Research Article

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High cell-density cultivation of phenolic acid decarboxylase-expressing *Escherichia coli* and 4-vinylguaiacol bioproduction from ferulic acid by whole-cell catalysis

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Abstract

BACKGROUND: 4-vinyl guaiacol (4-VG) is a high value-added product widely used in the cosmetic, pharmaceutical, and chemical industries. The practical bioproduction of 4-VG using phenolic acid decarboxylases has been limited by its relatively high biocatalyst cost and low yield and product concentration.

RESULTS: In the present study, high-cell density cultivation was employed to improve the activity and production of phenolic acid decarboxylase from *Bacillus licheniformis* (BLPAD) in recombinant *Escherichia coli*. The factors influencing enzyme production in *E. coli* such as the induction point and temperature for induction and feeding strategies were optimized. The highest BLPAD activity (531 U mL^{-1}) and productivity (20.4 U mL^{-1} h^{-1}), respectively, were achieved in a 5 L bioreactor using a glucose exponential feeding strategy with isopropyl β-D-thiogalactoside (IPTG) as inducer. Furthermore, a high BLPAD production level (512 U mL^{-1}) was achieved using lactose as an inducer and continuous lactose feeding. Using a biphasic emulsion system with equal volumes (1 L) of cyclohexane as a organic solvent and a substrate fed-batch strategy, the concentration and conversion yield of 4-VG reached 129.9 g L^{-1} (85.6%) in a 5 L bioreactor by whole-cell biocatalysis, which is the highest reported to date.

CONCLUSION: This study describes a strategy for large-scale 4-VG bioproduction using the biocatalytic method.

Keywords: phenolic acid decarboxylase; high cell-density cultivation; whole-cell catalysis; ferulic acid; 4-vinyl guaiacol

INTRODUCTION

4-vinyl guaiacol (4-VG), also known as 2-methoxy-4-vinyl phenol, is a high value-added product used in the food, cosmetic, pharmaceutical and chemical industries.[1–5] In addition, it can be converted through biocatalytic routes into products such as vanillin and ethyl guaiacol, which are widely used in perfumery.[6–8] 4-VG can also be utilized as the precursor for the synthesis of bio-based polymers such as oxygenated polystyrenes.[9,10] 4-VG has been commercially produced by chemical decarboxylation of ferulic acid (FA) in harsh conditions using metal catalysts. A promising alternative to the chemical route for 4-VG production is to employ phenolic acid decarboxylases (PAD) as biocatalysts to convert FA to 4-VG via decarboxylation.[3,11–13] Bioproduction of 4-VG has some advantages over the conventional chemical approach because of its high substrate specificity, mild reaction conditions, and eco-friendly procedures.[14,15] High levels of phenolic acid decarboxylase is a prerequisite for industrial scale 4-VG production. However, to date, the expression level of recombinant phenolic acid decarboxylase is relatively low, and the highest activity ever reported in *Escherichia coli* expression systems is <145 U mL^{-1}.[14,15] Furthermore, due to its toxicity and enzymatic inactivation, 4-VG production and final product concentration remain low for large-scale, commercial bioproduction.[8]

Previously, we reported a phenolic acid decarboxylase (BLPAD) with high organic solvent tolerance from *Bacillus licheniformis*, which exhibited outstanding catalytic activity in the bioconversion of FA into 4-VG at high substrate concentrations in an organic–aqueous two-phase system. These robust properties make it a promising biocatalyst for commercial bioproduction of 4-VG from FA.[15] Employing whole cells as biocatalysts circumvents steps such as cell lysis and enzyme purification, thereby significantly reducing production cost.[16] In this study, the fed-batch cultivation process was employed for high-cell density cultivation of phenolic acid decarboxylase-expressing *Escherichia coli*. The factors influencing enzyme expression in *E. coli* such as induction point and temperature as well as feeding strategies for induction were optimized. Furthermore, a biocompatible and effective two-phase reaction system using a substrate feeding strategy was employed for large-scale 4-VG bioproduction by whole-cell

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biocatalysis using a 5 L bioreactor. The aim of the present study was to resolve major drawbacks (high catalyst cost and low yield and product concentration) related to 4-VG bioproduction using phenolic acid decarboxylases.

MATERIALS AND METHODS

Bacterial strain

An *E. coli* Rosetta (DE3) strain harboring a pET28b (+) plasmid with the gene encoding a phenolic acid decarboxylase (BLPAD) from *B. licheniformis* with a Hisx6-tag at the C-terminus was used in all bioprocesses,[15] hereafter referred to as *E. coli* BLPAD. The host strain Rosetta (DE3) [genotype: F^- ompT hsdSB (rB^- mB^-) gal dcm (DE3) pRARE (Cam^R)] is a BL21 derivative that is designed to enhance the expression of proteins that contain codons rarely used in *E. coli*. This strain generates tRNAs for the AGG, AGA, AUA, CUA, CCC, GGA codons in a compatible chloramphenicol-resistant plasmid (Novagen, Madison, WI, USA).

Culture media

Luria-Bertani (LB) medium was used in shake flask cultivation, which contained 10 g L^{-1} NaCl, 10 g L^{-1} peptone, and 5 g L^{-1} yeast extract supplemented with 100 μg mL^{-1} of ampicillin (pH 7). Terrific Broth (TB) was used in seed culture, which contained 12 g L^{-1} tryptone, 24 g L^{-1} yeast extract, 5 g L^{-1} glycerol, 2.13 g L^{-1} KH_2PO_4, and 16.43 g L^{-1} $K_2HPO_4{\cdot}3H_2O$. The complex medium (CM) (pH 6.5) was used in high cell-density cultivation in a 5 L bioreactor (Sartorius BIOSTAT®Aplus), which contained 5 g L^{-1} glycerol, 2 g L^{-1} tryptone, 8 g L^{-1} yeast extract, 0.5 g L^{-1} NaCl, 2.5 g L^{-1} $(NH_4)_2SO_4$, 4 g L^{-1} $K_2HPO_4{\cdot}3H_2O$, 0.3 g L^{-1} ammonium ferric citrate, 2.1 g L^{-1} citric acid, and 0.5 g L^{-1} $MgSO_4{\cdot}3H_2O$ (pH 6.5). The glycerol feeding solution contained 10 g L^{-1} tryptone, 20 g L^{-1} yeast extract, and 400 g L^{-1} glycerol. All reagents were of analytical grade from various suppliers.

Fed-batch cultivation conditions

The fed-batch cultivation was performed in a 5 L bioreactor (Sartorius BIOSTAT®Aplus, 37079 Goettingen, Germany). Cultivation was initiated by inoculating 10% (v/v) of the seed culture into the CM medium (3 L) containing 100 μg mL^{-1} of kanamycin sulfate at a temperature of 37°C. The entire process was performed at pH 7 by automatic addition of 25% (v/v) ammonia. The dissolved oxygen (DO) level was maintained at 40% using a controlled pure O_2 aeration rate (2–4 L min^{-1}) and agitation rate (600–800 rpm). When the initial glycerol was completely used up as indicated by a sudden increase in DO, the feeding solution was added into the bioreactor at a rate of 10–40 mL L^{-1} h^{-1} (depending on culture time and DO level) to start the feeding phase. At the indicated induction phase, the temperature was lowered to the induction temperature, and 0.2 mmol L^{-1} of isopropyl β-D-thiogalactoside (ITPG) was added into the bioreactor to induce BLPAD production. To determine the effect of the induction point on BLPAD production, the cells were pre-cultured for 6 h, 10 h, 20 h (cell density reached an OD_{600nm} of around 10, 20, and 30, respectively), followed by the addition of 0.2 mmol L^{-1} IPTG to the bioreactor to induce BLPAD expression. To elucidate the effect of induction temperature on BLPAD production, the cells were pre-cultured for 10 h (cell density reached an OD_{600nm} of approximately 20), the culture temperature was decreased to a set temperature (28°C, 30°C, and 32°C), and 0.2 mmol L^{-1} IPTG was added to the bioreactor to induce BLPAD expression. When using glucose as carbon source, glucose was fed using a pH-stat or an exponential feeding strategy during the entire feeding stage. The pH-stat is based on the fact that pH rises when glucose is depleted. During the entire process, the glucose feeding solution was added as the pH increased to 7.1, and then stopped as pH decreased to 6.9. In the exponential fed-batch cultivation, the flow rate was increased based on an exponential feeding strategy to maintain cell growth at a specific growth rate of 0.125 h^{-1}. The exponential feeding rate was determined by a simple mass-balance equation of the cell and substrate.[17] A total of 780 mL of glucose feeding solution was added to the fermentor.

When using lactose as inducer, lactose was continuously added into the culture at a feeding speed of 12 mL L^{-1} h^{-1} to 15 mL L^{-1} h^{-1} as an inducer and carbon source, and maintained the concentration of lactose at >0.25 g L^{-1} for BLPAD production. BLPAD productivity (U mL^{-1} h^{-1}) was calculated by dividing BLPAD production (U mL^{-1}) by the induction time (h).

4-VG bioproduction in an organic–aqueous two-phase reaction system by whole-cell biocatalysis

The parameters of 4-VG bioproduction in a biphasic biotransformation system were first optimized in shaking tubes. The reaction was performed at 30°C and 200 rpm shaking for 30 min in 10 mL glass tubes with screw caps (Teflon seals) filled with 1.0 mL of different organic solvents and 1.0 mL of Na_2HPO_4–citric acid buffer (0.2 mol L^{-1}, pH 6.0) containing 50 mmol L^{-1} of FA and 1.4 mg of recombinant *E. coli* BLPAD cells. The bioconversion yield of 4-VG was determined in organic–aqueous two-phase reaction systems at different temperatures, pH levels, cell densities, and FA concentrations. The conversion yield was calculated by dividing the amount of product by the total amount of added reactant in molar ratio.

The 1 L scale 4-VG bioproduction using a substrate fed-batch strategy was performed in a 5 L bioreactor. Based on the flask experiments, the temperature, pH, and agitation speed (rpm) were set at 37°C, 7, and 200, respectively. The aqueous–organic two-phase biotransformation system consisted of 1 L of cyclohexane and 1 L of Na_2HPO_4-citric acid buffer (0.2 mol L^{-1}, pH 6) containing an initial 200 mmol L^{-1} of FA and 2.43 g L^{-1} of *E. coli* BLPAD. Because the maximum solubility of FA is about 200 mmol L^{-1} in the buffer, a defined amount of solid powder of FA was intermittently added into the aqueous phase after a certain time (30 g of FA at 60 min intervals) to obtain the desired product concentrations.

Assay methods

The phenolic acid decarboxylase activity was assayed as previously described.[15] Briefly, the standard assay was conducted in a mixture containing 0.8 mL of 200 mmol L^{-1} Na_2HPO_4–citric acid buffer (pH 6.0), 0.1 mL of 50 mmol L^{-1} FA, and 0.1 mL of diluted enzyme solution (1.7 μg) in a 10 mL glass tube with a screw cap (Teflon seals) at 37 °C for 5 min. One unit of enzymatic activity was defined as the enzyme required for the production of 1 μmol of 4-VG per min. For 4-VG bioproduction using biphasic biotransformation processes, the samples were collected from the organic phase and from the aqueous phase at different time intervals. The samples were further centrifuged at 20 800 × g for 5 min to separate the organic from the aqueous phase. The substrate and product formed were quantified by high-performance liquid chromatography (HPLC) as previously described.[15]

RESULTS AND DISCUSSION

Fed-batch cultivation of *E. coli* harboring phenolic acid decarboxylase

Effect of induction point on BLPAD production

Volumetric recombinant enzyme productivity mainly relied on cell density and cell-specific productivity during cultivation.[18] High-cell density cultures are usually achieved by fed-batch cultivation strategies; however, cell-specific productivity is significantly influenced by various cultivation parameters such as temperature, pH, and fed-batch feeding and induction strategies such as IPTG or lactose.[19,20]

Initial cell density plays an important role in the activity and productivity of recombinant enzymes using high-cell density cultivation.[19] Induction of protein expression using low cell density in the pre-induction phase can lead to certain metabolic and physiologic burdens in the post-induction phase, which in turn influences cell growth and protein expression.[18] In this study, 0.2 mmol L^{-1} IPTG was added at three time points (6 h, 10 h, and 20 h), at which the initial cell densities reached OD_{600nm} values of approximately 10, 20 and 30, respectively. Figure 1 shows that the growth of *E. coli* BLPAD was significantly inhibited, and the maximum production of BLPAD was <50 U mL^{-1} when induced at an initial cell density of $OD_{600nm} = 10$. When induced at $OD_{600nm} = 20$ and 30, final cell densities were $OD_{600nm} \sim 50$, indicating good cell growth. BLPAD peaked at 402 U mL^{-1} and 315 U mL^{-1}, respectively, suggesting that induction at $OD_{600nm} = 20$ is optimal for BLPAD production.

Effect of induction temperature on BLPAD production

Previous flask culture experiments have shown that lower induction temperatures (25–30°C) are associated with higher recombinant BLPAD production rates in *E. coli* compared with 37°C.[15] In many cases, low temperature is usually utilized to induce the expression of recombinant proteins and reduce the formation of inclusion bodies[21,22]; however, this particular condition also reduces the cell growth rates and prolongs induction time. Higher induction temperatures favor cell growth and protein synthesis, thereby improving recombinant protein expression by decreasing the induction time. In this study, three induction temperatures were employed to determine its influence on BLPAD production. Figure 2 shows that the application of induction temperatures of 28°C, 30°C, and 32°C results in a peak BLPAD production of 452 U mL^{-1}, 457 U mL^{-1}, and 522 U mL^{-1}, respectively, indicating that increasing the induction temperature up to 32°C enhances BLPAD production during fed-batch cultivation.

BLPAD production by feeding with glucose as carbon source

Glycerol has generally been used as a carbon source in fed-batch cultivation to improve recombinant protein production in *E. coli* to avoid acetic acid accumulation.[23] However, glucose has some advantages over glycerol in terms of plasmid stability and cell growth rate.[24,25] Acetate is an undesirable by-product for the fed-batch cultivation because it retards growth even at a low concentration and inhibits protein formation.[26] Previous studies have shown that pH-stat and exponential feeding promote cell growth and circumvent the accumulation of large amounts of acetate.[27–29] In this study, glycerol was replaced with glucose as the carbon source using the pH-stat or exponential feeding strategies to improve BLPAD production. Figure 3 shows that in both feeding strategies, BLPAD production reached >490 U mL^{-1} using glucose as carbon source, which is similar to that using glycerol as

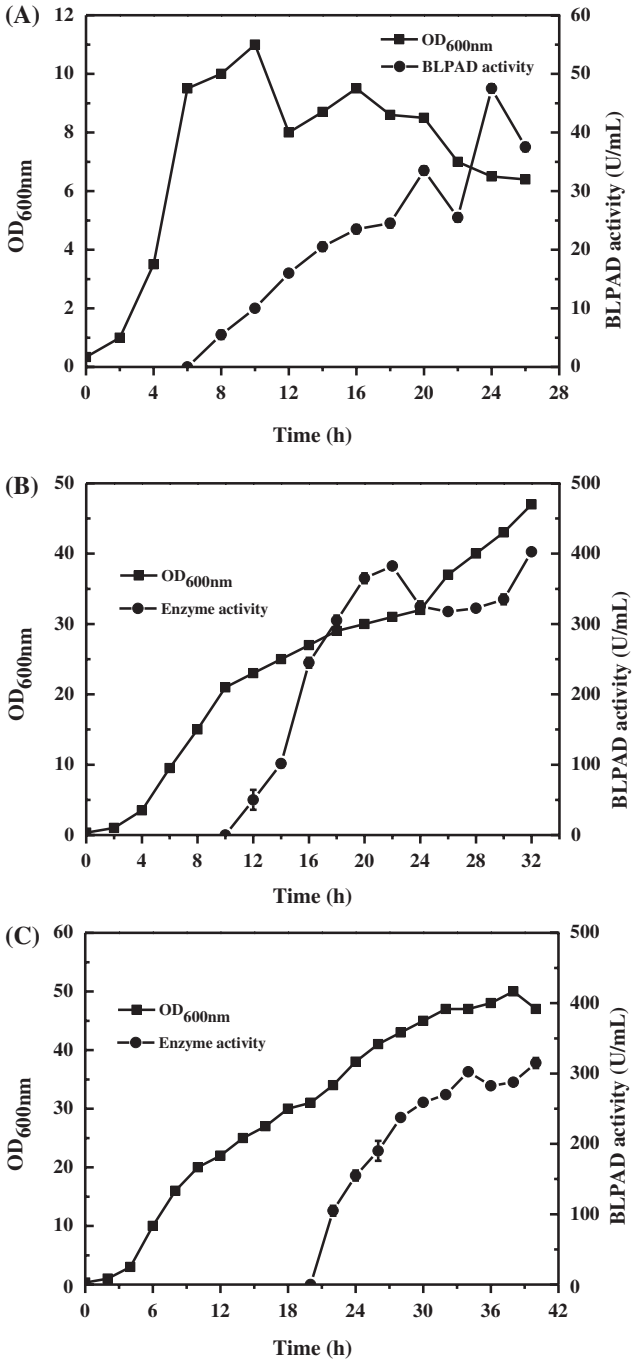

Figure 1. Effect of initial cell density on cell growth and BLPAD expression. A, $OD_{600nm} = 10$; B, initial cell density $OD_{600nm} = 20$; C, initial cell density $OD_{600nm} = 30$.

carbon source. However, in the exponential-feeding experiment, cell density and BLPAD production reached $OD_{600nm} > 60$ and >531 U mL^{-1}, respectively, after 24 h cultivation, indicating that the glucose exponential-feeding strategy significantly decreased fermentation time.

BLPAD production using lactose as inducer

The use of IPTG for large-scale production of recombinant proteins is generally considered inappropriate because of its toxicity and cost. IPTG can be replaced by lactose in fed-batch cultivation

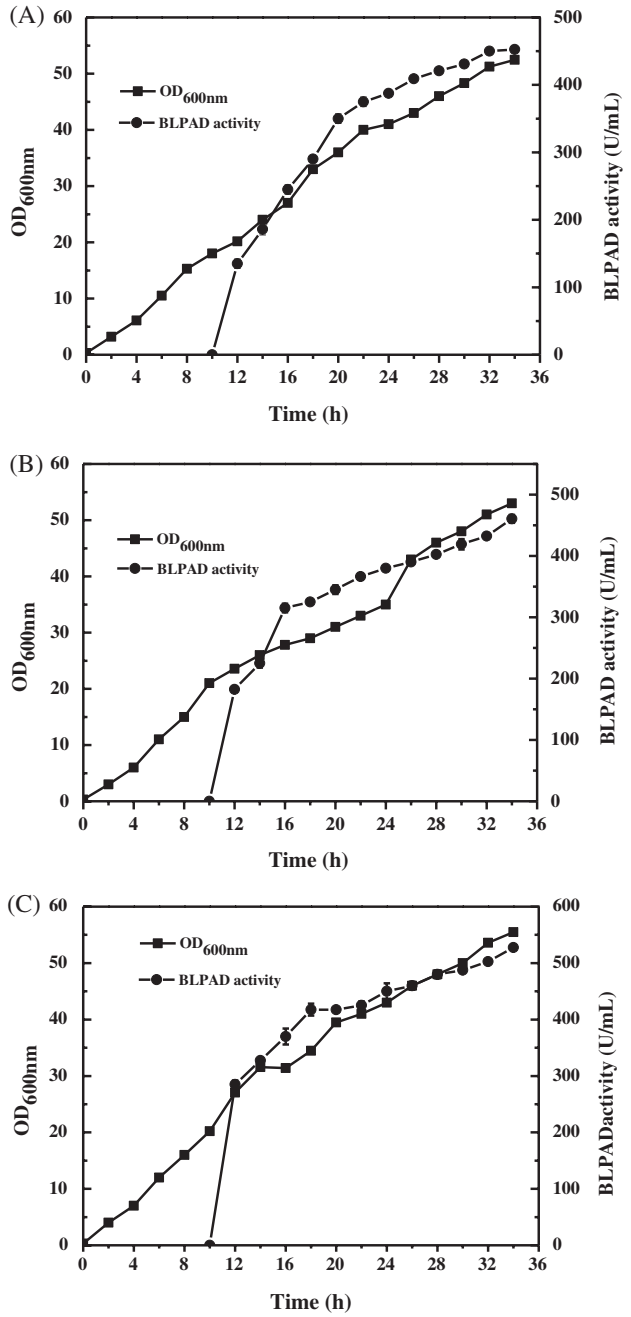

Figure 2. Effect of induction temperature on cell growth and BLPAD expression. A, 28°C; B, 30°C; C, 32°C.

processes to reduce its toxicity and cost.[30] However, the concentration of lactose that is used during induction of recombinant protein production is critical because it has a mild induction effect on foreign gene expression in *E*. coli.[18,31] The effect of lactose concentration on BLPAD production was initially investigated in shake flask cultures. As the initial lactose concentration reached 0.25 g L^{-1}, the induction effect was similar to that using IPTG at a concentration of 0.2 mmol L^{-1} (Table 1). Thus, in the subsequent fed-batch cultivation of *E. coli*, a continuous lactose feeding method was adopted to maintain the lactose concentration > 0.25% for the induction of BLPAD expression. Figure 4 shows that lactose induction results in BLPAD production of 512 U mL^{-1}, which is similar to that using IPTG.

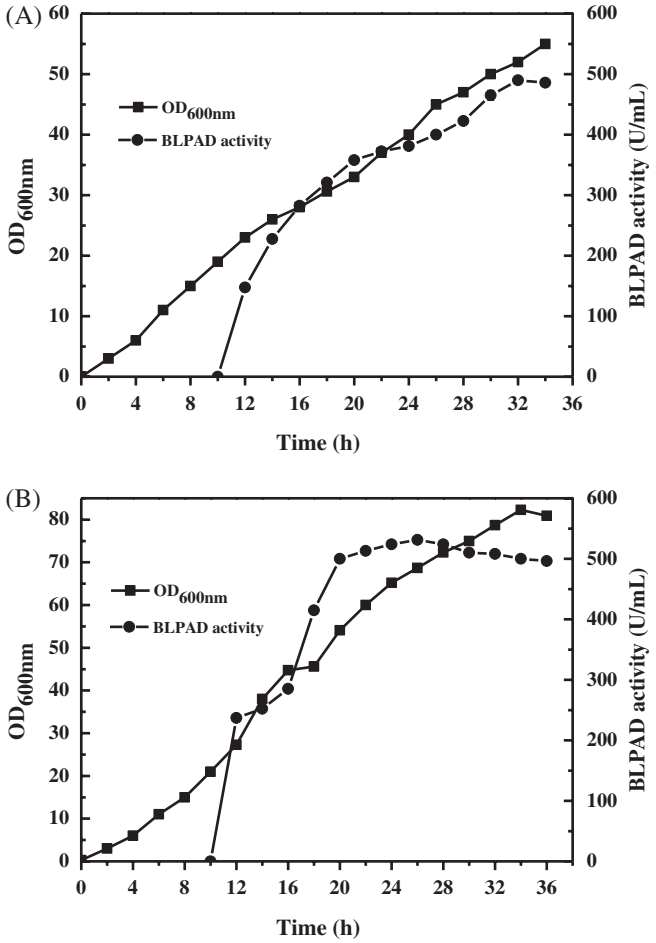

Figure 3. Effect of feeding strategy on cell growth and BLPAD expression using glucose as carbon source. A, pH-stat feeding strategy; B, Exponential-feeding strategy.

Table 1. Effect of lactose concentration on BLPAD expression

Lactose concentrations (g L^{-1})	BLPAD activity (U mL^{-1})
0.25	82.23 ± 1.23
0.5	85.02 ± 2.22
1	87.56 ± 0.24
2	83.23 ± 0.59
0.2 mmol L^{-1} (IPTG, reference)	80.99 ± 0.11

In our previous study, BLPAD production using flask cultures peaked at 144 U mL^{-1}.[15] In this study, the highest BLPAD activity and production in the fed-batch cultures was 531 U mL^{-1} and 20.4 U (mL h), respectively, using a glucose exponential-feeding strategy, which is 3.69-fold higher than that using flask cultures.

4-VG bioproduction in organic–aqueous two-phase bioreaction systems by whole-cell biocatalysis

Optimization of 4-VG bioproduction using biphasic systems in shake flasks

First, the effects of different organic solvents, temperatures, pH levels, cell densities, and FA concentrations on 4-VG bioproduction by whole-cell biocatalysis were investigated in shake flask cultures. Figure 5(A) shows that the highest 4-VG concentration

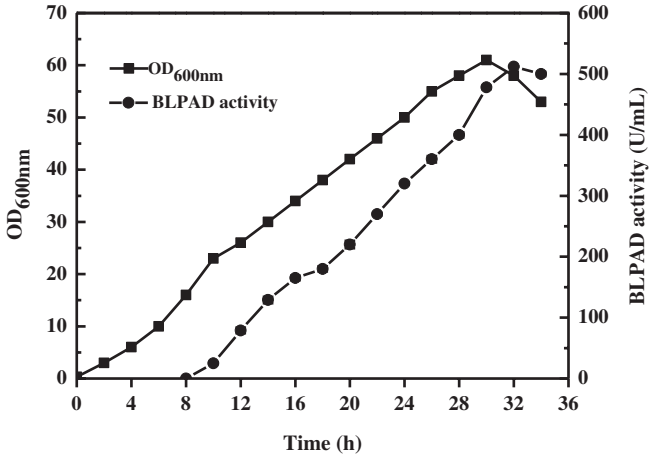

Figure 4. Time course of cell growth and BLPAD expression using lactose as inducer.

was obtained by using cyclohexane as solvent, followed by petroleum ether, toluene, 1-octane, and hexane. As expected, the concentration of 4-VG increased with cell density (Fig. 5(B)). The reaction temperature significantly influenced biocatalytic efficiency (Fig. 6(A)), and 4-VG concentration increased with temperature. The highest 4-VG concentration was obtained at 50°C, indicating that the optimal temperature for whole-cell biocatalysis is significantly higher than that for free BLPAD (37°C) due to the cell membrane protection.[15] However, at higher temperatures, BLPAD can be deactivated during biocatalysis,[15] and thus low temperature (37°C) was adopted in the 1 L biocatalysis system. Meanwhile, the optimal pH for whole cell biocatalysis was at 6–8, which is similar to that of free BLPAD (Fig. 6(B)). FA concentration also significantly affected 4-VG yield using a cyclohexane–aqueous two-phase biotransformation system. Table 2 shows that the conversion yield of 4-VG reached 96–99% after a 100- to 300-min reaction using an initial concentration of 100–200 mmol L^{-1}. Furthermore, 4-VG concentrations decreased over time (up to 20 h), which may possibly be due to its utilization by *E. coli*. However, 4-VG yield rapidly decreased as FA concentrations increased, so the initial concentration of FA was set at 200 mmol L^{-1} for the 1 L biocatalysis system in a 5 L bioreactor.

4-VG bioproduction in an organic–aqueous two-phase bioreaction system in a 5-L bioreactor

A scale-up experiment for 4-VG bioproduction was conducted in a 5 L bioreactor using *E. coli* BLPAD as the whole-cell biocatalyst. Three systems, including aqueous monophasic, biphasic with phase separation, and biphasic emulsion systems with a substrate fed-batch strategy, were compared in terms of 4-VG yield (Fig. 7). In the aqueous monophasic Na_2HPO_4–citric acid buffer system, 4-VG concentrations showed a slight increase after 120 h reaction, indicating that biocatalysis was significantly inhibited due to the accumulation and toxicity of both 4-VG and FA in the aqueous phase. Approximately 116 mmol L^{-1} (17.4 g L^{-1}, conversion yield: 38.7%) of 4-VG was obtained using 300 mmol L^{-1} of FA. In the biphasic system with phase separation, although the concentration of 4-VG gradually increased, the rate was significantly slower after 240 h of reaction, and FA substantially accumulated in the fed-batch substrate. Approximately 407 mmol L^{-1} (61.05 g L^{-1}, 40.7%) of 4-VG was obtained in the biphasic system with phase separation (Fig. 5). The concentration and conversion yield of 4-VG in the biphasic emulsion system with a substrate fed-batch strategy was the highest

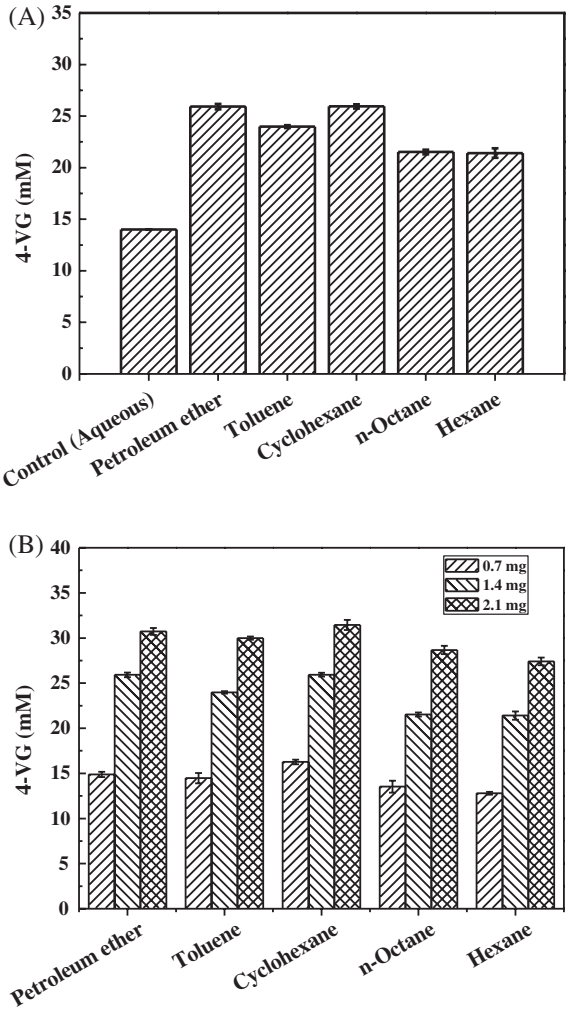

Figure 5. Effects of organic solvents (A) and cell densities (B) on 4-VG bioproduction by whole-cell biocatalysis in an organic–aqueous two-phase bioreaction system in shake flasks.

(866 mmol L^{-1}, 129.9 g L^{-1}, 85.6%) compared with that in the aqueous monophasic or in the biphasic system with phase separation. In the emulsion state, the continuous contact of the cells with the organic solvent may have permeabilized the cells, thereby facilitating substrate and product transport across the membranes, ultimately improving whole-cell biotransformation of 4-VG from FA using *E. coli* BLPAD.[14]

4-VG or 4-vinyl phenol (4-VP) bioproduction from phenolic acids using phenolic acid decarboxylases or whole cells with phenolic acid decarboxylase activity is generally associated with long reaction times, as well as low yield and product concentrations.[4,32] The observed low production rates of biocatalytic methods is partly due to the toxicity of 4-VG or 4-VP and enzymatic inactivation. A number of efforts had been made to improve final product concentrations and production using biphasic systems. A high 4-VG concentration (about 10 g L^{-1}) was achieved using whole cells of *Bacillus pumilus* as catalyst for the decarboxylation of FA to 4-VG in a hexane-buffered phosphate system.[33] Yang *et al.*[3] reported that 21.3 g of 4-VG (18.3 g in the organic phase, 3.0 g in the aqueous phase, 95% conversion yield in organic phase) was obtained in 1000 mL of an octane–aqueous two-phase biotransformation system using *E. coli* harboring a FA decarboxylase from *Bacillus*

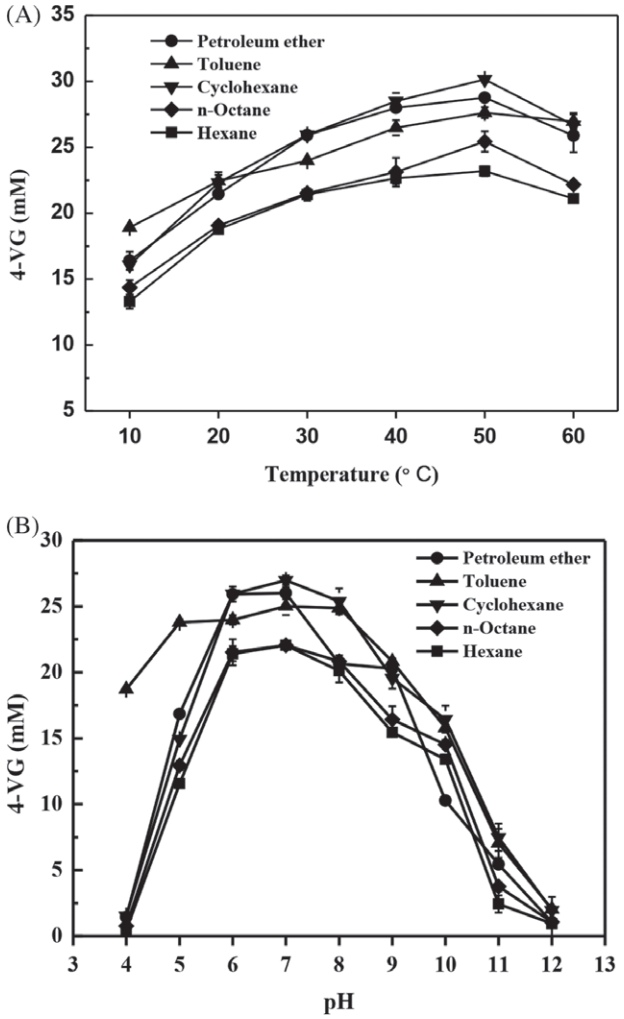

Figure 6. Effect of temperature (A) and pH (B) on 4-VG bioproduction by whole-cell biocatalysis in the organic–aqueous two-phase bioreaction system in shake flasks.

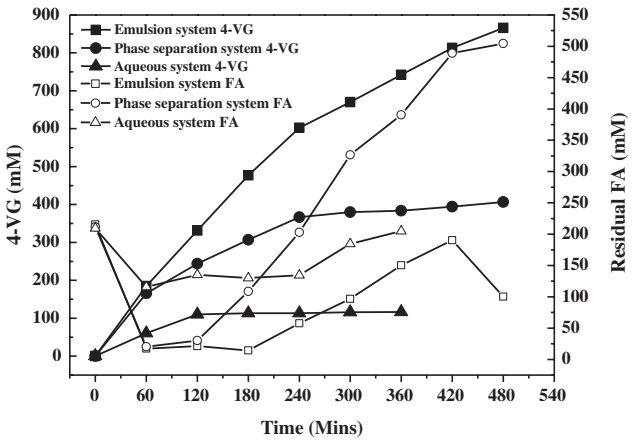

Figure 7. 4-VG bioproduction by whole-cell biocatalysis using three different organic–aqueous two-phase bioreaction systems in a 5-L bioreactor. The initial FA substrate concentration was 200 mmol L^{-1}, and recombinant *E. coli* harboring BLPAD was used with or without an equal volume of cyclohexane as organic solvent at 37 °C. The data are expressed as the mean of duplicate independent data sets. The total FA concentration added to the biocatalysis was 300 mmol L^{-1}, 1000 mmol L^{-1}, and 1000 mmol L^{-1} for aqueous monophasic, biphasic with phase separation, and biphasic emulsion systems, respectively.

pumilus. Ben-Bassat *et al.*[14] reported that 4-VP (17 g L^{-1}) was produced using the *para*-hydroxycinnamic acid decarboxylase (PDC) in 200 mL of a two-phase aqueous–organic solvent system. The present study describes an efficient and high-yield strategy for 4-VG bioproduction from FA using *E. coli* BLPAD that harbors a phenolic acid decarboxylase derived from *B. licheniformis* in a biphasic emulsion system using an equal volume ratio of cyclohexane to the Na_2HPO_4–citric acid buffer.

CONCLUSIONS

Fed-batch fermentation was employed for high-cell density cultivation of recombinant *E. coli* BLPAD harboring a phenolic acid decarboxylase gene from *B. licheniformis*. Using a glucose exponential-feeding strategy with IPTG as inducer, the highest BLPAD activity (531 U mL^{-1}) and production (20.4 U (mL h), respectively, were achieved in a 5 L bioreactor. Furthermore, high-level BLPAD activity (512 U mL^{-1}) was also achieved using lactose as inducer with the continuous lactose feeding strategy instead of IPTG as an inducer. Using a biphasic emulsion system with a substrate fed-batch strategy by whole-cell biocatalysis, the concentration and conversion yield of 4-VG peaked at 129.9 g L^{-1} and 85.6%, respectively, which are the highest values reported

Table 2. Effect of ferulic acid concentration on 4-VG bioproduction by whole-cell biocatalysis

Products and residual of substrates	Reaction time	Substrate concentration			
		200 mmol L^{-1}	300 mmol L^{-1}	400 mmol L^{-1}	500 mmol L^{-1}
Production of 4-VG (mmol L^{-1})	100 min	154.54 ± 2.51	229.39 ± 1.89	225.85 ± 1.69	221.13 ± 1.25
	200 min	198.99 ± 0.08	271.36 ± 1.08	266.96 ± 0.38	248.22 ± 3.75
	300 min	186.95 ± 0.86	288.73 ± 1.96	270.87 ± 1.59	250.71 ± 2.25
	12 h	178.73 ± 3.56	257.52 ± 1.95	224.59 ± 1.49	205.08 ± 2.31
Residual of ferulic acid (mmol L^{-1})	0 min	201.16 ± 1.16	300.72 ± 0.69	400.73 ± 0.71	501.51 ± 0.7
	100 min	16.71 ± 0.4	49.96 ± 1.35	167.45 ± 0.75	258.63 ± 1.97
	200 min	8.31 ± 0.4	29.49 ± 0.72	144.72 ± 0.53	238.69 ± 0.42
	300 min	4.89 ± 0.13	28.51 ± 0.71	142.58 ± 0.7	217.51 ± 0.54
	12 h	2.56 ± 0.62	21.62 ± 0.67	107.08 ± 1.2	176.66 ± 0.81
Overall conversion yields (%)		99.49 ± 0.12	96.24 ± 0.45	67.50 ± 0.89	50.14 ± 0.39

to date. This study provides a foundation for scaling up 4-VG bioproduction using a biocatalytic method.

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CONFLICTS OF INTEREST

None declared.

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