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High-cell-density fermentation and pilot-scale biocatalytic studies of an engineered yeast expressing the heterologous glycoside hydrolase of 7-β-xylosyltaxanes

Wen-Bo Yu · Xiao Liang · Ping Zhu

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Abstract The glycoside hydrolase of 7-β-xylosyltaxanes (designated as LXYL-P1-2) is encoded by *Lxyl-p1-2* isolated from *Lentinula edodes*. This hydrolase specifically removes C-7 xylose from 7-β-xylosyltaxanes to form 7-β-hydroxyltaxanes, which can be used for the semi-synthesis of paclitaxel or its analogues. In our present study, we established a high-cell-density fermentation of the recombinant *Pichia pastoris* harboring the *Lxyl-p1-2* gene. Moreover, we further optimized the fermentation conditions, including the initial cell density and the dissolved oxygen level in the induction phase. Under optimized conditions, the biomass of 312.3 g/l (wet cell weight, WCW) was obtained, and the biomass activity of the recombinant enzyme reached 6.55×10^4 U/g (WCW). The freeze-dried cells (32 g/l) were used to convert 7-β-xylosyltaxanes (10 g/l, 7-β-xylosyl-10-deacetyltaxol = 62.12 %) in a 5-l reaction volume, and a bioconversion rate about 80 % was achieved. The product purification was performed by ethyl acetate, silica gel chromatography, and preparative HPLC (prep-HPLC), yielding 15.13 g of 10-deacetyltaxol, 3.07 g of 10-deacetylcephalomanine, and 3.47 g of 10-deacetyltaxol C, respectively. In addition, the average recovery rate was around 70 %. Our work provided a foundation for the industrial utilization of the recombinant enzyme on the semi-synthesis of paclitaxel using 7-β-xylosyltaxanes.

W.-B. Yu · X. Liang · P. Zhu (✉)
State Key Laboratory of Bioactive Substance and Function of Natural Medicines and Ministry of Health Key Laboratory of Biosynthesis of Natural Products, Institute of Materia Medica, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing 100050, People's Republic of China
e-mail: zhuping@imm.ac.cn

Keywords Glycoside hydrolase of 7-β-xylosyltaxanes · *Pichia pastoris* · High-cell-density fermentation · Bioconversion · Paclitaxel

Introduction

Paclitaxel (Taxol, Bristol-Myers Squibb, New York, NY, USA), originally isolated from the bark of the yew (*Taxus brevifolia*), is one of the most effective anticancer drugs [17]. However, its content in the yew plant is extremely low (only ~0.02 % from the stem barks). Fortunately, the content of some 7-β-xylosyltaxanes, such as 7-β-xylosyl-10-deacetyltaxol (XDT) and its analogues, is relatively abundant (up to 0.5 %) [3]. Once their C-7 xylose is removed (Fig. 1), the products (7-β-hydroxyltaxanes) become potentially useful for semi-synthesis of paclitaxel or its analogues [5, 14].

Chemical [2, 3, 16] or biological [7, 8, 23, 27] methods are generally used to remove the C-7 xylose from 7-β-xylosyltaxanes. The obvious drawbacks of the chemical way include low specificity, environmental pollution, and relatively low yield. On the other hand, the biological method is an enzymatic catalysis process that removes the xylose from 7-β-xylosyltaxanes through specific β-xylosidase under a mild condition. Therefore, it is thought to be environmentally friendly. However, due to the ubiquitous low enzyme quantity in the cells, the low bioconversion yields have not been substantially improved when the selected microorganisms are directly used. In our previous study, we found that a fungal strain *Lentinula edodes* M95.33 could convert XDT into 10-deacetyltaxol (DT), but we encountered the same problems as mentioned above (unpublished result). We then successfully cloned a gene (*Lxyl-p1-2*) from the fungus encoding the enzyme

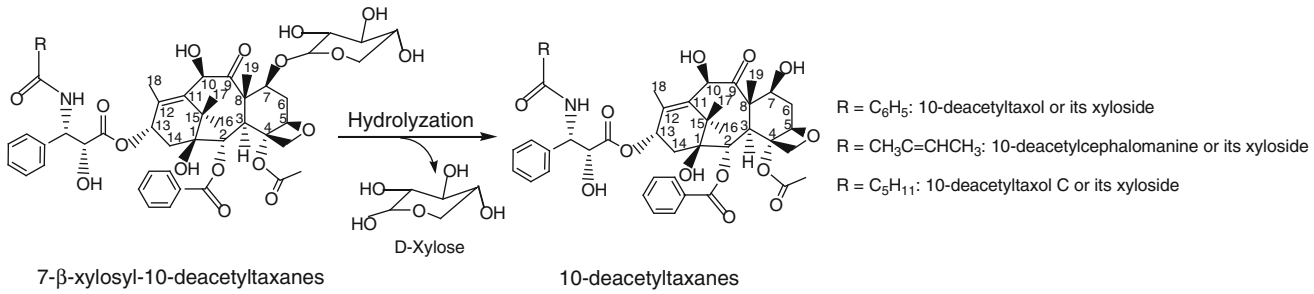

Fig. 1 C-7 xylose removing from 7-β-xylosyltaxanes

(designated as LXYL-P1-2) that could specifically remove the xylosyl group from XDT and its analogues. The gene could be expressed in the yeast host *Pichia pastoris*. However, the recombinant enzyme was mainly kept inside the host cells when the secretion vector pPIC9K was applied. Then the intracellular expression strategy was adopted, and the non-secretion vector pPIC3.5K was used to carry *Lxyl-p1-2* for the construction of the recombinant yeast GS115-3.5K-P1-2 (the work of the gene cloning and characterization will be published elsewhere). In this paper, we reported a high-cell-density fermentation of the recombinant yeast, the pilot-scale bioconversion of XDT and its analogues, and the purification of the products.

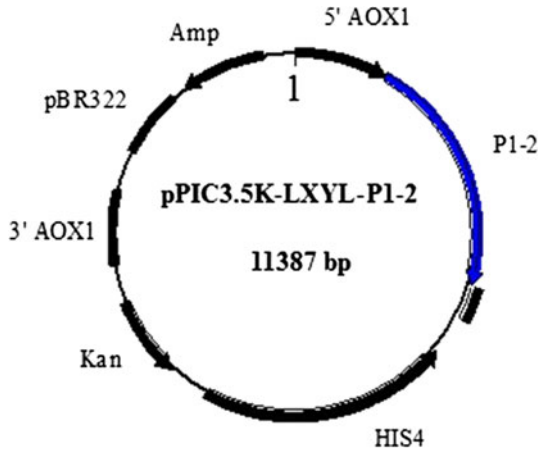

Fig. 2 Recombinant plasmid pPIC3.5K-LYYL-P1-2 harboring the sequence encoding LXYL-P1-2

Materials and methods

Strain

GS115-3.5K-P1-2 was constructed in our laboratory by transforming the host strain *P. pastoris* GS115 (Mut^+) with the recombinant plasmid pPIC3.5K-LXYL-P1-2 (intracellular expression) harboring the encoding sequence of LXYL-P1-2 (Fig. 2). Engineered strains were kept at −80 °C prior to use.

Media

Yeast extract peptone dextrose (YPD) agar plate (1 % yeast extract, 2 % peptone, 2 % glucose, and 1.5 % agar) containing 4 mg/ml G418 was used to cultivate the strain. Buffered glycerol-complex medium (BMGY) (1 % yeast extract, 2 % peptone, 1 % glycerol, 1.34 % YNB, 4×10^{-5} g/l biotin and 0.1 M potassium phosphate buffer, pH 6.0) was used for seed culture in a shake flask. FM22 with a PTM4 trace mineral solution was employed for high-density fermentation [22]. The FM22 recipe consisted of compounds as follows: 42.9 g/l KH_2PO_4, 5 g/l $(NH_4)_2SO_4$, 1.0 g/l $CaSO_4{\cdot}2H_2O$, 14.3 g/l K_2SO_4, 11.7 g/l $MgSO_4{\cdot}7H_2O$, and 40 g/l glycerol. The recipe for PTM4 was: 2.0 g/l $CuSO_4{\cdot}5H_2O$, 0.08 g/l NaI, 3.0 g/l $MnSO_4{\cdot}H_2O$, 0.2 g/l $Na_2MoO_4{\cdot}2H_2O$, 0.02 g/l H_3BO_3, 0.5 g/l $CaSO_4{\cdot}2H_2O$, 0.5 g/l $CoCl_2$, 7.0 g/l $ZnCl_2$, 22 g/l $FeSO_4{\cdot}7H_2O$, 0.2 g/l biotin and 1 ml/l H_2SO_4. In order to prepare the initial medium, 4.35 ml PTM4 was added in every liter of FM22. Glycerol solution (50 % w/v) and 100 % methanol containing PTM4 (12 ml/l) were added to the culture medium as feed medium for glycerol fed-batch phase and methanol fed-batch phase, respectively.

Cell density measurement

In order to determine the wet cell weight (WCW, g/l), 1 ml of cell broth was centrifuged at 12,000 rpm for 3 min, and then the pellet was collected and weighed.

Biomass and volumetric activity of β-xylosidase

β-Xylosidase enzymatic activity against the substrate *p*-nitrophenyl-β-D-xylopyranoside (PNP-Xyl, Sigma-Aldrich, St. Louis, MO, USA) is positively correlated with the recombinant enzyme amount. Moreover, the reaction is much easier to manipulate than the reaction using substrate XDT or its analogues. Therefore, the biomass and volumetric β-xylosidase activity (U/g or U/l) was determined by measuring the amount of *p*-nitrophenol released from

the substrate PNP-Xyl with spectrophotometry. Absorbance was recorded at a wavelength of 405 nm in a total volume of 60 μl, containing 10 μl of properly diluted fermentation broth and 50 μl 5 mM PNP-Xyl in 50 mM acetate buffer, pH 5.0. Reaction was performed at 50 °C for 20 min, and then it was terminated by adding 1 ml of saturated sodium tetraborate ($Na_2B_4O_7$) solution. One unit of enzyme activity was defined as the amount of enzyme required to release 1 nM *p*-nitrophenol per minute at 50 °C, pH 5.0.

Fermentation

High-density fermentation was carried out in a 10-l benchtop bioreactor (East China University of Science and Technology, Shanghai, China). Recombinant pPIC3.5K-LXYL-P1-2 strain was streaked on to an YPD agar plate containing 4 mg/ml G418, and then the plate was incubated at 30 °C for 72 h. A single colony was inoculated into five 500-ml shake flasks, and each flask contained 100 ml of BMGY medium. The flasks were incubated at 28 °C for 24 h in a shaking incubator (200 rpm), and then cell culture was used to inoculate the medium in the fermenter. Briefly, 10 % inoculum was used for inoculation of a 10-l fermenter containing 5 l of initial medium. Temperature was maintained at 28 °C. Dissolved oxygen (DO), unless otherwise indicated, was maintained above 20 % by controlling the aeration rate, impeller speed, and pure oxygen addition, when necessary. In addition, ammonia solution (28 %), which served as the nitrogen source at the same time, was used to maintain the medium pH (5.0). Antifoam agent (JXPF-1208) controlled by antifoam detector was used against foaming.

Growth phase

The batch culture was allowed to grow until the initial glycerol was completely consumed (indicated by the sudden rise of DO value to 100 %). Subsequently, glycerol-fed batch phase was initiated. The feeding medium contained glycerol (50 % w/v) and PTM4 (12 ml/l), and it was supplied at 18 ml/h/l until WCW reached the required level.

Induction phase

The fermentation procedure was performed according to the standard protocol (Invitrogen, Carlsbad, CA, USA). Glycerol feeding was terminated, and the methanol induction was then initiated by adding methanol solution (100 %) containing 12 ml/l PTM4. For methanol adaptation, the feeding rate was kept at 3.6 ml/h/l per initial fermentation volume for 2 h. After the transition phase, the feeding rate was increased to 7.3 ml/h/l per initial fermentation volume for 2 h, and then it was further increased and maintained to 10.9 ml/h/l per initial fermentation volume throughout the induction [11]. The biomass activity was monitored every 8 h.

Cell harvest

Fermentation broth was collected and centrifuged at 5,600 rpm for 15 min using a large-capacity centrifuge (Sigma 8K, Sigma, St. Louis, MO, USA). Due to the intracellular expression of the recombinant enzyme, the supernatant was discarded. The cell pellet was washed three times with distilled water, and then it was freeze-dried and stored at −20 °C prior to use.

Bioconversion of 7-β-xylosyltaxanes by recombinant cells

The freeze-dried cells were used to catalyze the removal of C-7 xylose from the partially purified XDT extract (or 7-β-xylosyltaxanes) (provided by Fujian South Biotechnology Co., Ltd., Fujian, China), which contained the following main components: XDT (62.12 %), 7-β-xylosyl-10-deacetylcephalomanine (XDC) (12.75 %) and 7-β-xylosyl-10-deacetyltaxol C (XDTC) (17.04 %). The XDT extract was dissolved in dimethyl sulfoxide (DMSO) as the stock solution (100 g/l). The reaction was performed in a 10-l impeller agitating bioreactor tank (350 rpm) with a reaction volume of 5 l. The reaction conditions were the optimized conditions obtained from the reaction volume of 200 ml in a 1-l glass beaker with magnetic stirring (this reaction volume was also used as a control). Freeze-dried cells (32 g/l) and substrates (10 g/l, it was an over-saturated concentration, as 7-β-xylosyltaxanes are slightly soluble in water) were mixed and suspended in 0.1 M acetate buffer (pH 4.0). The mixture was incubated at 45 °C for 24 h. In order to determine the bioconversion rate and the yield of 7-β-hydroxyltaxanes, sampling was performed at the beginning and the end of the reaction. Briefly, a 100-μl aliquot of the reaction broth was collected and added to 900 μl of methanol to determine the product yield and the remaining substrate. Samples were assayed by the analytical HPLC (Agilent 1200 series, USA). Analytical conditions were set as follows: column, Agilent Eclipse XDB-C18 (4.6 × 150 mm, 5 μm); mobile phase, acetonitrile (A)-water (B) (gradient, 0–12 min, VA 30–38%; 12–30 min, VA 38–52%); flow rate, 1 ml/min; column temperature, 28 °C; and detection wavelength, 230 nm. Bioconversion rate (%) of the substrate = (substrate peak area before reaction–substrate peak area after reaction)/substrate peak area before reaction × 100 %. Product yield (g/l) was calculated based on the product peak area using the linear regression equation.

Separation and purification of the products

The reaction broth was extracted by acetyl acetate. The products and the remaining substrates were separated by silica gel chromatography using the solution of CH_2Cl_2–MeOH (29:1, v/v) as the eluent. Reverse-phase preparative HPLC (prep-HPLC) (Shimadzu LC-6AD, Japan) was used to separate and purify the three main products: 10-deacetyltaxol (DT), 10-deacetylcephalomanine (DC), and 10-deacetyltaxol C (DTC). HPLC conditions were set as follows: column, GRACE Allsphere ODS-2 (22 × 250 mm, 5 μm); mobile phase, 42 % acetonitrile in water; flow rate, 10 ml/min; and detection wavelength, 230 nm. The purity of the three products was determined using the analytical HPLC mentioned above.

Results

Effect of the initial biomasses on the cell density and activity

Before this experiment, we observed the growth profile of the recombinant cells under the glycerol batch and glycerol fed-batch conditions. Figure 3 shows that the cell density continuously increased within 32 h until it reached 500 g/l (WCW), and then the cell density maintained this level.

Under the same DO value (20 %), we examined the effect of different initial biomasses (Table 1, batch 1 ~ 5) on the cell density and activity during the induction phase.

Figure 4a shows that different initial biomasses resulted in different cell-density profiles. Generally speaking, the higher maximum cell density was obtained when the higher initial biomass was applied. However, when the initial biomass was increased to 500 g/l (WCW), the cell density did not increase. Moreover, probably due to the increased number of the old cells, the recombinant enzyme activity dramatically decreased when the initial biomasses of 400

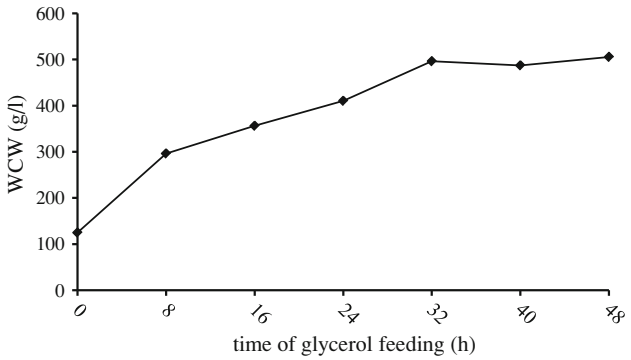

Fig. 3 Cell growth profile under glycerol batch and glycerol fed-batch conditions

Table 1 Initial biomass and DO level of induction phase for the six batches of experiments

Batches	Initial biomass WCW (g/l)	DO level (%)
1	200	20
2	250	20
3	300	20
4	400	20
5	500	20
6	300	5

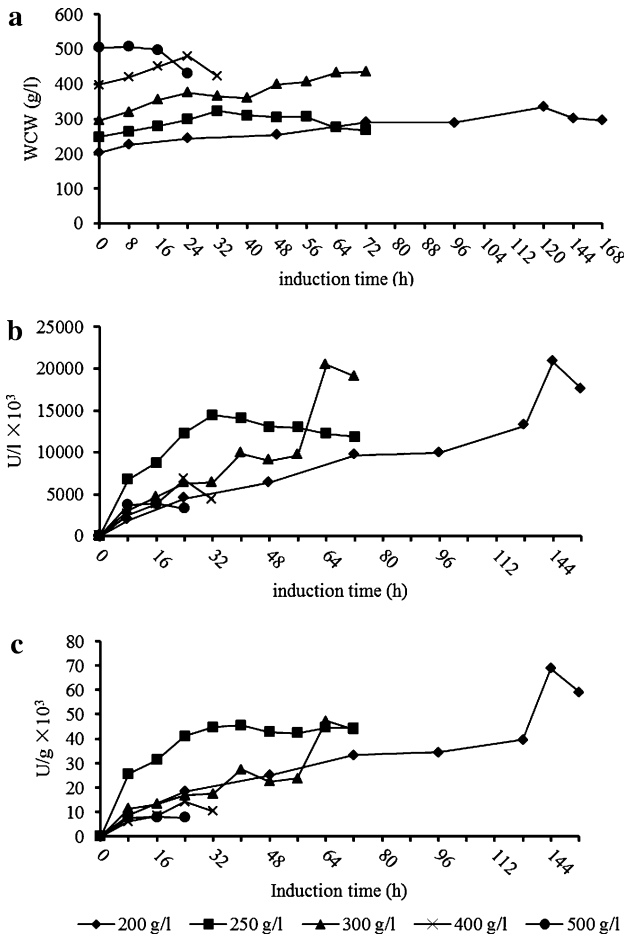

Fig. 4 Profiles of cell density (**a**), volumetric activity (**b**), and biomass activity (**c**) during the induction period

and 500 g/l were utilized. Except for the above two groups, the highest maximum cell density was 433.1 g/l when the initial biomass was 300 g/l, which also exhibited a higher enzyme activity (Fig. 4b, c).

Figure 4b shows that both initial biomasses of 200 and 300 g/l gave the highest maximum volumetric activities (2.08×10^7 U/l and 2.05×10^7 U/l, respectively). However, the peak value of the former was present at 144 h, which was over two times longer than that of the latter

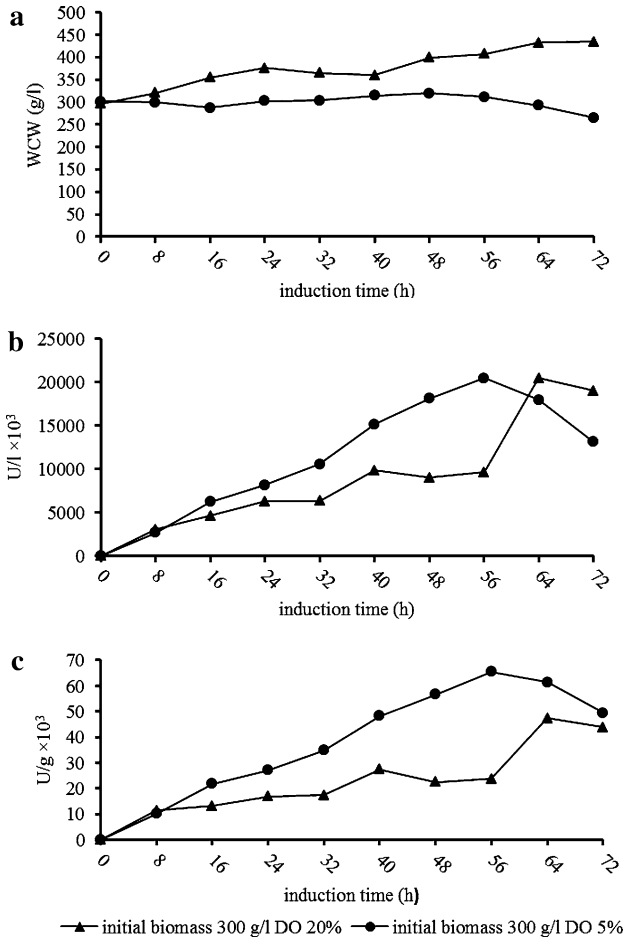

Fig. 5 Effects of the limited DO value (5 %) on the cell density (**a**), volumetric activity (**b**), and biomass activity (**c**) during the induction period

(64 h). When the initial biomasses were 200, 250, and 300 g/l, the corresponding higher maximum biomass activities were 6.88×10^4 (U/g), 4.54×10^4 (U/g), and 4.73×10^4 (U/g), respectively (Fig. 4c). Furthermore, their corresponding peak time was the as same as that shown in Fig. 4b. Higher biomass activity is required for bioconversion, as the recombinant enzyme is located in the cells, which will be directly used as the bio-catalyst. Since the highest cell density was obtained within 64 h, the initial biomass of 300 g/l was selected as an optimum factor for the high-cell-density fermentation.

Effect of the limited DO value on the cell density and activity

After the initial biomass (300 g/l) was optimized, we further examined the effect of the limited DO value (5 %, Table 1, batch 6) on the cell density and activity, and the DO value of 20 % (Table 1, batch 3) was used as the control (Fig. 5).

As expected, the net increase of biomass halted when the DO value was decreased to 5 %, keeping the cell density at the level of about 300 g/l within 56 h. At 64 h, its level was even decreased to 292.3 g/l (Fig. 5a). However, the maximum volumetric activity was not affected, and the peak time (56 h) appeared even earlier than that of the high DO value (20 %) (Fig. 5b). Furthermore, at this peak time, the maximum biomass activity (6.55×10^4 U/g) was obtained (Fig. 5c), which was much higher than that (4.73×10^4 U/g) of the high DO value (20 %) with the same initial cell density. This maximum biomass activity was close to the level of 6.88×10^4 U/g when the high DO value and the initial biomass of 200 g/l were applied (Fig. 4c).

The six batches of experiments are summarized in Table 2.

Bioconversion of 7-β-xylosyltaxanes

A total of 50 g substrates (the main components: 31.06 g of XDT, 6.38 g of XDC, 8.52 g of XDTC) were subjected to the bioconversion reaction in a volume of 5 l, and the average bioconversion rate was 81 % (Fig. 6a, b). The total yield of the three 7-β-hydroxyltaxanes was 6.17 g/l, including 0.87 g/l of DC, 4.23 g/l of DT, and 1.07 g/l of

Table 2 Summary of the six-batch high-cell-density fermentation

	Batch 1	Batch 2	Batch 3	Batch 4	Batch 5	Batch 6
Initial biomass (g/l)	200	250	300	400	500	300
DO level (%)	20	20	20	20	20	5
Peak time (h)	144	40	64	24	16	56
Maximum biomass activity (U/g)	6.88×10^4	4.54×10^4	4.73×10^4	1.41×10^4	0.79×10^4	6.55×10^4
Maximum volumetric activity (U/l)	2.08×10^7	1.41×10^7	2.05×10^7	0.68×10^7	0.39×10^7	2.05×10^7
Obtained biomass (g/l)	302.7	310.0	433.1	480.1	499.2	312.3

The results were the average of two duplicates

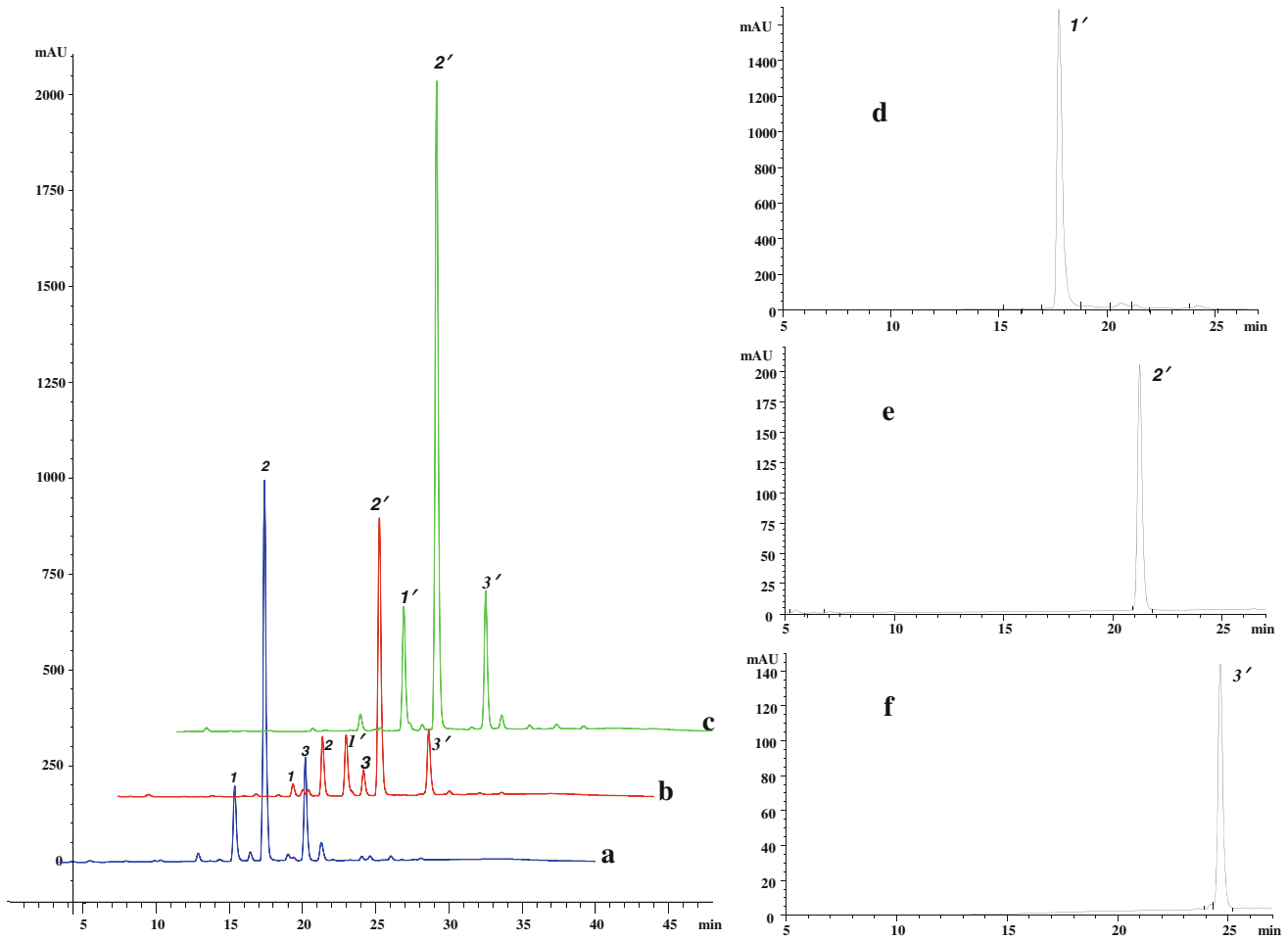

Fig. 6 Bioconversion of 7-β-xylosyltaxanes and separation of the three main products: **a** Before bioconversion, **b** after bioconversion, **c** after silica gel chromatography, and **d–f** after prep-HPLC. Peaks are: *1* XDC, *2*, XDT, *3* XDTC, *1′* DC, *2′* DT, *3′* DTC

Table 3 Bioconversion of 7-β-hydroxyltaxanes by GS115-3.5K-P1-2 obtained from high-cell-density fermentation

Reaction volume	Conversion rate (%)			Individual yield (g/l)			Total yield (g/l)
	XDC	XDT	XDTC	DC	DT	DTC	
200 ml (control)	81.4	85.4	84.4	0.84	4.09	1.12	6.05
5 l	81.4	81.4	69.4	0.87	4.23	1.07	6.17

DTC. It was similar to that of the 200-ml volume reaction, of which the average bioconversion rate was 85 % and the total yield was 6.05 g/l (Table 3).

Purification of the products

After the bioconversion solution was extracted with ethyl acetate, the extract was first subjected to silica gel chromatography to separate the products from the remaining substrates. Figure 6c shows that 7-β-hydroxyltaxanes (products) could be easily eluted from the column using the solution of CH_2Cl_2–MeOH. The remaining substrates were then washed off by methanol, yielding an amount of 10.36 g. The three main individual products (DC, DT, and DTC) could be further separated and purified by prep-HPLC. Product purity was determined by analytical HPLC (Fig. 6d, e, and f). Yields of the three products are summarized in Table 4.

Table 4 Separation results of the bioconversion products (total substrates: 50 g)

Types of the products	Yields after conversion* (g)	Yields after silica gel chromatography* (g)	Yields after prep-HPLC**(g)	Recovery rate (%)
DT	21.15	18.34	15.13	71.5
DC	4.34	3.75	3.04	71.2
DTC	5.15	4.06	3.47	68.4
Total	30.64	26.18	21.64	/

* Measured by HPLC, ** measured by weighing

Discussion

Pichia pastoris is a methylotrophic species of yeast that can grow on methanol as its sole carbon and energy source. The advantages of *P. pastoris* expression system include: rapid growth with high density on a defined medium with minimal basal salts [22]; efficient post-translational modifications; expressing secreted proteins with less secreted endogenous proteins; and a strong, well-regulated methanol-induced promoter. Due to these advantages, a multitude of heterologous proteins have been produced even through the approach of high-cell-density fermentation by the system [4, 15], and much attention has been paid to the volumetric activity when the proteins are secreted to the medium [20, 25]. In order to increase the volumetric activity, several strategies, such as the mixed feeding [1, 10, 19], appropriate initial biomass in methanol induction phase [9], growth rate control [6], pH control [18], and temperature control [24, 26], have been employed. In our present work, we established a high-cell-density fermentation of a recombinant yeast GS115-3.5K-P1-2 producing a heterologous protein LXYL-P1-2. LXYL-P1-2, a bifunctional β-xylosidase/β-glucosidase, is encoded by a gene isolated from a basidiomycete *L. edodes*, which specifically removes the xylosyl group from 7-β-xylosyl-10-deacetyltaxol and its analogues. As the recombinant enzyme was intracellularly expressed in the yeast host cells, we were very interested in the biomass activity as well as the biomass amount of the recombinant cells, which can be directly used as a general catalyst for the bioconversion of 7-β-xylosyltaxanes, while the volumetric activity is the secondary concern. Based upon the fermentation guideline given by Invitrogen [11], we further optimized the fermentation conditions, including the optimal initial cell density and the optimal limited DO level during the methanol induction phase. Preliminary results showed that the initial cell density of 300 g/l (WCW) was suitable for the recombinant cell production with higher biomass and higher maximum volumetric activity. Although its maximum biomass activity was slightly lower than that of the initial cell density of 200 g/l (Fig. 4), the peak value of the former was present at 64 h, which was much earlier than that of the latter (at 144 h). Although oxygen limitation should generally be avoided during the induction phase due to its adverse effects on foreign protein yields [12, 13], other reports also exhibited that successful protein production was achieved under the oxygen-depleted conditions [21]. In this study, we found that the limited DO level of 5 % was beneficial for higher biomass activity (Fig. 5), which was probably due to the relatively slow cell growth and enhanced expression of the heterologous protein under this DO condition. Similar phenomenon was also observed elsewhere [28]. Therefore, an ideal fermentation condition was defined as follows: the initial cell density of 300 g/l (WCW), DO level of 5 %, 28 °C, pH 5.0 and jar pressure of 0.05 MPa. Glycerol feeding before the induction phase was 18 ml/h/l. During the induction phase, the methanol feeding was 3.6 ml/h/l in the initial 2 h, 7.3 ml/h/l in the next 2 h and then 10.9 ml/h/l until the end of the fermentation. The fermentation period was 5 days, and the biomass activity reached 6.55×10^4 U/g (WCW) with the biomass of 312.3 g/l.

The freeze-dried cells were used to catalyze the reaction of hydrolyzing 7-β-xylosyltaxanes. Generally, the conversion rate (%) of 7-β-xylosyltaxanes is negatively correlated with the substrate concentration under the same conditions. To achieve a higher yield (g/l), the balance between the conversion rate and substrate concentration needs to be considered. Sometimes, the obtained yield is quite desirable although the conversion rate is not high enough. Before the pilot-scale bioconversion, we determined that the optimized 7-β-xylosyltaxanes concentration was 10 g/l (main substrate XDT), which was an over-saturated concentration in a smaller reaction volume. This over-saturated concentration was selected for the higher volumetric reaction. The conversion rate was about 81 % when the reaction volume reached 5 l. In fact, we found that as long as the recombinant cells sufficiently contacted with the substrates (the reaction volume from 2 to 200 ml) under proper conditions, the reaction volume was not a limited factor for a higher conversion rate (data not shown). In addition, we established a reliable purification method of the converted products with high yields and high quality in this work, and the average recovery rate was 70 %.

Paclitaxel is a well-known anticancer drug, but its natural content in the yew plant is very low. However, its byproducts, 7-β-xylosyltaxanes, are very abundant, from which the yield of XDT is as much as 0.5 %, over 20 times higher than that of paclitaxel. Once their C-7 xylose is removed, these byproducts become potentially useful in the semi-synthesis of paclitaxel or its analogues. Our work included the high-cell-density fermentation (in a 10-l fermenter) of the engineered yeast GS115-3.5K-P1-2, its efficient bioconversion of 7-β-xylosyltaxanes to 7-β-hydroxyltaxanes at higher substrate concentration (10 g/l) and in higher reaction volume (5 l), as well as the reliable purification process of the products. Our data provided a concrete foundation for largely improving the utilization of the *Taxus* resource and mitigating the imbalance between supply and demand of this drug.

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