solute and resin and thus further affected the adsorption capacity (Jia and Lu, 2008). Fig. 4A showed that the PPD adsorption capacity stayed at a high level when liquid flow rate was set below 3 BV/h. Therefore, 3 BV/h was selected as the optimal flow speed. Under this condition, the processing volume of PPD solution was approximately 10 BV, and the absorption capacity was 61.6 mg/mL. For desorption operation, the pale yellow pigment can be washed by 2 BV of 30% ethanol solution at a flow rate of 2 BV/h (Fig. 4A). Then PPD was washed by 90% ethanol solution at rate of 1, 2 and 3 BV/h, respectively. PPD were totally desorbed in 2, 3 and 5

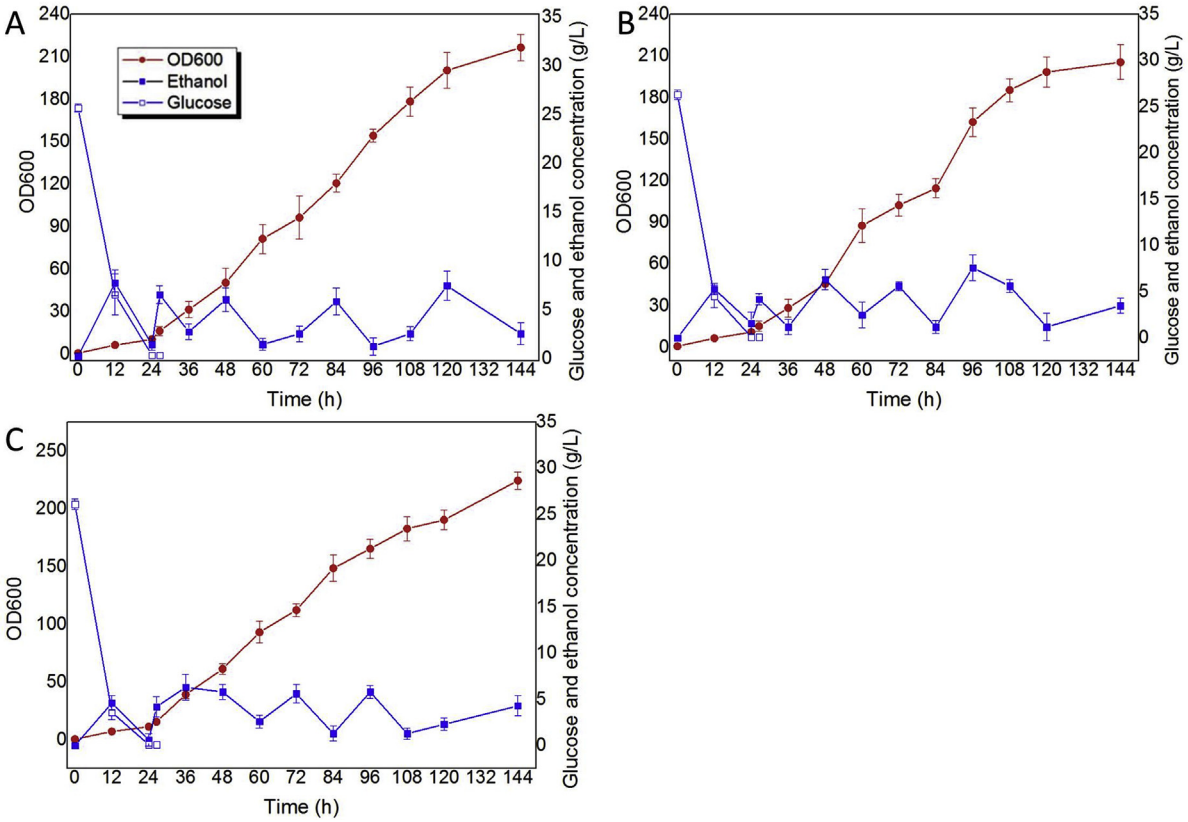

Fig. 5. Fermentation results of 3 batch productions.

Table 4
Results of 3-batch production.

	Batch 1	Batch 2	Batch 3
PPD_{pro} (g)	10.81 ± 1.12 g	12.58 ± 0.84 g	13.56 ± 1.30 g
Purity of PPD product	92.3 ± 1.2%	91.2 ± 2.2%	90.4 ± 2.6%
PPD_{tot} (g)	13.08 ± 0.81 g	12.96 ± 1.09 g	13.27 ± 1.40 g

BV ethanol solution (90%) at the flow rate of 1, 2 and 3 BV/h, respectively. This result indicated that the lower desorption flow rate made the more concentrated products. Thus, 1 BV/h was selected as the proper desorption flow rate with a desorption ratio of 90.5%.

PPD is stable under high temperature (Liang, 2011). After column chromatography, the purified PPD sample was subjected to a rotary evaporator at 80 °C, and PPD product was obtained at a yield of 88.2% with the purity of 91.5%. The ethanol solution used in PPD elution and resin regeneration was collected and the overall ethanol recovery efficiency was 86.3%.

3.5. The integrated bioprocess

In general, several factors make the integrated bioprocess possible: 1) extracellular secretion of the product PPD; 2) high-intensive foaming ability of the PPD; and 3) the multiple applications of ethanol as extractant, chromatography eluent and yeast carbon source. After finishing the optimization of unit processes, 3 batched-fermentations were conducted to verify the feasibility of the integrated bioprocess. For the first batched-fermentation (Batch 1), absolute ethanol was used as feeding carbon source. During fermentation, the PPD adhered on tank B was extracted by 50% ethanol solution at 60 h, 96 h, and 144 h, respectively. The extraction solution was filtered and flowed past to 80 mL pre-treated HP-20 resin. The ethanol used for PPD elution was recycled as yeast carbon source. For the second batched-fermentation (Batch 2) and the third batched-fermentation (Batch 3), recycled ethanol from Batch 1 and Batch 2 was used as fed carbon source, respectively.

Ethanol consumption for PPD elution and carbon source were listed in Table 3. Generally, all of the recycled ethanol from resin chromatography process could be used completely as carbon source. This means that, in a long-term production process, the integrated bioprocess will produce PPD without solvent discharge. In chemical and biological processes for bulk chemicals productions, raw materials have the major part in the total costs: 40%–60%. In 3 batched productions, the recycled ethanol (1667.1 mL) could compensate 81.3% of the ethanol used as carbon source in fermentation (2050.9 mL), which decreased 30%–50% of the raw material cost.

OD600 of the 3 batched fermentations reached about 210 at 144 h (Fig. 5), and the productions of 3-batched fermentation were 13.08 ± 0.81, 12.96 ± 1.09 and 13.27 ± 1.40 g, respectively (Table 4). This date was almost the same with the result in section 3.1 which indicated that the recycled ethanol solution could be used as carbon source without any negative effect on yeast growth and PPD production. During 3-batched fermentations, the lost PPD during chromatography elution was returned into the next batch together with recycled ethanol, therefore we got increased amount of PPD product (PPD_{pro}) in continuously batched fermentations (10.81 ± 1.12 g, 12.58 ± 0.84 g, and 13.56 ± 1.30 g PPD_{pro} for Batch 1, Batch 2 and Batch 3, respectively (Table 4)). The yield of Batch 1 production (YB1P) was 76.3%. The overall yield of 3 batched productions (Y3BP) was 85.78%, which was about 10% higher than YB1P.

After 3 batched productions, a total of 36.95 g PPD product was obtained. After chromatographic purification, the purity of the PPD product improved to about 91% (Table 4). However, because of the similar molecular structure, DMD could not be removed by resin purification and the ratio of PPD and DMD was almost not changed (Fig. S1 C and D).

4. Conclusion

Organic solvent recovery and reutilization in downstream bioprocessing is a huge challenge for cost-effective and eco-friendly bioprocess construction. Ethanol is a widely used solvent for many compounds, and fortunately, it was turned out to be an optimized carbon source for PPD production in *S. cerevisiae*. In this study, the integrated bioprocess not only removed the PPD timely by foam separation, but also coupled the fermentation process and purification process by ethanol reutilization. One of the major merits of this integrated bioprocess is the complete reutilization of recovered solvent, which can compensate 81.3% of the ethanol used in fermentation. Another commendable point of this integrated process is the high production yield in multi-batch production. The overall yield of 3 batched productions was 10% higher than the yield of single batch production (Batch 1), which verified the high-yield assumption of this integrated bioprocess. In recent years, synthetic biology technology rendered *S. cerevisiae* as one of the commonly used host for production of triterpene compounds, for example, saikoside (Moses et al., 2014), beta-amyrin (Liu et al., 2014), betulinic acid (Li and Zhang, 2014), ginsenoside CK (Yan et al., 2014) and Rh2 (Wang et al., 2015). Therefore, except PPD, this integrated bioprocess can also be appropriate for other triterpene compounds production.

Conflicts of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

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