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BIOCATALYSIS

Pilot studies on scale-up biocatalysis of 7-β-xylosyl-10-deacetyltaxol and its analogues by an engineered yeast

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Abstract Paclitaxel content in yew tree is extremely low, causing a worldwide shortage of this important anticancer drug. Yew tree can also produce abundant 7-β-xylosyl-10-deacetyltaxol that can be bio-converted into 10-deacetyltaxol for semi-synthesis of paclitaxel. However, the bio-conversion by the screened natural microorganisms was inefficient. We have constructed the recombinant yeast with a glycoside hydrolase gene from *Lentinula edodes* and explored the bioconversion. Based on previously established reaction conditions, the bioconversion of 7-β-xylosyl-10-deacetyltaxol or its extract was further optimized and scaled up with the engineered yeast harvested from 200-L scale high-cell-density fermentation. The optimization included the freeze-dried cell amount, dimethyl sulfoxide concentration, addition of 0.5 % antifoam supplement, and substrate concentration. A 93–95 % bioconversion and 83 % bioconversion of 10 and 15 g/L 7-β-xylosyltaxanes in 10 L reaction volume were achieved, respectively. The yield of 10-deacetyltaxol reached 10.58 g/L in 1 L volume with 15 g/L 7-β-xylosyl-10-deacetyltaxol. The conversion efficiencies were not only much higher than those of other reports and our previous work, but also realized in 10 L reaction volume. A pilot-scale product purification was also established. Our study bridges the gap between the basic research and commercial utilization of 7-β-xylosyl-10-deacetyltaxol for the industrial production of semi-synthetic paclitaxel.

Keywords Bioconversion · Scale-up · Engineered yeast · 7-β-Xylosyl-10-deacetyltaxol · Paclitaxel

Introduction

Bioactive agent harvested from natural sources often does not provide adequate quantities of interested product in a sustainable manner. On the other hand, bioconversion or biocatalysis of abundant, low-value, and renewable natural organic materials to high value-added products presents substantial interest for scientists, and the use of microbial or enzymatic catalysis offers a route to natural-equivalent products which resonate with green industrial technologies [15, 17]. Product yield and conversion rate, especially the former, are the two main concerns of bioconversion process, because they directly affect the economic viability of products. Moreover, to quickly obtain highly purified target compound from bioconversion products is of great significance [5]. Effective and versatile purification process for products not only saves time and cost, but also achieves a real practical sense of bioconversion technology.

Paclitaxel (Taxol®), a kind of first-line natural broad-spectrum antitumor drug, was severely restricted in its early stage of exploitation by the low yields from the dried bark of yew tree (typically 0.02 % by weight) [27], which hampered its preclinical and clinical development [7, 11]. Since the relatively abundant 10-deacetylbaccatin III was found present in the leaves of *Taxus baccata*, semi-synthesis of paclitaxel from this precursor became one of the major

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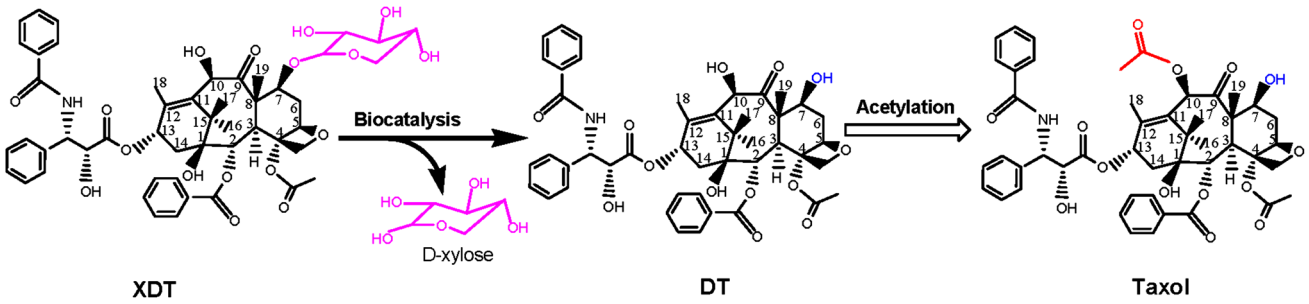

Scheme 1 Bioconversion of 7-β-xylosyl-10-deacetyltaxol to 10-deacetyltaxol for semisynthesis of paclitaxel. XDT, 7-β-xylosyl-10-deacetyltaxol; DT, 10-deacetyltaxol

sources of paclitaxel [6, 14], in addition to the yew plant nursery cultivation and the plant cell cultures, especially the cultured cambial meristematic cells [13]. 7-β-Xylosyl-10-deacetyltaxol (XDT) is an analogue of paclitaxel that can be obtained with a yield of as much as 0.5 % from the dried bark of yew tree [3]. If XDT's xylosyl moiety at C7 is removed, the resultant 10-deacetyltaxol (DT) can also be used for semi-synthesis of paclitaxel by acetylation at C10 position [3, 4, 18, 19] (Scheme 1). Studies showed that the efficiency of bioconversion from XDT to DT by screened natural microorganisms was not high enough (the maximum yield: 0.76 mg/mL in 25 mL reaction volume) [9, 10, 26, 29], due to the ubiquitously low amount of β-xylosidase within these microbial cells. To overcome this limitation, we cloned a novel glycoside hydrolase gene from *Lentinula edodes*, which can remove the xylosyl group from XDT. The process included the natural enzyme purification, LC–MS/MS de novo sequencing of the purified enzyme to obtain some of the oligopeptide sequences, and the gene mining by RT-PCR and RACE techniques [4]. The cloned *Lxyl-p1-2* gene was introduced into the methylotrophic yeast *Pichia pastoris,* and the engineered yeast efficiently transformed XDT into DT with the yield of over 8 mg/mL in 2 mL reaction volume and the reaction volume was scaled up to 200 mL with over 84–88 % conversion of 10 mg/mL 7-β-xylosyltaxanes [4]. Then, we completed 10-L scale high-cell-density fermentation of the recombinant strain and 5 L reaction volume of biocatalysis of 7-β-xylosyltaxanes using the harvested high-cell-density fermentation cells with about 69–81 % conversion of 10 g/L 7-β-xylosyltaxanes [30].

The main goal of the present study was to further optimize the conditions of XDT biocatalysis for larger scale reaction volume up to 10 L using the engineered yeast cells (G200 cells, freeze-dried) harvested from 200-L scale fermentation process. Impact factors including the dry cell amount, concentration of co-solvent dimethyl sulfoxide (DMSO), and substrate concentration were assessed. 0.5 % antifoam supplement was also added to the reaction solution (volume ≥200 mL). Then, the biocatalysis of 7-β-xylosyltaxanes [XDT extract, designated as XDT_{ex}, which contained 71.08 % XDT, 10.67 % 7-β-xylosyl-10-deacetylcepholamanine (XDC), and 12.05 % 7-β-xylosyl-10-deacetyltaxol C (XDTC)] was scaled up under the reaction volumes of 1, 5, and 10 L, respectively, with the magnetic stirring mode. Finally, we conducted a pilot-scale purification of DT from the XDT_{ex}-converted DT extract (DT_{ex}) by medium-pressure liquid chromatography (MPLC).

Materials and methods

Strain, media, and substrates

The engineered strain GS115-3.5 K-P1-2 was constructed in our laboratory by transforming the host strain *Pichia Pastoris* GS115 (Mut^+) with the recombinant plasmid pPIC3.5 K-LXYL-P1-2 (intracellular expression) harboring the sequence encoding LXYL-P1-2 [4], and cryopreserved at −80 °C prior to use. The details of media including yeast extract peptone dextrose (YPD) (containing 4 mg/mL of G418), buffered minimal glycerol complex medium (BMGY), and FM22 (containing 0.435 % of PTM4 salts) were reported elsewhere [23, 31]. *Pichia* trace minerals 4 (PTM4) salts solution, 50 % of glycerol solution (containing 1.2 % of PTM4 salts), methanol (containing 1.2 % of PTM4 salts), and the antifoam solvent (polyether type, v/v) were as reported previously [30]. *p*-Nitrophenyl-β-D-xylopyranoside (PNP-Xyl, artificial chromogenic substrate) was purchased from Sigma-Aldrich (St. Louis, MO, USA). XDT_{ex} was purchased from Fujian South Pharmaceutical Co., Ltd, (Fujian, China), which contained 71.08 % of XDT, 10.67 % of XDC, and 12.05 % of XDTC. The fully purified XDT was prepared from XDT_{ex} by MPLC method (LC2000, equipped with ultraviolet detector, Separation technology Co., Ltd, Beijing, China) established by this lab. All other chemicals were of analytical grade unless otherwise indicated.

High-cell-density fermentation

The procedure established previously in our lab [30] based on the protocol described in the manufacturer's handbook (Invitrogen, USA) was followed for the large-scale high-cell-density fermentation in a bench-top 200-L scale fermenter (30 S, Yangzhong Weikete Bioengineering Equipment Co. Ltd, China). Briefly, 500 mL seed culture from the shake flasks was used as inoculum for a 30-L fermenter (30 S, Yangzhong Weikete Bioengineering Equipment Co., Ltd, China) containing 10 L of FM22 plus 4.35 mL/L PTM4 salts. When the second seed culture reached a cell density of 110 g/L WCW (wet cell weight), 6.5 L of the culture were directly added into 65 L of FM22 medium in a 200-L fermenter. Then the glycerol batch phase was started, and the fermentation conditions were maintained at 30 °C, pH 5.0, 0.05 MPa, and DO of 20 % saturation. After all the glycerol was consumed, the process was converted to a glycerol fed-batch phase, with feeding of 50 % (v/v) glycerol containing 1.2 % (v/v) of PTM4 salts at a constant rate of 18 mL/L/h. After the desired biomasses of 300 g/L were reached, the methanol fed-batch phase was initiated, during which the culture was supplied with 100 % (v/v) of methanol plus 1.2 % (v/v) of PTM4 salts, started at 3 mL/L/h for 2 h, then increased to 7 mL/L/h for 3 h, and finally kept at 10 mL/L/h. The DO value was semi-automatically controlled by adjusting agitation and/or pure oxygen supplement, and impeller speed and agitation rate varied within the range of 10–270 rpm and 20–200 L/min, respectively. Time-course samples were withdrawn from fermenter for biomass analysis and enzyme assay with the chromogenic substrate PNP-Xyl. The WCW was measured for biomass analysis, and the enzyme activity was evaluated by calculating both U/L (volumetric activity) and U/g (biomass activity) as described in the literature [30], in which 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. At the end of fermentation, the fermentation broth was timely collected and centrifuged at 20,000 rpm for 15 min using a large-capacity tubular centrifuge (GQ145, Shanghai Pudong Tianben Centrifuge Co., Ltd, Shanghai, China), and the cell pellet was washed three times with distilled water, freeze-dried (GZLY-2, Beijing Suyuan Zhongtian Scientific Inc, Beijing, China), and stored at −20 °C prior to use.

Optimization of the bioconversion

XDT or XDT_{ex} was used for bioconversion by G200 cells as described previously [4, 30]. Briefly, G200 cells were suspended in 0.1 M sodium acetate buffer (pH 4.0); then XDT or XDT_{ex} stock solution (dissolved in DMSO) was added to the buffer to a final concentration of 10–20 mg/mL; If necessary, 0.5 % of antifoam solvent (polyether type, v/v) was supplemented to eliminate the foam formation during the reaction process, and the mixture was incubated at 45 °C for 24 h. A sealed 10 mL glass vial was used for the volume of 2 mL reaction (in a water bath shaker), while a 2-L glass beaker was used for the volume of 200 mL reaction (mixed by magnetic stirring), and a 20-L glass bottle was used for the volume of ≥5 L reaction (mixed by magnetic stirring).

The substrate conversion rate and product yield were quantitatively analyzed by analytical HPLC (Agilent 1200 series, US) with the Agilent XDB-C18 column (5 μm, 4.6 × 150 mm) as described elsewhere [4, 30]. Briefly, analytical conditions were set as follows: mobile phase, acetonitrile (A)–water (B) (gradient, 0–15 min, VA 28–40 %; 15–30 min, VA 40 %; 30–35 min, VA 40–28 %; and 35–45 min, VA 28 %); flow rate, 1 mL/min; column temperature, 28 °C; and detection wavelength, 230 nm. The measurements were performed at least in duplicate. Conversion 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.

Pilot-scale purification of DT

After bioconversion, the reaction mixture was fractioned for three times with ethyl acetate in the ratio of 1:3 (v/v). The combined organic layer was dehydrated with sodium sulfate and dried by vacuum rotary evaporation (Eyela NVC-2100, equipped with MLS-3750 low temperature circulating pump, Tokyo Rikakikai Co., Ltd, Tokyo, Japan) at 45–50 °C. The resulting DT extract (DT_{ex}) was purified by HPLC, semi-prep HPLC or MPLC. For HPLC or semi-prep HPLC, DT_{ex} was dissolved in DMSO (~100 mg/mL), and the DMSO solution was directly analyzed after filtering through a 4.5-μm membrane. For MPLC, DT_{ex} was first dissolved in DMSO (~100 mg/mL) and the DMSO solution was treated as mentioned above. Alternatively, DT_{ex} was dissolved in THF, and an appropriate amount of methanol was added to the THF solution to a certain THF/methanol ratio to obtain the maximal concentration of DT_{ex}. Then the THF-methanol solution was mixed with about ten times of ODS and evaporated at ~50 °C to remove the organic solvents. The resulting mixture of ODS and DT_{ex} was subjected to a preparative ODS column (45–60 μm, 26 × 310 or 49 × 460 mm) pre-equilibrated with the standard mobile phase consisting of acetonitrile (A)–water (B) at a flow rate of 15–25 mL/min. The preparation conditions were as follows: elution gradient, 0–30 min, VA 30 %, >30 min, VA 41 %; flow rate, 15–25 mL/min; column temperature, room temperature (~25 °C); and detection wavelength, 230 nm.

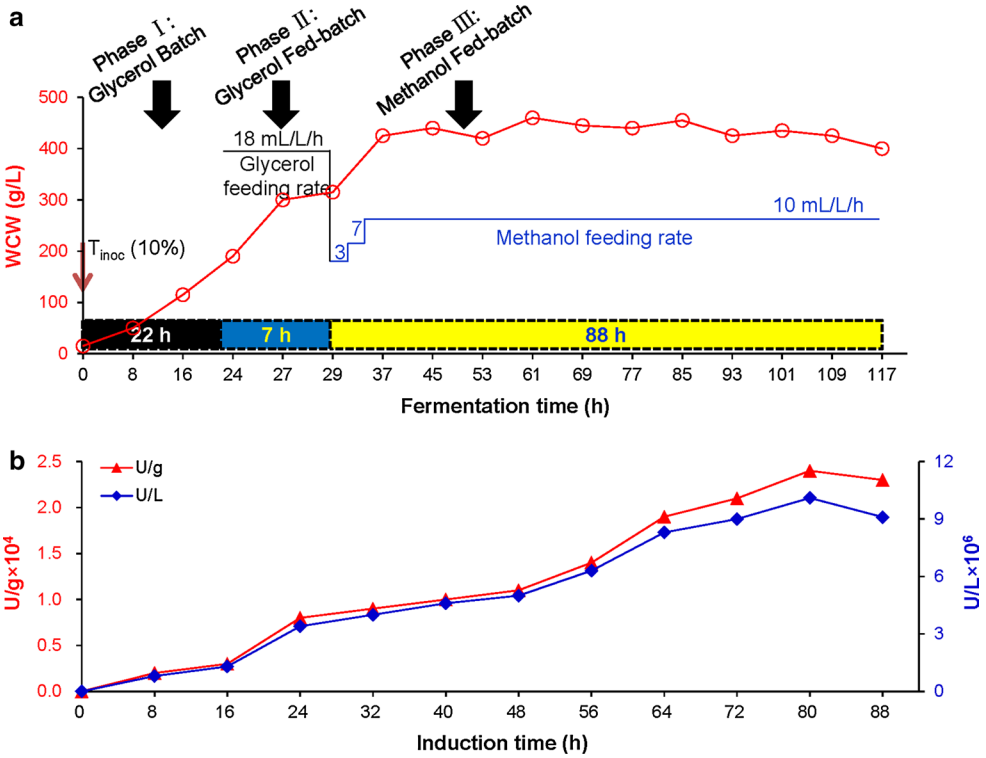

Fig. 1 Profile of the yeast high-cell-density fermentation in the 200-L scale fermenter. **a** The fermentation strategy and biomass-time curve. **b** The cell enzymatic activity-time curves during the induction phase. The wet cell weight (*WCW*), biomass activity (U/g, *triangle*), and volumetric activity (U/L, *diamond*) were measured at an interval of 8 h

The pooled unbound peak fraction of DT obtained from the preparative ODS column was concentrated by rotary evaporation at 45–50 °C, and the chromatographic purity and recovery yield of DT were analyzed by analytical HPLC using aforementioned HPLC methods.

Results

High-cell-density fermentation with the recombinant strain

Based on the procedure established previously in our lab [30], the high-cell-density fermentation of the engineered *Pichia pastoris* was conducted in the 200-L scale fermenter with dissolved oxygen (DO)-stat strategy. The procedure continued for 117 h which comprised glycerol culture phase of 29 h and methanol induction phase of 88 h (Fig. 1a). Glycerol fed-batch culture with 50 % glycerol (v/v) addition was started after the initial amount of glycerol had been exhausted, as evident by the abrupt increase in the DO level, and continued for 7 h including 2 h of starvation at the end until the second DO spike and up to 300 g/L wet cell weight (WCW). At the biomass accumulation stage (including the glycerol batch culture and glycerol fed-batch culture), the DO value was mainly kept at 20 % level. Then, methanol was added, with the DO level kept at 5 %. The WCW continuously increased to 425 g/L at induction time of 8 h and then basically kept constant until enzyme activity reached its maximum of 1.0×10^7 (U/L, volumetric activity) and 2.4×10^4 (U/g, biomass activity) at induction time of 80 h (Fig. 1b). Finally, about 13.7 kg of the freeze-dried recombinant cells were obtained from ~140 L fermentation broth.

Optimization of the bioconversion

The impact of dry cell amount on bioconversion

The relationship between dry cell amount and conversion rate was studied under the reaction volume of 2 mL in which the XDT concentration was first fixed at 10 mg/mL (DMSO concentration: 5 %, v/v) (Fig. 2a). The successive increase in the conversion rate of XDT was observed from 70 % up to the maximum 96 % when the dry cells were increased from 16 to ≥80 mg/mL.

The impact of DMSO concentration on bioconversion

Taxoid compounds are dissolved well in DMSO but slightly in water. Certain amount of co-solvent DMSO is beneficial to the dispersion of these compounds in water solution and the bioconversion of 7-β-xylosyltaxanes. DMSO was also selected as a relatively optimal co-solvent in our previous

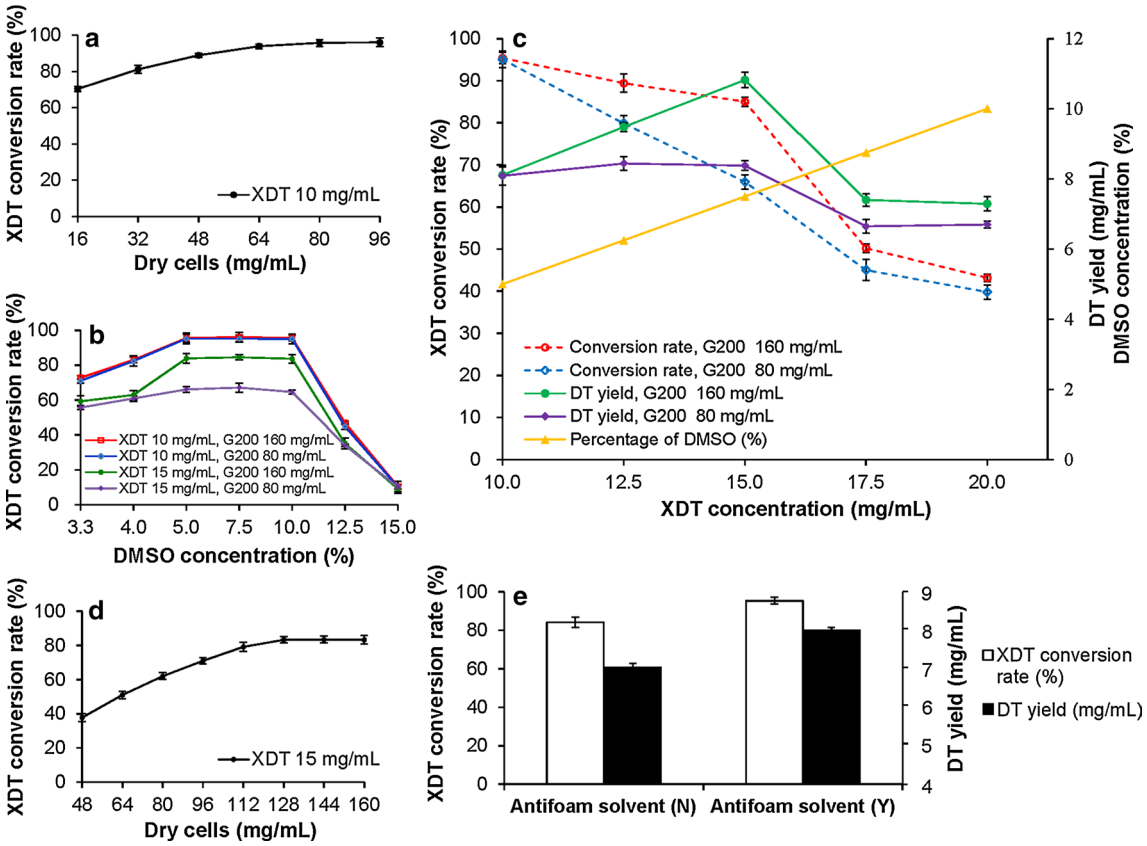

Fig. 2 Optimization of XDT bioconversion ($n = 3$, *error bars* show SD). **a** The amount of dry cells-XDT conversion curve under the substrate concentration of 10 mg/mL (reaction volume: 2 mL). **b** the impact of DMSO concentration on the bioconversion (reaction volume: 2 mL). **c** The impact of substrate concentration on the bioconversion under 80 and 160 mg/mL dry cells, respectively (reaction volume: 2 mL). **d** The amount of dry cells-XDT bioconversion curve under the substrate concentration of 15 mg/mL (reaction volume: 2 mL). **e** The impact of adding antifoam solvent on the bioconversion under 80 mg/mL dry cells and 10 mg/mL XDT (reaction volume: 200 mL)

work [4, 30], also see supplementary additional file 1: Fig. S1.

Higher concentration of DMSO is needed for the dispersion of larger amount of 7-β-xylosyltaxanes in a fixed reaction volume. However, the high concentration of DMSO may cause negative effect on the enzyme activity. The tolerance of the recombinant enzyme against DMSO was observed in a 2-mL reaction volume with different concentrations (from 3.3 to 15 %) of DMSO, 80 and 160 mg/mL G200 cells, and 10 and 15 mg/mL XDT, in four combinations (Fig. 2b). Our results showed that the conversion rate of XDT was linearly increased in the DMSO concentration range of 3.3–5 %. The conversion rate was kept at nearly the same high level until the DMSO concentration reached 10 %; then the conversion rate was sharply decreased. Other combinations of G200 cells and XDT also showed similar trends (supplementary additional file 2: Table S1). These results suggest that the DMSO content in the reaction should be controlled in the range of 5–10 % (v/v).

The impact of substrate concentration on product yield

Generally, the conversion rate is negatively correlated with increased substrate concentration under the same conditions. To achieve a higher yield (g/L), the balance between the two factors needs to be considered [30]. Sometimes, the high yield has more practical implication than the high conversion rate for the industrial purpose. Therefore, we examined the DT yield at different substrate concentrations from 10 to 20 mg/mL (with the DMSO concentrations from 5 to 10 %, v/v) in 2 mL reaction volume (Fig. 2c) with identical reaction conditions as mentioned above. It was observed that for the 80 mg/mL G200 cells, the conversion rate declined continuously with the increase of XDT concentration. Similar yields were obtained at the substrate concentrations in the range of 10–15 mg/mL, and the highest DT yield was 8.4 mg/mL at the substrate concentration of 12.5 mg/mL; the DT yield dropped dramatically when XDT concentration surpassed 15 mg/mL. For the 160 mg/mL G200 cells, although the conversion rate declined as

the XDT concentration increased, the decline was not so sharp before the substrate concentration approached 15 mg/mL, leading to a continuous increase of the DT yield. The highest DT yield reached 10.82 mg/mL with 85 % bioconversion at the substrate concentration of 15 mg/mL.

The cell amount-XDT bioconversion curve was further detected under 15 mg/mL XDT and different G200 cells amounts ranged from 48 to 160 mg/mL. As shown in Fig. 2d, nearly equal conversion rates of 83, 84, and 83 % were achieved for cell amounts of 128, 144, and 160 mg/mL, respectively. So, 128 mg/mL G200 cells and 15 mg/mL XDT may be the combination of choice for the bioconversion to obtain a higher yield.

The effect of antifoam supplement on bioconversion

Mixing the reaction solution by magnetic stirring or impeller is often performed during bioconversion process, especially in large reaction volume, which frequently leads to the formation of foams on the solution surface. The foams may influence the bioconversion efficiency. According to the fermentation process, antifoam solvent (polyether type, 0.5 % v/v) was supplemented to 200 mL reaction solution (G200 cells: 80 mg/mL; XDT: 10 mg/mL) prior to the bioconversion, which increased the conversion rate from 84 % (without antifoam solvent) to 95 % (with antifoam solvent) (Fig. 2e). The latter was even comparable to the 96 % maximum conversion rate in 2 mL reaction volume. Thus, the antifoam solvent was used as a routine supplement in the following reaction process of large scale bioconversion.

Scale-up bioconversion

The conditions for scale-up bioconversion process were as follows: 80 mg/mL (g/L) G200 cells plus 10 mg/mL (g/L) XDT or XDT_{ex}; 128 mg/mL (g/L) G200 cells plus 15 mg/mL (g/L) XDT or XDT_{ex}. First, the bioconversion was scaled up from 2 through 200 mL to 1 L with 80 mg/mL G200 cells plus 10 mg/mL XDT and 128 mg/mL G200 cells plus 15 mg/mL XDT. In the 80 mg/mL G200 cells plus 10 mg/mL XDT group, a conversion rate of about 95 % was achieved in the reaction volumes from 2 mL to 1 L (Table 1), reproducing the same bioconversion efficiency as mentioned above (Fig. 2a). In the 128 mg/mL G200 cells plus 15 mg/mL XDT group, the conversion rate was around 84 %, and the maximal DT yield reached 10.58 g/L in 1 L volume, exhibiting the same capacity as that in smaller volumes (Table 1).

Then, the bioconversion was further scaled up from 1 L through 5–10 L with G200 cells and XDT_{ex} containing about 71 % XDT, 11 % XDC, and 12 % XDTC. Instead of the specialized reaction tank, a large glass bottle mixed by magnetic stirring was applied when the reaction volumes were ≥5 L (Fig. 3a). The results are summarized in Table 1, and partial results are shown in Fig. 3b. In the 80 g/L G200 cells plus 10 g/L XDT_{ex} group, ≥93 % of the conversion rates for each of the 3 main substrates were obtained in 1, 5, and 10 L reaction volumes, respectively, in which about 95 % XDT bioconversion was obtained, reproducing the same bioconversion efficiency in smaller volumes. In the 128 g/L G200 cells plus 15 g/L XDT_{ex} group, besides the XDC and XDTC substrates, the XDT conversion rate in 1, 5, and 10 L reaction volumes reached 83 %, respectively, showing the same bioconversion efficacy as that in the 128 mg/mL G200 cells plus 15 mg/mL XDT group (from 2 mL to 1 L reaction volume). The total yield of the three main products [DT, 10-deacetylcepholamanine (DC), and 10-deacetyltaxol C (DTC)] reached 9.8 g/L in 10 L reaction volume.

Pilot-scale purification of DT

A sufficient amount of purified DT is beneficial to the semi-synthesis of paclitaxel. We have reported a primary DT purification process using general silica gel column chromatography followed by a semi-preparative reverse phase high-pressure liquid chromatography (HPLC) [30]. However, the purification efficacy was restricted by a relatively lower loading capacity of the octadecylsilane (ODS) column. To overcome this restriction, we further explored the MPLC method and purified DT from XDT_{ex}-converted DT_{ex} (containing 8.1 % DC, 67.5 % DT, 9.3 % DTC, and 7.1 % other substrates) using the acetonitrile–water mobile phase [30]. Herein, two main factors were considered: solvent of DT_{ex} and size of ODS column. For the solvent, because of its high viscosity and non-volatility, DMSO was replaced with the binary solution tetrahydrofuran (THF)-methanol, as DT_{ex} also showed a reasonable solubility (~120 mg/mL) in THF-methanol at the ratio of 4:1 (v/v) (other ratios: THF/methanol = 6:1, solubility ~90 mg/mL; THF/methanol = 2:1, solubility ~75 mg/mL; THF/methanol = 1:1, solubility ~45 mg/mL). For the size of ODS column, the loading capacity was upgraded step by step from analytical HPLC to MPLC scales. Partial results are summarized in Table 2. About 7 g of purified DT with 78 % recovery was obtained within approximately 4.5 h when 13 g DT_{ex} was subjected to the MPLC preparation, and the other products (DC and DTC) were also properly separated (Fig. 4).

Discussion

Several literatures have provided examples of bioconversion of XDT to DT by the screened natural microorganisms [9, 10, 26, 29]. However, the reported conversion levels

Table 1 Bioconversion of XDT and XDT_{ex} by G200 cells (2 mL–10 L)

Volume[a] and mixing mode	Substrate and concentration (g/L)	Dry cells (g/L)	Conversion rate (%)	Yield (g/L)
2 mL[b]	XDT, 10	80	95.2 ± 1.2	8.02 ± 0.03
200 mL[c]	XDT, 10	80	94.9 ± 0.8	8.00 ± 0.06
1 L[c]	XDT, 10	80	94.5 ± 1.1	7.97 ± 0.05
2 mL[b]	XDT, 15	128	83.3 ± 1.4	10.51 ± 0.17
200 mL[c]	XDT, 15	128	84.1 ± 1.3	10.65 ± 0.09
1 L[c]	XDT, 15	128	83.3 ± 0.9	10.58 ± 0.06
1 L[c]	XDT_{ex}, 10	80	95.1 ± 1.2 (XDT) 94.5 ± 1.4 (XDC) 93.7 ± 0.6 (XDTC)	5.73 ± 0.05 (DT) 0.76 ± 0.02 (DC) 0.86 ± 0.03 (DTC) 7.35 ± 0.05 (total)
5 L[c]	XDT_{ex}, 10	80	94.5 ± 1.1 (XDT) 93.6 ± 0.7 (XDC) 93.1 ± 0.9 (XDTC)	5.67 ± 0.09 (DT) 0.75 ± 0.10 (DC) 0.84 ± 0.06 (DTC) 7.26 ± 0.08 (Total)
10 L[c]	XDT_{ex}, 10	80	95.3 ± 1.2 (XDT) 94.1 ± 0.8 (XDC) 93.0 ± 0.7 (XDTC)	5.75 ± 0.08 (DT) 0.76 ± 0.05 (DC) 0.84 ± 0.05 (DTC) 7.35 ± 0.10 (total)
1 L[c]	XDT_{ex}, 15	128	83.0 ± 1.1 (XDT) 82.3 ± 1.5 (XDC) 81.9 ± 0.6 (XDTC)	7.52 ± 0.11 (DT) 1.02 ± 0.01 (DC) 1.20 ± 0.07 (DTC) 9.74 ± 0.16 (total)
5 L[c]	XDT_{ex}, 15	128	83.2 ± 0.8 (XDT) 82.4 ± 0.7 (XDC) 81.2 ± 0.7 (XDTC)	7.53 ± 0.07 (DT) 1.02 ± 0.05 (DC) 1.18 ± 0.06 (DTC) 9.73 ± 0.11 (total)
10 L[c]	XDT_{ex}, 15	128	83.1 ± 0.8 (XDT) 83.0 ± 1.1 (XDC) 82.8 ± 1.8 (XDTC)	7.54 ± 0.02 (DT) 1.05 ± 0.11 (DC) 1.23 ± 0.14 (DTC) 9.81 ± 0.16 (total)

XDT_{ex}, 7-β-xylosyl-10-deacetyltaxol extract (containing 71.08 % XDT, 10.67 % XDC and 12.05 % XDTC); XDT, 7-β-xylosyl-10-deacetyltaxol; XDC, 7-β-xylosyl-10-deacetylcepholamanine; XDTC, 7-β-xylosyl-10-deacetyltaxol C; DT, 10-deacetyltaxol; DC, 10-deacetylcepholamanine; DTC, 10-deacetyltaxol C

[a] The values of bioconversion were mean ± SD of triplicate or duplicate from 2 mL to 1 L ($n = 3$), or from 5 to 10 L ($n = 2$)

[b] Mixed by water bath shaking

[c] Mixed by magnetic stirring

are far from the yields and/or the catalytic reaction volumes needed for an economically viable commercial-scale production. To efficiently remove the sugar by biological approach, which is considered to be more environmental friendly than the chemical method, we established a system using recombinant yeast that harbors the novel specific glycoside hydrolase gene from *L. edodes* [4]. Based on the results of 10-L scale high-cell-density fermentation [30], 200-L scale high-cell-density fermentation using the low-cost FM22 basic salts medium was implemented in this work (Fig. 1a). Due to the limits of the equipment, such as the not highly accurate DO value control, the maximum biomass activity was not as high as that of the 10-L scale high-cell-density fermentation, though the biomass amount at the activity peak time was higher than that of the latter (415 vs. 312 g/L, WCW). However, the lower biomass activity can be compensated by adding more dry cells during the bioconversion process (Fig. 2a, d). In fact, under 10 mg/mL XDT, over 95 % XDT conversion rates were achieved when the cell amounts were ≥80 mg/mL. Furthermore, over 10 kg of the freeze-dried recombinant cells obtained from the large-scale fermentation made it possible to carry out systematic trials of scale-up bioconversion up to 10 L. Further improvement of the biomass activity in large-scale fermentation may reduce the cell amount used in the bioconversion. Certain amount of co-solvent could increase the solubility of hydrophobic substrates in aqueous system [22]. DMSO, a so-called "universal" co-solvent, was often used in dissolving the hydrophobic compounds. Rather et al. [20] concluded

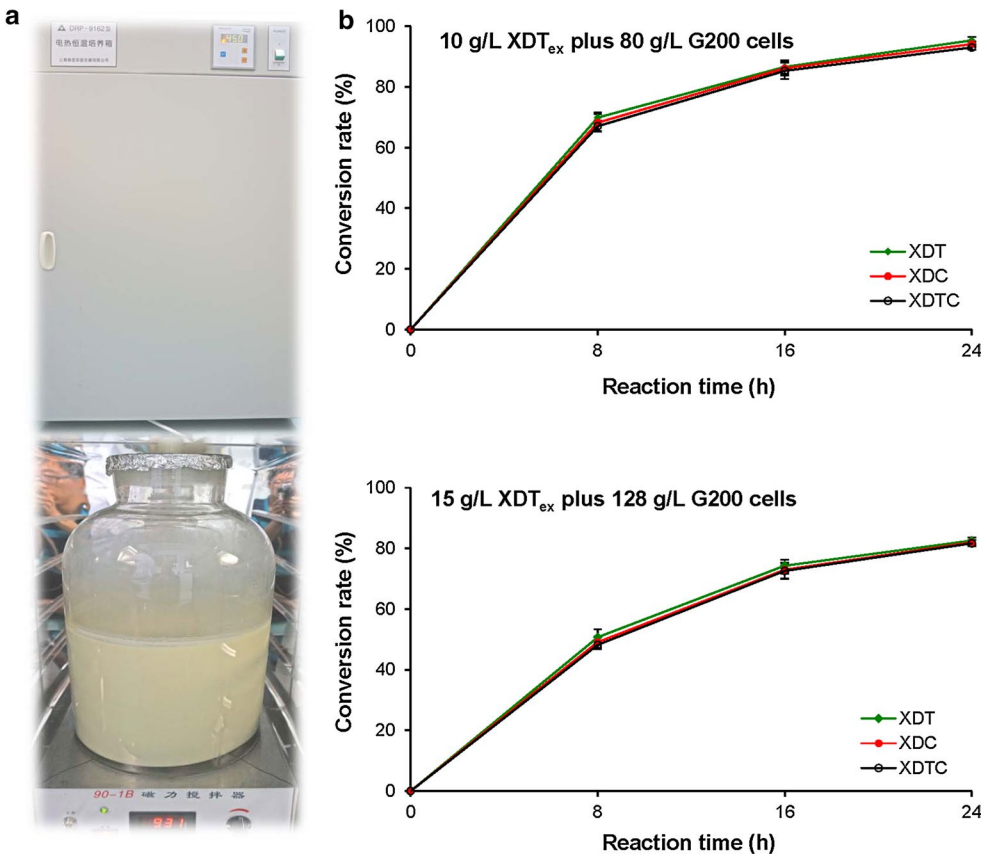

Fig. 3 10 L volume bioconversion and the conversion rate-time curves. **a** The apparatus showing the incubator, magnetic stirrer, and 20-L glass bottle. **b** Conversion rate-time curves based on the HPLC analysis ($n = 3$, *error bars* show SD). XDT$_{ex}$, 7-β-xylosyl-10-deacetyltaxol extract; XDT, 7-β-xylosyl-10-deacetyltaxol; XDC, 7-β-xylosyl-10-deacetylcepholamanine; XDTC, 7-β-xylosyl-10-deacetyltaxol C

Table 2 Scale-up of DT purification from DT$_{ex}$ by HPLC and MPLC methods

Purification scale	Separation conditions			Purification efficiency		
	DT$_{ex}$ solvent	ODS column	Flow rate (mL/min)	DT$_{ex}$ amount	DT amount	DT recovery (%)
HPLC	DMSO	5 μm, 4.6 × 250 mm	1	20 μg	13.0 μg	96.3
Semi-prep HPLC	DMSO	10 μm, 10 × 250 mm	5	35 mg	20.2 mg	85.7
MPLC	DMSO	45–60 μm, 26 × 310 mm	10	3.1 g	154.8 mg	7.4
MPLC	THF-methanol (4:1, v/v)	45–60 μm, 26 × 310 mm	15	3.0 g	1.7 g	81.6
MPLC	THF-methanol (4:1, v/v)	45–60 μm, 49 × 460 mm	25	13.0 g	6.9 g	78.4

that nearly total activity of the β-glucosidase against the substrate methyl-β-D-glucopyranoside was retained in the presence of 20 % (v/v) of DMSO. Uzan et al. [25] found that the laccases were resistant to DMSO at the concentrations as high as 50 % (v/v). Girhard et al. [8] reported that addition of 2 % DMSO in a two-liquid-phase system could enhance the regioselective oxidation of (+)-valencene to (+)-nootkatone, a high-value ingredient for the flavor and fragrance industry, by a recombinant *E. coli*. In our previous work, we also chose DMSO as the optimal XDT co-solvent for the bioconversion. In this study, the optimal range of DMSO concentrations (5–10 %) in the reaction solution was determined (Fig. 2b). Based on this information, we further observed the impact of XDT concentration on the conversion rate and product yield. Actually, 15 mg/mL XDT (in 7.5 % DMSO) was chosen to achieve the higher DT yield (maximum: 10.82 mg/mL) with the maximum conversion rate of 85 %, when the cell amounts were more than 128 mg/mL (Fig. 2c, d). Additionally, to keep the higher conversion rate, 0.5 % antifoam solvent, which is frequently used in bioreactors, was added to the reaction solution once the foams were formed by the mechanical mixing process. Adding antifoam solvent to the reaction solution may be extended to other substrate bioconversion to raise yield (Fig. 2e). The higher conversion efficiency was retained when the reaction volume was increased from 2 mL to 1 L with the combination of 128 mg/mL (g/L) G200 cells plus 15 mg/mL (g/L) XDT (DT yield: 10.58 g/L, with 83 % XDT conversion rate in 1 L reaction volume) (Table 1).

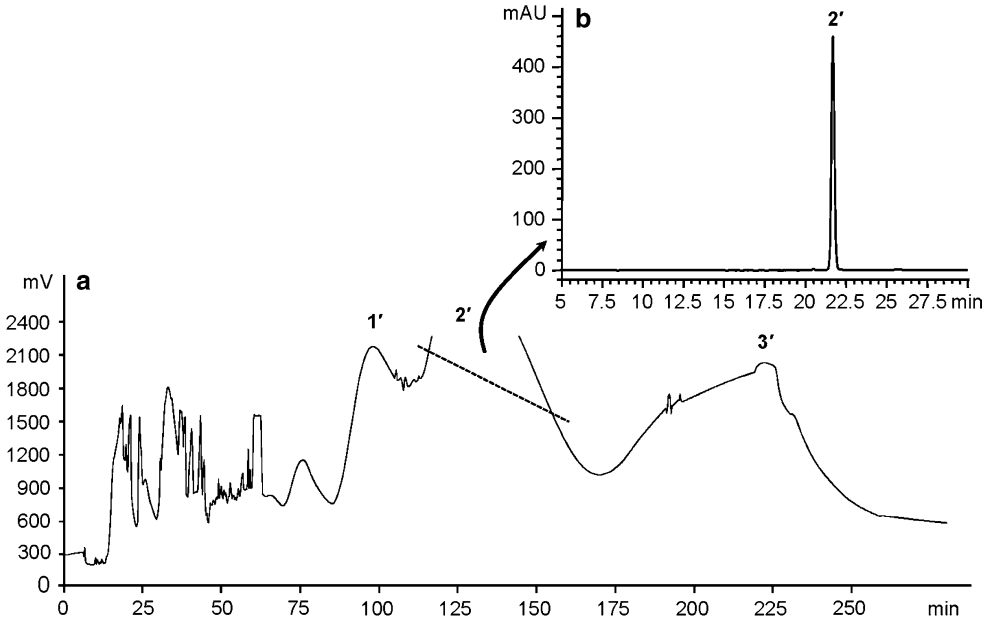

Fig. 4 Pilot-scale purification of DT from DT_{ex}. **a** MPLC profile of DT_{ex} separation. **b** DT purified from DT_{ex} by MPLC and analyzed by HPLC. The peaks 1′, 2′ and 3′ indicate DC, DT, and DTC, respectively

Then the reaction volume was scaled up from 1 L through 5 to 10 L with the substrate XDT_{ex}, in which the XDT content was about 71 %, together with the other two main components: ~11 % XDC and 12 % XDTC. Similarly, high conversion rates with high yield were also observed with these reaction volumes. For example, in the combination of 80 g/L G200 cells plus 10 g/L XDT_{ex}, besides the other two main substrates XDC and XDTC, XDT showed a conversion rate up to 95 % with about 5.8 g/L of the main product DT in 10 L reaction volume; in the same reaction volume the XDT conversion rate reached 83 % with 7.5 g/L of the main product DT when the combination of 128 g/L G200 cells plus 15 g/L XDT_{ex} was applied (Table 1). The high bioconversion efficiency in 10 L reaction volume is of industrial significance. Normally, specialized reaction tank is needed for large reaction volume. Instead, we tried to use the large glass bottle with magnetic stirring for the large-scale bioconversion. Because of its sufficient mixing effect, this apparatus could easily reproduce the same bioconversion efficiency as that in smaller volumes. The bioconversion efficiency was even higher than that in the fermenter tank with the same reaction volume (data not shown). Moreover, the apparatus is quite simple, inexpensive, and energy efficient (only an incubator and a magnetic stirrer are needed). Our results demonstrated again that the reaction volumes are not the restriction factor for the large scale bioconversion of XDT and its analogues.

Scientists have developed various chromatographic methods to isolate taxoid compounds [1, 2, 12, 16, 21, 24, 28], and a sufficient amount of purified DT is beneficial to the semi-synthesis of paclitaxel. Rao [19] introduced a reverse phase chromatography on a preparative scale column (38 × 300 mm) eluted with acetonitrile in water at a flow rate of 6–12 mL/min. We also reported a primary DT purification process using general silica gel column chromatography followed by a semi-preparative reverse phase HPLC [30]. However, the chromatography still needs to be improved. In this work, we used a larger ODS column (45–60 μm, 49 × 460 mm) and faster flow rates (15–25 mL/min) with an acetonitrile–water system for the reverse phase MPLC. Our approach is proven to be efficient for large-scale purification of DT or XDT and may be scaled up for the industrial purpose (e.g., adding the column number or enlarging the column size). Furthermore, the waste acetonitrile can be recycled to reduce the production cost and the potential environmental pollution.

With the engineered yeast G200 cells, we further optimized and scaled up the bioconversion of XDT or XDT_{ex}, especially based on magnetic stirring mode. The DT yield reached about 10.6 g/L in 1 L volume with 15 g/L XDT. The XDT conversion rates reached 95 and 83 % in 10 L volume, when 10 and 15 g/L XDT_{ex} were treated, respectively, reproducing the same efficacy as that in the smaller volumes. The conversion efficiencies were not only much higher than those of other reports (DT yield: 10.58 g/L vs. 0.23–0.76 mg/mL) [9, 10, 26] and our previous work [30], but also realized in large reaction volume. A pilot-scale MPLC method was also set up. Our study bridges the gap between the basic research and commercial utilization of XDT for the industrial production of semi-synthetic paclitaxel.

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Conflict of interest The authors declare that they have no competing interests.

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