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Bioproduction of lauryl lactone and 4-vinyl guaiacol as value-added chemicals in two-phase biotransformation systems

Jianzhong Yang · Shaozhao Wang ·
Marie-Josée Lorrain · Denis Rho · Kofi Abokitse ·
Peter C. K. Lau

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Abstract Recombinant *Escherichia coli* whole-cell biocatalysts harboring either a Baeyer–Villiger monooxygenase or ferulic acid decarboxylase were employed in organic-aqueous two-phase bioreactor systems. The feasibility of the bioproduction of water-insoluble products, viz., lauryl lactone from cyclododecanone and 4-vinyl guaiacol from ferulic acid were examined. Using hexadecane as the organic phase, 10–16 g of lauryl lactone were produced in a 3-l bioreactor that operated in a semicontinuous mode compared to 2.4 g of product in a batch mode. For the decarboxylation of ferulic acid, a new recombinant biocatalyst, ferulic acid decarboxylase derived from *Bacillus pumilus*, was constructed. Selected solvents as well as other parameters for in situ recovery of vinyl guaiacol were investigated. Up to 13.8 g vinyl guaiacol (purity of 98.4%) were obtained from 25 g of ferulic acid in a 2-l working volume bioreactor by using octane as organic phase. These selected examples highlight the superiority of the two-phase biotransformations systems over the conventional batch mode.

Keywords Ferulic acid · Baeyer–Villiger monooxygenase · Phenolic acid decarboxylase · Two-phase biotransformation · Green chemistry

Introduction

Technological advances in biocatalyst discovery, bioprocess optimization, and product recovery are important consid-

erations for the impact of industrial or white biotechnology that promotes a wider use of microbial or bio-based production of goods and services (Hermann and Patel 2007; Caesar 2008). In this study, we carried out whole-cell biocatalysis in organic-aqueous two-phase partitioning bioreactors (TPPB) systems to test the feasibility of bioproduction of two water-insoluble products. As examples, we chose the production of lauryl lactone (LL) through the enzymatic Baeyer–Villiger oxidation of cyclododecanone (CDD) using a previously described cyclopentadecanone monooxygenase (Iwaki et al. 2006) and the decarboxylation of ferulic acid (FA) to yield 4-vinyl guaiacol (VG) utilizing a new recombinant biocatalyst described in this work.

A typical TPPB setup consists of an organic phase and an aqueous phase that contains the biocatalytic microorganism and growth media. The TPPB systems have been used as a platform for treatment of environmental pollutants, fermentations as well as biocatalysis (Deziel et al. 1999; Daugulis 2001; Malinowski 2001; Schmid et al. 2001; Heipieper et al. 2007; Muñoz et al. 2007). By and large, the TPPB systems offer the prospect of increased mass transfer, alleviation of possible substrate and product toxicity, and hence, enhanced biotransformations and simplified product recovery.

Besides being a valuable pharmaceutical building block, large-ring lactones such as the C₁₂ LL is potentially useful in the functionalization of cellulose as thermoplastic polysaccharides. The C₆ ϵ -caprolactone, for example, has been grafted onto cellulose by polymerization in various solvent systems (Warth et al. 1997; Ikeda et al. 2003). LL is presently not readily available by industrial-scale chemical synthesis (Thomas et al. 2002). Although the chemical Baeyer–Villiger reaction would represent a possible production route, it is dependent on

J. Yang · S. Wang · M.-J. Lorrain · D. Rho · K. Abokitse ·
P. C. K. Lau (✉)
Biotechnology Research Institute,
National Research Council Canada,
6100 Royalmount Avenue,
Montreal, QC H4P 2R2, Canada
e-mail: peter.lau@cnrc-nrc.gc.ca

chlorinated solvents and, therefore, not environmentally friendly (Constable et al. 2007).

VG is a high-value product for the flavor, fragrance, and perfume industry, and it is extensively used in the food and alcoholic beverage sectors. Although chemical decarboxylation of FA is the most widely used method for preparing VG or styrene type compounds, a typical reaction is carried out by heating under reflux at 200–300°C for 4–5 h in quinoline in the presence of copper powder (Griengl et al. 1969). Microwave-assisted and base-catalyzed decarboxylation reactions of FA have also been studied (Nomura et al. 2005; Bernini et al. 2007).

A promising alternative to the chemical production of VG is the use of biocatalysis. Several bacteria and fungi have been shown to be able to convert FA to VG via decarboxylation by a ferulic acid decarboxylase (Fdc) also known as phenolic acid decarboxylase (Huang et al. 1993, 1994; Rosazza et al. 1995; Cavin et al. 1998; Barthelmebs et al. 2001; Tsujiyama and Ueno 2008). Several of the Fdc or Pad-encoding genes from organisms, e.g., *Lactobacillus plantarum*, *Pediococcus pentosaceus*, *Bacillus* sp. BP-7, *B. subtilis*, and *B. pumilus* strain PS231 and ATCC 14884, have been cloned and sequenced (Zago et al. 1995; Cavin et al. 1997, 1998; Barthelmebs et al. 2001; Prim et al. 2003). The first purified Fdc, coming from the *Bacillus pumilus* (formerly identified as *Pseudomonas fluorescens*) strain UI-670, was characterized to be a homodimeric 20.4-kDa subunit protein, and it appeared to be produced constitutively (Huang et al. 1993, 1994; Rosazza et al. 1995). As part of this study, the *fdc* gene from *B. pumilus* UI-670 was cloned in *E. coli* for its use in biotransformation experiments for the recovery of VG from FA in the TPPB system. The results are compared to the experiments carried out by Lee et al. (1998), who also employed a two-phase bioreactor but using a different wild-type *B. pumilus* strain (DRV52131/NRRL 14942) and different parameters.

Materials and methods

Chemicals

Cyclododecanone (C₁₂H₂₂O), lauryl lactone (oxacyclotridecan-2-one, C₁₂H₂₂O₂), isopropyl-β-D-thiogalactopyranoside (IPTG), *trans*-ferulic acid (*trans*-4-hydroxy-3-methoxy-cinnamic acid, 99%) were purchased from Aldrich Chemical, Canada. 4-Vinyl guaiacol (2-methoxy-4-vinylphenol, 97%) was obtained from Alfa Aesar (Heysham Lancashire, UK). Alkanes (hexane, hexadecane and octane), HPLC-grade methanol, and other chemicals of analytical grade were purchased from J.T. Baker, USA and used without further purification.

Bacterial strains

E. coli BL21[pCD201] cells containing a cloned cyclopentadecanone monooxygenase (CPDMO) gene from *Pseudomonas* sp. strain HI-70 in an IPTG-inducible plasmid pSD80 were used as previously described (Iwaki et al. 2006). *Bacillus pumilus* UI-670 (also referred to as AM-670) was kindly provided by Prof. John P. N. Rosazza (Center for Biocatalysis and Bioprocessing, University of Iowa, USA). *E. coli* JM109 [pKFAD] cells carrying a cloned ferulic acid decarboxylase (*fdc*) from *B. pumilus* UI-670 is described below.

Recombinant DNA techniques and sequencing

Standard methods used for the isolation of plasmid DNA, restriction digestion, ligation, cloning and transformation, and bacterial growth media were according to Sambrook et al. (1989). A ~830-bp fragment containing the putative *fdc* sequence and promoter region from *B. pumilus* UI-670 was amplified according to the *fdc* sequence of a related *B. pumilus* strain PS231 (Zago et al. 1995). The forward and reverse primers were 5'-CGTCTGCAGTCCTGCATCAGTGATTTG-3' and 5'-CGCAAGCTTCTTTTCGTCAGCTCTG-3'. The underlined sequences are *Pst*I and *Hind*III restriction sites that are used in the cloning in the *E. coli* pKK223-3 vector (Brosius 1984) (Amersham Pharmacia Biotech). The culture with the recombinant plasmid was designated *E. coli* JM109[pKFAD]. The DNA sequence of the cloned insert was determined on both strands by an automated DNA sequencer (Model 377, ABI prism) using a Big Dye DNA sequencing kit (Applied Biosystems). The DNA and predicted amino acid sequence of Fdc of strain UI-670 have been deposited in GenBank with accession number FJ825140.

Solvent selection and resting cell assays for CDD biotransformation

Four hydrocarbon solvents (octane, decane, dodecane, hexadecane) were examined as possible solvent for CDD biotransformation using resting cell-activity assays in 100 ml scale. *E. coli* BL21[pCD201] cells were cultured in glucose-containing LB medium (10 g/l each of yeast extract and tryptone peptone, 5 g/l NaCl, 20 g/l glucose, and 50 mg/l ampicillin) in 2-l shake flasks, and the enzyme expression was induced with 0.1 mM IPTG for 5 h after the cells reached an OD of 1. Cells were harvested by centrifugation, and the cell pellet was resuspended in 0.05 M phosphate buffer (pH=7, 2.15 g DCW/l, 20 ml) containing glucose in 100-ml flasks. The dry-cell weight (DCW) was determined after drying the washed cells at 100°C for 48 h. A coefficient of 0.43 g DCW/l per OD₆₀₀

unit was used to calculate the biomass concentration. Biotransformation was started by mixing in 5 ml of organic solvent containing 50 mM of CDD, and the reaction was carried out at 30°C with orbital shaking at 250 rpm for 1 h. Sample concentrations of CDD and LL in the organic phase were analyzed using ReactIR 4000 (Mettler Toledo, ASI Applied Systems, USA; Yang et al. 2006).

Fed-batch biotransformation of CDD

The cell preculture was prepared in 500-ml Erlenmeyer flask as described above. The cell pellet was recovered by centrifugation and inoculated in a 3-l bioreactor (Biobundles, Applikon Inc, USA) that contains two Rushton turbine impellers and four baffles. The 1-l culture medium contains 4.0 g Na_2HPO_4 ; 2.0 g KH_2PO_4 ; 3.0 g $(\text{NH}_4)_2\text{SO}_4$; 0.5 g NaCl; 1.0 g casamino acid; 0.12 g MgSO_4 ; 58.0 mg $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$; 50.0 mg thiamine; 50.0 mg ampicillin; 6.0 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$; 20 g glucose, and 4.5 ml trace element solution as described by Panke et al. (2002). An additional carbon source (glucose 500 g/l) was fed at a rate of 10 ml/h when the OD_{600} reached a value of 10. The fermentation was carried out under the following conditions: 30°C; pH 7.0 adjusted by the addition of 2 M KOH; the reactor was aerated at 1 VVM via a submerged sparger and the agitation rate was controlled between 600 and 1,000 rpm in order to maintain the dissolved oxygen concentration above 30%. Antifoam (Mazu DF 204, BASF, 5% (w/w)) was added to control foaming of broth. The dissolved oxygen tension, feed rate and base consumption, were monitored and recorded with a LabVIEW software. When the OD_{600}

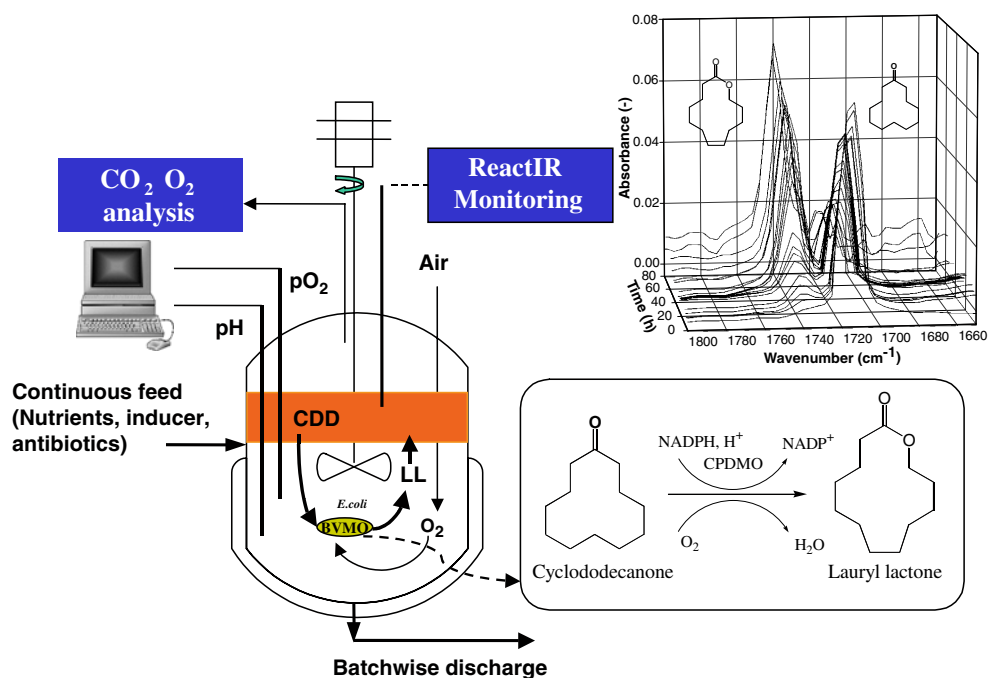
reached 1, IPTG (1 ml, 100 mM) was added to induce the CPDMO expression. After 1-h induction, CDD (5.0 g) dissolved in 20 ml hexadecane was dispersed into the fermentation medium. Ten-milliliter samples were withdrawn from the reactor during the biotransformation process and were immediately centrifuged at $20,800 \times g$ for 6 min to separate the organic phase from the aqueous phase for monitoring the progress of biotransformation.

Two-phase semicontinuous reactor system

A variation of TPPB system is the two-phase semicontinuous reactor (TPSCR; an integration of reactor configuration with a new operational mode shown in Fig. 1), in which the cells are grown in the aqueous phase by continuously feeding nutrients and inducer in order to maintain the enzyme expression. The organic phase plays a dual function: as carrier for dispersion of water-insoluble reactant into the aqueous phase and as an extraction medium for in situ removal of product from the aqueous phase. In the operational mode, mixing and aeration were interrupted for a 20-min period to allow separation of the aqueous layer (bottom) from the lighter organic (e.g., hexadecane, specific gravity=0.773) layer. A portion of the aqueous phase containing aged cells, metabolites and debris of dying cells, was discharged from the bottom of the reactor while maintaining the organic phase at the top layer of the bioreactor.

The initial working volumes of the aqueous and organic phases were 1 l and 375 ml, respectively. The organic solvent, containing 120 mM CDD, was dispersed into the aqueous phase under turbulent and aeration conditions. Feeding rate of nutrients in the complete medium contain-

Fig. 1 TPSCR for the biotransformation of CDD to LL. *E. coli* BL21 [pCD201] was used to express Baeyer–Villiger mono-oxygenase and ReactIR 4000 spectroscopy was used to provide a fingerprint of the progress of biotransformation, as previously described (Yang et al. 2006)



ing IPTG (0.1 mM) was at 75 ml/h. When the total volume increased to about 2.5 l, approximately 1 l of old cells was removed from the bottom-layer medium. The remaining cells continued to grow using freshly added medium to allow biotransformation to proceed continuously. Samples were taken periodically to monitor the progress of biotransformation as described before.

Decarboxylation of FA and analysis

Seven solvents (see Table 1) were screened using decarboxylation activity assay in 20-ml scale. *E. coli* JM109 [pFKAD] cells were cultivated in glucose-containing LB medium in 2-l shake flasks and harvested at the end of the exponential growth phase. The cell pellet was resuspended in 0.1 M phosphate buffer (0.86 g DCW/l) and mixed with an equal volume of organic solvent in 20-ml seal-capped bottles. The biotransformation activities were measured in 2-ml reaction mixture by directly adding the FA solution, which was prepared under basic condition, pH 8. The initial FA concentration was 12 mM, and the bioconversion conditions were controlled at 30°C with orbital shaking at 250 rpm. After 30-min incubation, the reaction was stopped by the addition of 20 µl of 50% trichloroacetic acid. The 2-ml reaction mixture was diluted with 18 ml of methanol, and the FA and VG concentrations were analyzed in duplicate by HPLC (Waters, USA) equipped with a reverse-phase C18 column (Discovery® 250×4.6 mm, particle size 5 µm) and a UV diode array detector. The mobile phase consisted of methanol/water solution, 70:30, v/v; acidified with phosphoric acid to pH 3, and operated at a flow rate of 0.8 ml/min. Eluates were monitored at 279 nm. The quantification was performed using authentic VG standard.

Table 1 Activity of *E. coli* JM109 [pKFAD] and *B. pumilus* UI-670 cells in organic and aqueous two-phase system

Solvents	Partition* coefficient		Fdc Activity (µmol/min/g DCW)	Log P* octanol/water
	FA	VG		
Phosphate buffer			21.8	
Chloroform	0.1	167	229.6	1.97
Ethyl acetate	0.4	120	111.2	0.73
Cyclohexane	<0.01	6	234.4	3.44
<i>n</i> -Hexane	<0.01	7	235.6	4.0
<i>n</i> -Octane	<0.01	< 6	227.1	5.15
<i>n</i> -Dodecane	<0.01	< 6	202.9	5.6
<i>n</i> -Hexadecane	<0.01	< 6	200.1	8.25

*Partition coefficients of reactant and product in water/solvent and Log P values of solvents in octanol/water

Growth rate and biocatalytic activity of native *B. pumilus* strain UI-670 and recombinant *E. coli* whole cells in bioreactor

E. coli JM109[pKFAD] and *B. pumilus* UI-670 were cultured respectively in a 3-l bioreactor (Biobundles, Applikon Inc, USA). Precultures in shaking flask were inoculated (10%, v/v) into fresh Iowa medium (Lee et al. 1998) in the case of *B. pumilus* and LB medium containing 20 g/l glucose in the case of *E. coli*. The cell culture was carried out under following conditions: temperature 30°C; pH 6.8 adjusted by the addition of 2 M KOH; dissolved oxygen was controlled at above 30% air saturation by adjustment of the agitation; and foaming was controlled by addition of 5% defoamer (Mazu DF 204, BASF). The *B. pumilus* UI-670 cells were induced using FA (0.5 g/l) at OD₆₀₀=0.5 for 24 h. Samples were taken to monitor the growth curve by measuring the OD at 600 nm. The whole cells from each sample were harvested by centrifugation at 4°C, and the pellet was washed with 0.1 M phosphate buffer pH 6.8, for subsequently analysis of activity and 10% SDS-PAGE by conventional method (Sambrook et al. 1989).

The decarboxylation activity of FA in the whole cells was performed in 20-ml seal-capped bottles as previously described for the solvent screening. Here, octane was used, and the initial FA concentration was 25 mM. Control experiments were carried out under the same conditions with boiled cells or *E. coli* JM109 without the cloned plasmid. The specific activities of decarboxylation were calculated and expressed as mmol VG/h/g DCW.

VG production in TPPB and product recovery

Batch biotransformation was carried out in a 3-l Applikon bioreactor. *E. coli* JM109 [pKFAD] cells were cultured under the following conditions: 30°C, pH 6.8 adjusted by the addition of 2 M KOH; dissolved oxygen was controlled at above 30% air saturation by adjustment of the agitation, and foaming was controlled by addition of Mazu DF 204 defoamer (BASF). At the end of the exponential growth phase, octane solvent and FA powder were directly added into the bioreactor. Samples were taken at regular intervals from the organic phase and from the aqueous phase. The collected samples were centrifuged in an Eppendorf 5417R at 20,800×g, 30°C for 6 min in order to separate the organic from the aqueous phase. The samples were diluted using methanol and both the content of FA and VG were determined by HPLC.

After the biotransformation, the organic phase was separated by centrifugation then dried over anhydrous Na₂SO₄. The organic solution was filtered and evaporated at 60°C using a vacuum rotary evaporator (Buchi Rotavapor R-250) to give VG as a light yellow clear liquid.

The structure was confirmed by gas chromatographic-mass spectrometric analyses. The purity of the product was calculated from the GC chromatogram. The MS analyses were performed using a commercial quadrupole system (Agilent 5973). Chromatographic separations were carried out on an Agilent 6890 DB-5ms capillary column (50 m × 0.2 mm i.d. × 0.33 nm). The temperature was controlled following program at 70°C for 1.4 min, raised to 275°C, 2.5°C/min, and maintained at this level for 1 min. Helium was the carrier gas. The temperatures of the injector and the flame ionization detector were maintained at 250°C and 290°C, respectively. The mass spectrometer was operated in the electron impact mode under the following conditions: ion source temperature, 200°C; electron energy, 70 eV; emission, 300 pA. Mass spectra were recorded during each chromatographic run by scanning the mass spectrometer from m/z 50 to m/z 500 at a scan speed of 500 amu/s.

Results

Solvent choice for the biotransformation of CDD

Among four apolar solvents (hexadecane, dodecane, decane and octane), hexadecane with a logP value of 8.25 was chosen as a carrier solvent for all subsequent experiments. Hexadecane was able to dissolve 1 mol/l of CDD at 30°C. Its high-boiling point (289°C) would minimize stripping or solvent evaporation from the bioreactor under aeration and agitation conditions. This phenomenon and the effect on the quantitative analysis of CDD was found to be negligible during a 24-h period at 1 VVM of aeration and 1,000 rpm of agitation at 30°C. Also, the bioactivity of the whole cells was found to be higher when hexadecane was used compared to other solvents (data not shown).

The capacity for product recovery is another considered parameter. According to the partition coefficient ($\text{Log}K(i)_{\text{hw}} = 1.21 * \log K(i)_{\text{ow}} - 0.43$, www.envsci.rutgers.edu/~totten/522/kow.ppt), lauryl lactone (LL) would be mainly absorbed into the organic phase. At 1 M LL in the organic phase, the corresponding equilibrium concentration of LL in the aqueous phase is less than 0.5 mM. Saturated concentration or solubility of LL in hexadecane was analyzed at 30°C. Depending on the phase ratio, the maximum recoverable quantity of LL was calculated to range between 30 g at ratio of 1/10 and 74 g at ratio of 1/4 in a liter of total working volume (organic phase + aqueous phase).

Batch process for CDD biotransformation

Bioconversion results in a batch process and the productivity of LL are shown in Fig. 2. After IPTG induction, LL production increased according to the growth phase,

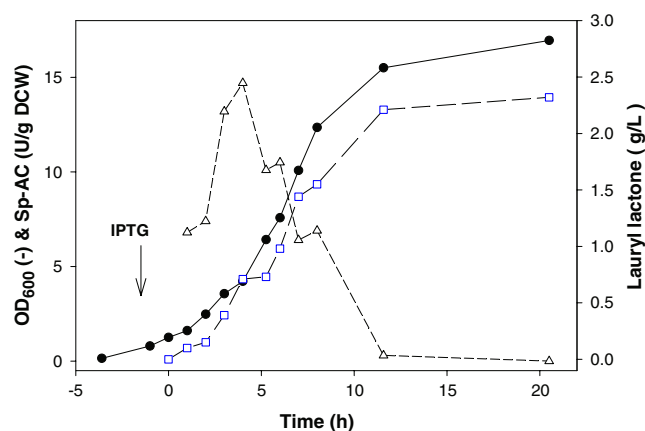


Fig. 2 Bioprocess kinetics of the production of lauryl lactone during cell-growth process in a batch system; the specific activity was calculated by determining the rate of product formation per gram of DCW as the average between two data points; *filled circles* growth curve; *empty squares* lauryl lactone production; *empty triangles* specific activity

plateauing at about 2.4 g/l after 10 h. A specific productivity of 15 U/g DCW of the biocatalytic system was achieved after 4 h of growth, but the productivity dropped sharply in the following 6 h.

LL production under TPSCR conditions

We sought to prolong the biocatalytic activity of the system by operating the TPSCR way (Fig. 3). In this regime, used cells were periodically discharged (ten times) over a period of 132 h, and LL production was seen to plateau after 72 h with a concomitant decrease of CDD (Fig. 3a, b). The concentration of CDD in the organic phase decreased from 126 to 24 mM representing 80% conversion; 99 mM (7.3 g) of LL was produced and recovered in the organic phase (Fig. 3a). The biomass increased from 4 to 10 g DCW/l at the expense of glucose consumption and ammonium ions (Fig. 3c). Sugar was supplied to maintain the supply of cofactor and ADP regeneration.

Effectively, the TPSCR operating mode extended the productivity of LL over a prolonged period compared to that of a batch mode. Further enhancement of the productivity was realized by increasing the initial CDD concentration from 216 to 840 mM. As a result, 11 g of LL were produced within 72 h (Fig. 4).

Cloned *fdc* in *E. coli* JM109

The *fdc* gene sequence of *B. pumilus* strain UI-670 was determined to code for 161 amino acids and found to be identical to that reported for *B. pumilus* strain PS231 (Zago et al. 1995) and 98.5% identical to *B. pumilus* ATCC 14884 (Barthelmebs et al. 2001; please note that the original

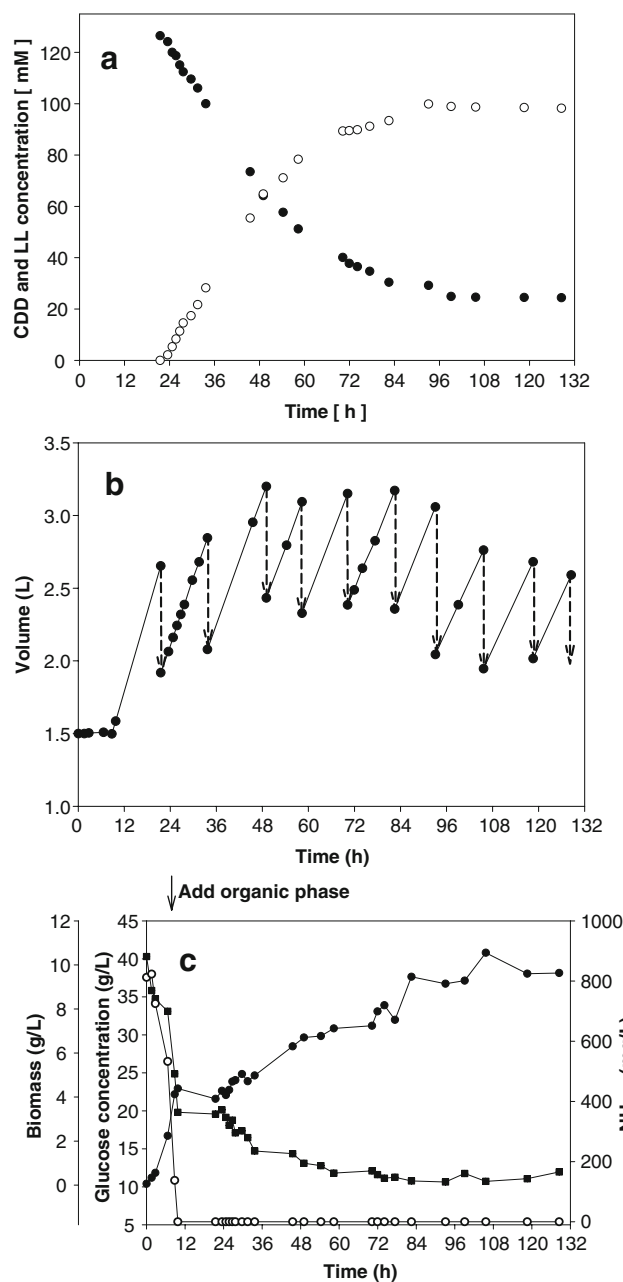


Fig. 3 **a** Bioconversion of cyclododecanone to lauryl lactone using TPSCR system. *filled circles* CDD; *empty circles* LL; **b** Volume change in the bioreactor; **c** Biomass, glucose, and ammonium concentration; *filled circles* biomass; *filled squares* glucose; *empty circles* ammonium

ATCC number reported in this reference is incorrect). Figure 5b shows the presence of the Fdc protein band of the expected molecular mass (23 kDa) expressed in the *E. coli* JM109[pKFAD] cells that were sampled over a 27-h period. A qualitative analysis indicated that the biocatalyst was relatively stable even after the exponential growth phase. In this time frame, the specific biotransformation activity for VG production was found to be in the range of 88–115 mmol/g DCW/h (Fig. 5a). Operationally, this means

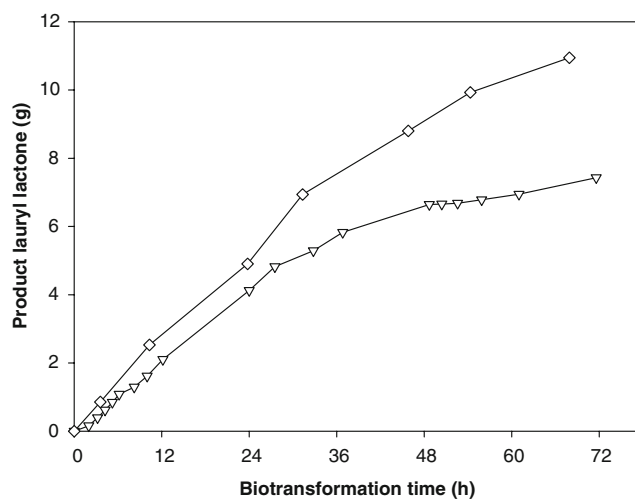


Fig. 4 Process optimization of TPSCR; *empty inverted triangles* TPSCR I, *empty diamonds* TPSCR II. In TPSCR II, CDD concentration in the organic phase was increased from 126 to 845 mM, and the phase ratio was reduced from 1:4 to 1:10

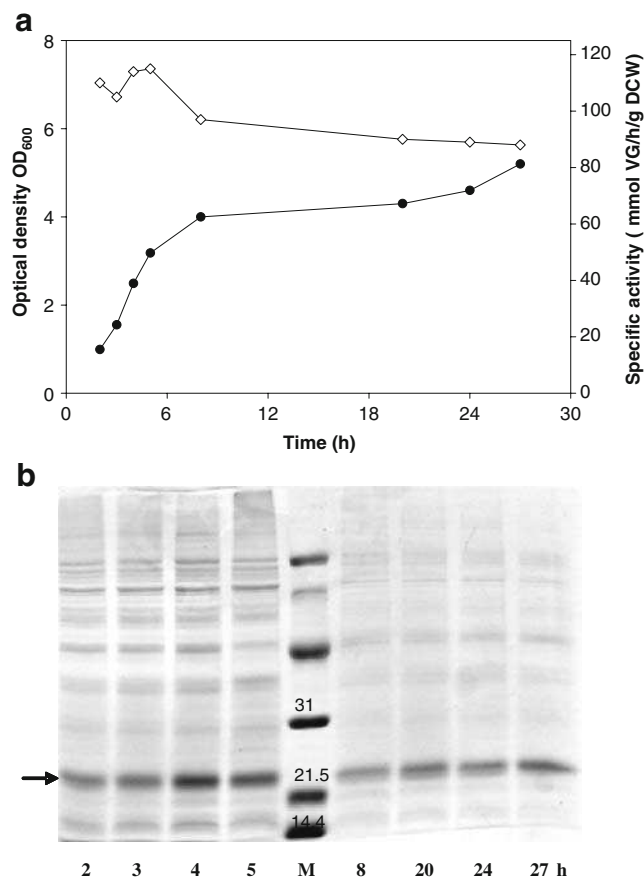


Fig. 5 **a** Specific activity (*empty diamonds*) during cell growth (*filled circles*) in bioreactor and **b** Expression of the FDC

Table 2 Summary of bioproduction of VG in two-phase bioreactor using the recombinant *E. coli* biocatalyst

Octane	FA added (g)	FA conversion in bioreactor (%)	VG yield in organic phase (%)	VG recovered in octane	Purified VG and purity	Overall production yield (%)
250 ml O/W=1:4	10.0	97	81.5	5.0 g in 230 ml	4.65 g (97.5%)	59
1000 ml O/W=1:1	25.0	>99	95	15.3 g in 850 ml	13.8 g (98.4%)	70

O/W octane/water ratio

that the whole-cell biocatalyst can be harvested at any time for biotransformation.

Characteristics of native *Bacillus pumilus* UI-670 and cloned *E. coli* JM109 [pKFAD]

Experiments characterized that the growth rate μ and the cell doubling time for the native *B. pumilus* UI-670 were 0.22 h^{-1} and 3.15 h, respectively. Those of the *E. coli* JM109[pKFAD] cells were 0.48 h^{-1} and 1.44 h, respectively. Specific activity was found to be 6–8 mmol/h/gDCW compared to 88–115 mmol/h/gDCW of the latter. Therefore, the volumetric productivity of VG of the cloned *E. coli* system (28–36 g/l/h) was at least ten times over the native *Bacillus* (2–2.6 g/l/h).

Octane as solvent for the two-phase biotransformation of FA

Table 1 shows the FA biotransformation activity in several solvents of different logP values. Polar solvents such as ethyl acetate resulted in half of the biotransformation rate compared to those of nonpolar solvents. Considering other criteria such as solvent separation, reutilization, manipulation safety, and product recovery, octane was selected as the solvent of choice for the desired biotransformation.

VG production, recovery, and purification

Two experimental results of FA biotransformation to VG are summarized in Table 2. The performance of varying ratio of organic to aqueous phase (1:4 and 1:1) was compared. Cultured cells at the end of growth phase were directly used for biotransformation. FA (25 g) was almost completely converted within 75 min (Fig. 6). A total amount of 21.3 g of VG (18.3 g in the organic phase, 3.0 g in the aqueous phase) was produced in the bioreactor.

After the biotransformation, the organic phase was recovered and separated by decanting and centrifugation. A total volume of 850 ml of octane containing VG (15.3 g, determined by HPLC) was collected; the remaining 150 ml octane trapped in a water-octane emulsion was discarded. The organic solvent was dried by addition of anhydrous Na_2SO_4 then filtered and evaporated using a vacuum rotary

evaporator. Final product, 13.8 g of VG, was obtained with a purity of 98.4%. Based on the initial 25 g FA starting material, an overall molar yield of 70 % was achieved.

Discussion

The feasibility of LL production in gram quantity through BVMO technology was demonstrated in a TPSCR system. Effectively, this growing cell mode ensures an adequate supply of NADPH cofactor that is needed for the bioconversion. One advantage of the TPSCR compared to a batch mode is the capacity to maintain biocatalytic stability over an extended period, thus, resulting in higher volumetric productivity and product yield (Staijen et al. 2000; Buhler and Schmid 2004; Gennaro et al. 2008). Supplementing biotransformation process with NADPH regenerating system is another strategy to possibly increase product yield.

To date, there is no available literature on the bioproduction of LL for a direct comparison. However, the production of lower ring-sized lactones such as C_6 ϵ -caprolactone from cyclohexanone by the widely studied *Acinetobacter*-derived cyclohexanone monooxygenase (CHMO) under nongrowing conditions was found to be about 0.8 g/l/h (Walton and Stewart 2002). Lee et al (2007)

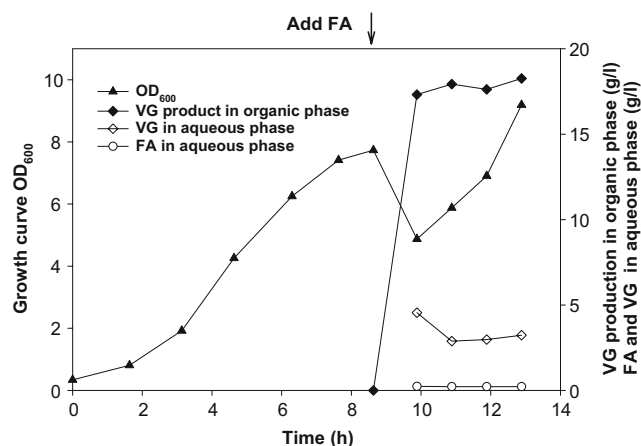


Fig. 6 Bioproduction of 4-vinylgaiacol in two-phase bioreactor. Filled triangles OD₆₀₀; filled diamonds VG product in organic phase; empty diamonds VG in aqueous phase; empty circles FA in aqueous phase

reported a 15.3-g/l yield of ϵ -caprolactone in a glucose-limited fed-batch reaction when glucose-6-phosphate dehydrogenase was co-expressed with CHMO. Perhaps, the best example of a scale-up BV biotransformation came from the strategy of in situ substrate feeding and product removal, known as SFPR (Hilker et al. 2004a, b). This methodology is resin-based where both the substrate and product can be released or adsorbed onto a resin (e.g., Amberlite XAD, Dowex Optipore L-493), thus, avoiding the substrate and product accumulation (inhibition) in the aqueous phase. Together with a glycerol feeding regime and a special bubble column to improve oxygenation, pH control, etc., near-kilogram amount of the targeted chiral lactone was obtained in >75% yield and high enantiopure form (*ee* >98%; Hilker et al. 2005, 2008). The biocatalyst employed in these studies (and others, e.g., (Baldwin and Woodley 2006)) was whole cells *E. coli* TOP10 (pQR239) that express *Acinetobacter* CHMO in an inducible manner by arabinose (Doig et al. 2001).

High substrate concentration, oxygen supply, product inhibition, and biocatalytic stability are the major issues that have been addressed in various BVMO bioprocess development (Baldwin and Woodley 2006; Kim et al. 2007; Hilker et al. 2008). However, in an industrial setting such as the pharmaceutical industry, BVMO technology is poised to play an increasingly important role in its special contribution to asymmetric synthesis of chiral compounds that meets the requirement of green chemistry and environmental sustainability (ten Brink et al. 2004; Constable et al. 2007; Woodley 2008). Based on substrate profiling with reference to chiral lactone formation, the CPDMO biocatalytic system appears to be best applied to the synthesis of 7-methyl-2-oxepanone lactone that can act as a surrogate building block for ω -2-hydroxyl heptanoic acid toward the production of a pheromone called daumone, that has an antiaging effect in worms (Jeong et al. 2005; Iwaki et al. 2006). In addition, an exciting and new avenue of opportunity arose recently by the capacity of CPDMO to oxidize 3- and 17-ketosteroids with full control of the regiochemistry of the resulting lactones and a recovery yield of up to 42% (Beneventi et al. 2009).

Decarboxylation of FA to VG is a potentially profitable value addition to the starting material especially if the FA can be extracted efficiently from its bound polysaccharides in the biomass (Rosazza et al. 1995). VG is priced some 40 times more than FA, and it can be biotransformed further to acetovanillone, ethylguaiacol, and vanillin (Rosazza et al. 1995; Mathew and Abraham 2006). As a styrene-type molecule, VG can be polymerized; the resultant oligomer [poly(3-methoxy-4-hydroxystyrene)] was found to be easily biodegradable (Hatakeyama et al. 1977).

Whole cells of *E. coli* JM109[pKAD] described in this study appeared to be an efficient biocatalyst under the

operating conditions in the TPPB system. The fact that the decarboxylation reaction does not need cofactor and the recombinant Fdc is also produced constitutively in *E. coli* make it potentially a versatile industrial biocatalyst. Also, immobilized cells can possibly contribute to prolonged stability and/or reuseability of the biocatalyst.

Lee et al (1998) carried out the decarboxylation of FA to VG by *B. pumilus* NRRL 14942 (DRV 52131) using hexane and a buffered phosphate system. The highest VG concentration achieved was about 10 g/l, the product was not recovered. The biochemical properties of the *B. pumilus* NRRL 14942 Fdc and UI-670 Fdc were reported to be different (Huang et al. 1994; Lee et al. 1998). The temperature optimum, pH optimum, and K_m towards FA were 37°C, 6.8, and 0.31 mM and 27–30°C, 7.3, and 7.9 mM, respectively. Several other Fdc/Pad-encoding genes from *L. plantarum*, *P. pentosaceus*, *B. subtilis*, and *B. pumilus* have been cloned and expressed in *E. coli* as well (Cavin et al. 1998; Barthelmebs et al. 2001; Rodriguez et al. 2008). These studies were, however, focused on structure–function relationship of the proteins. Interestingly, the Fdc or Pad is a relatively conserved group of proteins (60% identity); most of the amino acid substitutions that may be responsible for substrate specificity are located in the C-termini. The Fdc from *B. pumilus* UI-670 has been shown to decarboxylate *p*-coumaric acid (4-hydroxycinnamic acid) but not 2- or 3-hydroxycinnamic acid indicating that a 4-hydroxyl group is essential for the decarboxylation reaction (Rosazza et al. 1995).

In summary, this study reiterated the usefulness of the TPPB system in biocatalysis application in the production of two water-insoluble products, both of which have commercial potential. The challenge ahead is scalability of the respective chemical bioproduction and, most of all, if the source of the substrates can be derived from a renewable feedstock, e.g., agriculture residues or crops. Whereas an immediate bioresource of acyclic compounds such as cyclododecanone is not obvious, the cyclododecane structure is believed to be present in coal (Schumacher and Fakoussa 1999). The situation is, however, very different for ferulic acid. This aromatic acid is abundantly present in plant cell wall materials from both monocots and dicots, corn bran (3% w/w) in particular (Rosazza et al. 1995; Mathew and Abraham 2004). Work is in progress in our laboratory for the enzymatic extraction of FA from plant-derived biomass, such as, triticale, a manmade hybrid of wheat and rye, and a nonfood forage crop of economic importance in Canada and abroad.

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