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Enhanced 3-methylcatechol production by *Pseudomonas putida* TODE1 in a two-phase biotransformation system

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In this study, genetically engineered *Pseudomonas putida* TODE1 served as a biocatalyst for the bioproduction of valuable 3-methylcatechol (3MC) from toluene in an aqueous-organic two-phase system. The two-phase system was used as an approach to increase the biocatalyst efficiency. Among the organic solvent tested, *n*-decanol offered several benefits including having the highest partitioning of 3MC, with a high 3MC yield and low cell toxicity. The effect of media supplementation with carbon/energy sources (glucose, glycerol, acetate and succinate), divalent metal cations (Mg^{2+}, Ca^{2+}, Mn^{2+} and Fe^{2+}), and short-chain alcohols (ethanol, *n*-propanol and *n*-butanol) as a cofactor regeneration system on the toluene dioxygenase (TDO) activity, cell viability, and overall 3MC yield were evaluated. Along with the two-step cell preparation protocol, supplementation of the medium with 4 mM glycerol as a carbon/energy source, and 0.4 mM Fe^{2+} as a cofactor for TDO significantly enhanced the 3MC production level. When in combination with the use of *n*-decanol and *n*-butanol as the organic phase, a maximum overall 3MC concentration of 31.8 mM (166 mM in the organic phase) was obtained in a small-scale production, while it was at 160.5 mM (333.2 mM in the organic phase) in a 2-L scale. To our knowledge, this is the highest 3MC yield obtained from a TDO-based system so far.

Key words: an aqueous-organic two-phase fermentation; bioproduction; 3-methylcatechol; *Pseudomonas putida* TODE1; toluene; toluene dioxygenase

Introduction

3-Methylcatechol (3MC) is an important precursor for the production of valuable pharmaceutical compounds such as barbatusol and L-DOPA analogues, as well as synthetic food flavors (Husken et al., 2002; Shirai, 1986). Due to difficulties and relatively low overall production yield from chemical synthesis (Held et al., 1999), 3MC production mainly relies on an economic and efficient biotechnological process, in which toluene is employed as a substrate for a bioconversion to 3MC using bacterial whole-cells as biocatalysts (Faizal et al., 2007; Wery et al., 2000). However, since toluene and 3MC, even at concentrations as low as 1% (v/v), detrimentally affect microbial survival and their catalytic activity, their toxicity, especially to 3MC generated and accumulated in the fermentation system, is the main constraint of the production process (de Smet et al., 1978; Fillet et al., 2012; Husken et al., 2001). Two common approaches to overcoming the toxicity limitation are 1) the use of an organic solvent-tolerant (OST) bacteria, which can thrive in relative high concentrations of organic compounds, as a biocatalyst, and 2) the use of a two-liquid (organic-aqueous) phase biotransformation system (referred to as a two-phase system hereafter), where the immiscible organic phase with microbial compatibility acts effectively as a source and a sink for the particular organic substrate and product (Heipieper et al., 2007).

3MC is typically produced by a genetically engineered bacterial host from a direct bioconversion of toluene by the action of *todC1C2BA* and *todD* genes encoding toluene dioxygenase (TDO), and *cis*-toluene dihydrodiol dehydrogenase, respectively. Since these genes are present in most, though not all, *Pseudomonas putida* strains (Faizal et al., 2007; Hüsken et al., 2001; Wery et al., 2000), genetic modifications of OST *P. putida* such as strain MC2 and strain MC1

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were conducted to increase gene dosage or gene expression level in order to enhance 3MC production yield (Hüsken et al., 2001; Wery et al., 2000). In addition, the 3MC production system could be improved by optimization of two-phase bioconversion conditions, e.g. the type of organic-solvent second phase, and the adjustment of bioproduction mode, i.e. batch or fed-batch, in a bioreactor (Prpich and Daugulis, 2007).

Upon employment of the abovementioned strategies for 3MC production, an organic-solvent tolerant *P. putida* T57 was previously genetically engineered creating the TODE1 mutant where the *todE* gene encoding methylcatechol 2,3-dioxygenase was disrupted to prevent further oxidation and thus facilitate the accumulation of 3MC (Faizal et al., 2007). A small-scale (10-ml scale) production of 3MC by the TODE1 mutant was then investigated in a single-phase and a two-phase system where glucose (2 g/L) and oleyl alcohol were provided as a bacterial carbon source and the organic phase in a two-phase fermentation system, respectively. Under their optimized production conditions using the TODE1 mutant, the 3MC production level at 24.4 mM was reported at the time as the highest yield (Faizal et al., 2007). However, it was stated that their biotransformation system was critically hindered from carbon catabolite repression in which half of TDO activity was repressed in the presence of glucose. As a consequence, the amelioration of 3MC production conditions remains to be investigated in order to enhance 3MC production by TODE1.

In this study, we aimed to enhance 3MC production in a two-phase system using TODE1 by focusing on the optimization of production conditions that either conserve or enhance TODE1 TDO activity. Moreover, to minimize the detrimental effect to cells from 3MC toxicity, and to achieve the highest 3MC production yield, various production parameters as well as cell preparation protocols were also investigated. This study not only strengthened the biotransformation concept for chemical synthesis using an organic-solvent tolerant bacterium in a two-liquid (organic-aqueous) phase fermentation system, but also successfully demonstrated the highest 3MC production yield reported so far.

Materials and Methods

Bacterial strain, cultivation medium and chemicals. *P. putida* TODE1 is a 3MC-accumulating mutant derived from OST *P. putida* T-57 in which the methylcatechol 2,3-dioxygenase gene (*todE*) was disrupted by a kanamycin cassette insertion (Faizal et al., 2007). The bacterial cultivation medium was either Luria-Bertani (LB) medium or minimal salt basal medium (MSB) (Faizal et al., 2005) as indicated. When MSB was supplemented with glucose (11 mM), it was referred to as MSBG, and with glycerol (4 mM) and $FeSO_4$ (0.4 mM), it was referred to as "the optimized medium" (OM-1 or OM-2, as indicated). All chemicals were analytical grade (chemical components for cultivation medium from Scharlau Chemie, Barcelona, Spain; toluene and aliphatic alcohols from Sigma-Aldrich, St. Louis, MO).

Partitioning of toluene and 3MC between aqueous and organic phases. The partitioning of 3MC between the aqueous and immiscible organic phases was examined under conditions resembling the production conditions. A sterile culture medium (10 ml), a test alcohol as an organic phase (1 ml), and a known concentration of 3MC were put together in a 70-ml screw-capped glass vial and incubated at 30°C on a shaker at 200 rpm for 20 h, and, then the content was centrifuged to separate the two phases. The 3MC concentration in each phase was analyzed using high performance liquid chromatography (HPLC) as described in the analytical method section, and then the partition coefficient was calculated (Wery et al., 2000). The test was conducted in a similar fashion to determine the partition coefficient of toluene.

Optimization of a small-scale 3MC production. TODE1 cells were initially grown as a seed prior to cell induction. The overnight-grown culture in LB medium was used as a seed to inoculate (1%, v/v) into MSB medium supplemented with 11 mM glucose (MSBG). After a 3-h incubation at 30°C to an OD_{600} of 0.5, toluene vapor was provided for the TDO induction. The incubation was continued for 7 h under the same conditions to reach an OD_{600} of 1.0.

The small-scale optimization of 3MC batch production was carried out in a 70-ml screw-capped vial. The volume ratio between cell suspension in the selected medium and the selected organic phase was initially tested (10 : 1, 10 : 3, and 10 : 5), and thereafter kept at the optimal volume ratio (indicated later on as 10 : 1). Toluene vapor was constantly provided. The influences of chemical and production conditions on 3MC production were then examined as follows. The effect of short-chain alcohol (ethanol, *n*-propanol, and *n*-butanol) was examined by providing it as a vapor phase by placing a small glass tube containing each chemical inside the vial. Effects of agitation speed (150, 200, and 280 rpm), initial pH (6.5, 7.0, 7.5, and 8.0), and temperature (28, 30, 35, and 37°C) were investigated. The 3MC concentration, [3MC], in the medium and in the organic phase was determined using HPLC. Then, the overall 3MC concentration was calculated accordingly: ([3MC] in aqueous phase × volume of aqueous phase + [3MC] in organic phase × volume of organic phase) / (volume of aqueous phase + volume of organic phase).

Effects of carbon/energy sources and metal divalent cations on the 3MC production level were tested as follows. Cells were grown as described above. The addition of acetate, glucose, glycerol, or succinate to the MSB medium as a carbon/energy source for the cells during the bioproduction was tested at various concentrations (as indicated in Table 1). Likewise, the effect of different concentrations (as indicated in Table 1) of the divalent metal ions: Mg^{2+}, Ca^{2+}, Mn^{2+} and Fe^{2+}, derived from $MgCl_2$, $CaCl_2$, $MnCl_2$ and $FeSO_4$, respectively, to the MSB medium, was tested. Cell survival, TDO activity, and 3MC production level were determined at 20 h of incubation. Then, the response surface methodology (RSM) with a central composite design (CCD) (Minitab, version 15, State College, PA) was used to optimize the formulation of the medium for 3MC production. Afterwards, the experiments were carried out to confirm the optimized production conditions.

Optimization of 3MC production in a 2-L bioreactor. A time-course analysis of the fed-batch 3MC production was conducted in a 2-L bioreactor (Model MDS-U, Marubishi Bioengineering Co., Ltd., Tokyo, Japan). The system

Table 1. Effects of carbon/energy source and metal ion on 3MC production in a two-phase system by *P. putida* strain TODE1.

Factors		TDO specific activity (μg indigo/min/OD_{600})[a]	3MC production (mM)[b]			Cell viability (log CFU/ml)[b]
			Aqueous phase	Organic phase	Overall	
Carbon and energy (mM)						
Glucose	3	4.75 ± 0.03	3.0 ± 0.8	76.2 ± 3.1	11.6 ± 1.0	7.36
	6	3.21 ± 0.02	3.1 ± 0.0	76.3 ± 7.7	12.7 ± 0.7	7.38
	11 (control)	2.43 ± 0.01	3.5 ± 0.2	69.3 ± 6.5	11.4 ± 0.8	7.43
Glycerol	3	4.20 ± 0.03	14.6 ± 0.8	131.0 ± 3.1	25.2 ± 1.0	7.63
	6	4.17 ± 0.02	13.1 ± 3.6	125.7 ± 9.2	22.6 ± 5.1	7.36
	11	3.87 ± 0.02	10.7 ± 0.8	103.9 ± 1.2	21.1 ± 0.8	7.29
Acetate	5	4.72 ± 0.02	9.8 ± 0.4	99.4 ± 3.1	17.9 ± 1.0	7.23
	10	4.87 ± 0.01	8.1 ± 0.7	86.7 ± 0.1	15.2 ± 0.6	6.90
	20	4.73 ± 0.02	8.1 ± 1.2	91.5 ± 6.6	15.4 ± 2.1	6.71
Succinate	5	5.57 ± 0.01	5.3 ± 4.2	89.8 ± 1.2	8.9 ± 2.0	7.04
	10	5.72 ± 0.02	11.0 ± 1.5	108.3 ± 8.1	19.8 ± 2.1	7.41
	20	4.29 ± 0.01	9.8 ± 0.5	121.7 ± 1.4	20.0 ± 0.3	7.46
Metal divalent ion (mM)						
Mg^{2+}	5	4.61 ± 0.02	8.8 ± 1.1	71.8 ± 4.2	14.5 ± 1.4	7.27
	10	4.63 ± 0.01	18.9 ± 1.5	98.2 ± 1.7	25.3 ± 2.6	7.16
	20	4.54 ± 0.01	12.3 ± 1.0	106.2 ± 3.8	20.9 ± 1.3	7.08
Ca^{2+}	1	3.73 ± 0.00	3.0 ± 0.5	76.2 ± 5.9	11.6 ± 0.1	7.38
	5	3.80 ± 0.01	4.1 ± 0.0	78.3 ± 7.7	12.9 ± 0.7	7.57
	10	3.87 ± 0.02	3.5 ± 0.2	69.3 ± 6.5	11.4 ± 0.8	6.80
Fe^{2+}	0.05	4.69 ± 0.03	12.2 ± 0.4	110.2 ± 2.0	21.2 ± 0.6	7.43
	0.10	4.67 ± 0.05	13.6 ± 0.7	123.5 ± 3.2	23.6 ± 1.0	7.56
	0.50	4.45 ± 0.04	12.3 ± 3.3	120.4 ± 9.7	21.4 ± 5.0	7.01
Mn^{2+}	0.01	4.65 ± 0.04	13.7 ± 0.3	124.7 ± 2.2	23.8 ± 0.5	7.25
	0.025	4.69 ± 0.03	15.3 ± 0.7	132.3 ± 5.0	25.9 ± 1.1	7.27
	0.05	4.24 ± 0.04	13.6 ± 1.7	107.2 ± 7.7	22.1 ± 0.8	7.37

[a]TODE1 cells were prepared as described in MATERIALS AND METHODS. Then, cells were resuspended and grown in MSB medium supplemented with various types and concentrations of carbon/energy sources and metal ions. When the initial cell number was at 7.0×10^6 CFU/ml (or log CFU/ml of 6.84), toluene was provided to induce TDO activity and to start the production. The production medium (11-mM-glucose containing MSB) reported by Faizal et al. (2007) was used as a control for comparison.

[b]3MC production was conducted in a small-scale two-phase system where *n*-decanol was used as an organic phase. Toluene and *n*-butanol vapor were provided as described in MATERIALS AND METHODS. The initial cell number was at log CFU/ml 6.84. After a 20-h production, cell viability and 3MC levels in aqueous and organic phases were determined prior to the calculation for overall 3MC production. Results are expressed as means ± standard deviation from at least three experiments.

comprised toluene-induced cells in 800 ml of either OM-1 or OM-2 medium (as described in the text), *n*-butanol and toluene at the indicated volume and concentrations, and *n*-decanol (80 ml) as the organic phase. The bioconversion period ran for 50 h with the temperature maintained at 30°C, the agitation rate between 300 and 500 rpm, and without the addition of an antifoam reagent under various conditions, as follows: toluene feeding (every 3, 6, or 10 h after initiating reaction), pH (6.5, 7.0 or 7.5; adjusted using 2 M NaOH) and aeration (0.5, 1.0, and 1.5 vvm).

Analytical methods. Cell growth measurement: Cell density in the aqueous phase was measured using a spectrophotometer at 600 nm (OD_{600}). Cell number measurement: The number of cells was assessed by colony counting of the serially diluted culture using 0.85% (w/v) saline on an LB agar plate after a 12-h incubation at 30°C, and expressed as colony forming units per milliliter (CFU/ml).

Toxicity effect of chemicals to TODE1: Cell survival after exposure to the test alcohol acting as an organic phase was determined by incubating cell suspension (OD_{600} at 1.0; approximately 10^{13} CFU/ml) (10 ml) with the test alcohol (1 ml) in a 70-ml screw-capped glass vial at 30°C on a shaker at 200 rpm for 20 h. Then, the number of viable cells was determined by colony counting, and compared to the number of cells without alcohol exposure. Cell survival after 3MC exposure was determined by incubating cell suspension with various concentrations of 3MC prior to colony counting analysis.

Enzyme assay: Toluene dioxygenase assay was determined by the formation of indigo from indole as previously described (Faizal et al., 2007).

Glucose level was determined using a glucose oxidase assay kit (Human Diagnostics Worldwide, Wiesbaden, Germany).

The analysis of toluene and 3MC concentration: The concentrations of toluene and 3MC in the aqueous and organic phase were determined by reverse phase HPLC with a UV detector at 240 nm. The separation was performed using Hyperclone 5 μ BDS C18 HPLC column (Phenomenex, Torrance, CA) with a mixture of acetonitrile and water (70 : 30, v/v) as a mobile phase at a flow rate of 1 ml/min. The injection volume was 20 μl. The HPLC retention time under the test conditions of toluene and 3MC was 5.4 min, and 3.0 min, respectively. The quantitative analysis was performed using a calibration curve generated from toluene or 3MC standard (Sigma-Aldrich).

Statistical analysis. All experiments were conducted with at least 3 replicates. The data reported are the average expressed with the standard deviation. Statistical analysis was carried out using one-way ANOVA with the Student-Newman-Keuls post-test method (GraphPad Instat 3

Software, San Diego, CA).

Results and Discussion

3MC toxicity and the selection of the organic phase of a two-phase biotransformation system.

It was previously reported that OST *P. putida* could tolerate 3MC up to 5.5 mM (Hüsken et al., 2001), while this study showed that the *P. putida* T57 wild-type strain as well as the TODE1 mutant strain could tolerate up to 7 mM 3MC when tested under growing-cell conditions. In this study, as the production goal was to produce 3MC at higher concentrations, the use of a high-density OST resting-cell-based bioproduction was employed instead of a growing-cell based one.

When a two-phase system is used, it should be remembered that bacterial cells can also be damaged by long-term exposure to an immiscible organic phase; therefore, it was essential to identify a suitable one. Previous studies showed that among aliphatic alkanes, alkenes, alcohols, esters, and oils tested for 3MC partitioning, aliphatic alcohols with relatively higher polarity and a log P_{ow} value between 2 and 7 demonstrated higher affinity to 3MC than less-polar solvents such as alkanes or esters with a log P_{ow} value larger than 8 (Prpich and Daugulis, 2007). In addition, in comparison to common bacteria, although OST bacteria typically exhibit relatively high tolerance to organic solvents, the tolerance level is likely to be organic solvent-type dependent and strain-dependent (Isken and Bont, 1998). Accordingly, in this study, the effects of five medium chain-to-long chain aliphatic alcohols on partitioning capacity towards toluene and 3MC and on TODE1 biocompatibility were tested. In this work, since toluene is the biotransformation substrate, a moderate amount of toluene should remain in the aqueous phase for cell uptake. Consequently, the preferred organic phase should only fairly allow toluene partitioning, while facilitating better partitioning of 3MC, which is more toxic to cells.

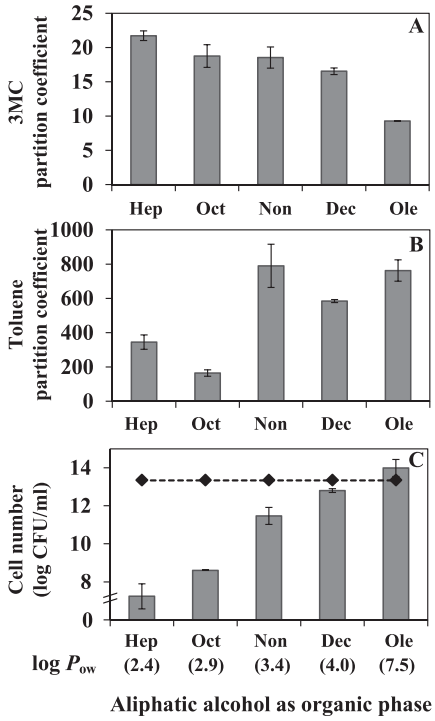

Fig. 1. Characteristics of alcohols as the organic phase in a two-phase 3MC bioproduction system.

The test alcohols included *n*-heptanol (Hep), *n*-octanol (Oct), *n*-nonanol (Non), *n*-decanol (Dec), and oleyl alcohol (Ole). Affinity of the organic phase to 3MC (A), and toluene (B) was determined and expressed as the partitioning coefficient of each target chemical. Biocompatibility of the test alcohols as the organic phase to TODE1 was examined (C). The dashed line represents the initial cell number (2.17×10^{13} CFU/ml) prior to the alcohol exposure test. Error bars represent the mean ± standard error from three determinations.

Among the five alcohols tested as the organic phase, the alcohols with a log P_{ow} value between 2.4 and 4.0 (from *n*-heptanol to *n*-decanol) exhibited affinity to 3MC (Fig. 1A), while those with a log P_{ow} value over 3.4 and under 7.5 (from *n*-nonanol to oleyl alcohol) showed affinity to toluene (Fig. 1B). This result agreed with the previous prediction (Prpich and Daugulis, 2007) in that 3MC has higher polarity with a log P_{ow} value of 1.6, so it could be partitioned well into the organic phase with relatively higher polarity, whereas it is *vice versa* for toluene. Further determination of an organic phase biocompatibity to TODE1 illustrated that *n*-nonanol and *n*-decanol were biocompatible with cells as they were viable up to 70% and 92%, respectively (Fig. 1C). Although oleyl alcohol was also shown to be biocompatible, it could be consumed to support cell growth, which may make it a poor candidate for economic reasons and may cause difficulty for cell biomass control in the bioconversion system. As a consequence, judging from 3MC and toluene partitioning affinities and TODE1 biocompatibity, *n*-decanol was selected as an organic phase for the bioconversion system.

Influence of short-chain alcohol on 3MC production by TODE1

It has been stated that oxygenase-based biocatalytic reactions are often faced with problems of high energy demand and redox cofactor consumption (Ebert et al., 2011). This adverse effect has been accentuated in OST bacteria because they require additionally high energy and a high NADH level to sustain the solvent tolerance property (Kuhn et al., 2010). In most cases, the problem can be minimized by coupling the system with in situ cofactor regeneration in which a common substrate, such as glucose, is provided for NADH regeneration (Weckbecker et al., 2010). The efficiency of 3MC production by TODE1 relied on toluene dioxygenase activity, but it was markedly inhibited by carbon catabolite repression when glucose was added (Faizal et al., 2007). Alternatively, since a dehydrogenase system of short-chain alcohol, such as ethanol, *n*-propanol, or *n*-butanol, was previously reported as an available system for in situ cofactor regeneration in *Pseudomonas* sp. (Hummel, 1997; Wichmann and Vasic-Racki, 2005), in this study, the effect of the indicated short-chain alcohol on TODE1 performance was examined and expressed as overall 3MC yield. Although the positive effect of the addition of short-chain alcohol on cell energy generation was theorized, the increase of TDO activity was not observed in this study (data not shown). On the other hand, a further test showed that the presence of each of the test short-chain alcohol was beneficial for the system in that it increased toluene partitioning into the medium by 25−75%. Regardless of the type of organic phase used, the positive effect was obviously shown

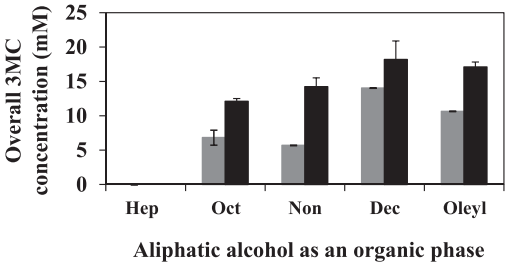

Fig. 2. Effect of *n*-butanol on 3MC production in a two-phase (aqueous-organic) system.

The organic phase included *n*-heptanol (Hep), *n*-octanol (Oct), *n*-nonanol (Non), *n*-decanol (Dec), and oleyl alcohol (Ole). 3MC production levels in the systems without *n*-butanol vapor (grey bars), and with *n*-butanol vapor (black bars) are shown. Error bars represent the mean ± standard error from three determinations.

with *n*-butanol addition (Fig. 2). In the presence of *n*-butanol, especially with *n*-decanol as an organic phase, 3MC overall production was significantly enhanced by 25% from 14.0 ± 0.3 mM to 17.0 ± 0.5 mM (Fig. 2). This positive effect was in agreement with the previous report where short-chain alcohol was used as a co-solvent in a two-phase system to reduce toluene-water interfacial tension and thus enhance toluene solubility in the aqueous phase (Lee and Peters, 2004). Because *n*-butanol was advantageous for 3MC production, it was incorporated into the two-phase production system thereafter.

Factors promoting overall production of 3MC in a two-phase system

The efficiency of 3MC bioproduction mainly depends on the catalytic activity of the TODE1 biocatalyst. The biocatalyst requires energy to function and metal ion cofactors to support the TOD enzyme function. Nevertheless, since *P. putida* strain TODE1 is unable to mineralize toluene as a growth substrate because it lacks the *tod*E gene, it is necessary to provide an alternative carbon/energy source to maintain cell activities as well as for efficient 3MC production. Glucose as a common and simple substrate cannot be used in this case because glucose at high concentration suppresses TDO activity in TODE1 (Faizal et al., 2007); thus the effects of various supplemented nutrients (four carbon/energy sources and four divalent metal ions) on the 3MC production level and cell viability were determined (Table 1).

The nutrient concentration ranges used in this study were chosen based on previous reports (Faizal et al., 2007; Husken et al., 2002; Wery et al., 2000). To determine the optimal carbon/energy source for 3MC production and for cell maintenance, the effects of succinate, glycerol and acetate supplementation were evaluated, while those in 11-mM glucose containing MSB (MSBG), which was previously reported by Faizal et al. (2007), were also evaluated as a control for comparison. Supplementation of the medium with acetate clearly promoted TDO activity and overall 3MC production level by approximately 2 and 1.5 fold, respectively, but at the cost of a reduced cell viability. Supplementation with succinate promoted an increased TDO activity (up to 2 fold) with a moderate increase in the overall 3MC production level. Interestingly, the presence of glycerol at 3−6 mM had no observable effect on cell viability, but promoted an increased TDO activity by 2 fold, and an increased overall 3MC production level to a maximum of approximately 25 mM with the increased production rate of 1.3 mM/h, while it was 0.6 mM/h of the control (Table 1).

Metal ions generally play essential roles as cofactors in the activities of enzymes as well as whole-cell biocatalysts. In particular, the role of divalent metal ions on TDO activity was previously noted (Zylstra and Gibson, 1989). To improve 3MC bioproduction by TODE1, we investigated the influence of four different divalent metal ions (Fe^{2+}, Mn^{2+}, Ca^{2+} and Mg^{2+}) on the TDO activity, 3MC production yield and cell viability (Table 1). These four divalent metal ions were selected based on specific functions in TDO enzyme activity, and their Gram-negative bacterial solvent-tolerance mechanism. Calcium (Ca^{2+}) has an important role in organic solvent tolerance of several OST bacteria, including the Gram-negative *Pseudomonas* and *Escherichia coli* K-12 (Isken and Bont, 1998). The decreased TDO activity in the presence of Ca^{2+} agreed with the low overall 3MC concentrations. Magnesium (Mg^{2+}) has been reported to improve cell tolerance towards organic solvents in *Pseudomonas* (Faizal et al., 2005; Isken and Bont, 1998). Although Mg^{2+} supplementation promoted a higher TDO activity (~2 fold), an increase in the overall 3MC yield was only observed at high concentrations of Mg^{2+} (10 and 20 mM). Mn^{2+} plays an important role in central carbon metabolism (Kehres and Maguire, 2003), while Fe^{2+} serves as a unique active site component of the TDO enzyme system in *P. putida*. Without Fe^{2+}, the enzyme structure may undergo a conformation change to an inactive state (Lee et al., 2005; Wackett and Gibson, 1988). All concentrations of Mn^{2+} and Fe^{2+} tested in this study resulted in an enhanced TDO activity and significantly increased the 3MC concentrations by 1.9- and 2.0-fold for Fe^{2+} and Mn^{2+}, respectively, compared to the control (Table 1).

Supplementation of the MSB medium with a different carbon/energy sources or divalent metal cations demonstrated the potential importance of the production medium on the 3MC production level by the TODE1 strain in a two-phase system. When added alone, glycerol, Fe^{2+} and Mn^{2+} had the greatest effect upon the 3MC production and so were selected for further optimization based on the obtained overall 3MC yield and TDO activity level.

To determine the optimal concentrations of glycerol, Fe^{2+} and Mn^{2+}, we employed a RSM approach with a CCD. The central points chosen for the experimental design were 4 mM glycerol, 0.2 mM Fe^{2+} and 0.025 mM Mn^{2+}, and the overall 3MC production level at 20 h was used as the response. In summary, all three selected components (glycerol, Fe^{2+} and Mn^{2+}) had a significant effect on the 3MC production level and a significant interaction between the glycerol and Fe^{2+} concentration was found. The optimal concentrations of glycerol and Fe^{2+} were predicted at 4 mM and 0.4 mM, respectively, while the combination of Mn^{2+} with other factors (either Mn^{2+} and glycerol, or Mn^{2+} and Fe^{2+}) did not enhance 3MC production and thus Mn^{2+} should not be added. Based on these results, the optimized medium (OM-1) for 3MC production by the TODE1 strain is MSB medium supplemented with 4 mM glycerol and 0.4 mM $FeSO_4$.

To confirm the prediction by the RSM approach with a CCD, four test systems for 3MC production were set up, all

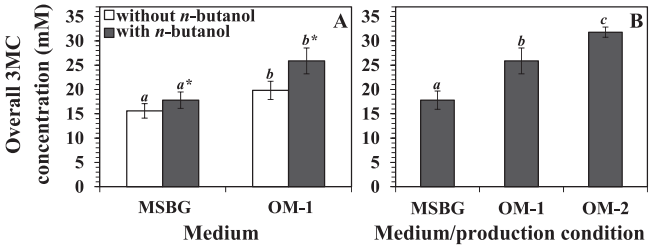

Fig. 3. 3MC production by TODE1 in a two-phase solvent system.

Overall 3MC level was determined at 20 h of production.The effect of *n*-butanol supplementation was examined on 3MC production that was carried out in two types of medium: an original medium MSBG, and the optimized medium OM-1 (A). When a two-step cell preparation protocol was developed, batch productions of 3MC were conducted, all in the presence of *n*-butanol, using three types of medium/production conditions: MSBG, OM-1, and OM-2 (B). Data are shown as means ± standard deviation and are derived from at least three independent experiments. Asterisks (*) and italic letters indicate a significant difference ($p < 0.05$) within the same and between groups, respectively, according to the Student-Newman-Keuls post-test.

having toluene vapor added as a substrate. Then, the production in two-phase systems with and without supplementation of *n*-butanol vapor was compared between the non-optimized MSBG and the optimized medium (OM-1), where glucose was completely replaced by glycerol (Fig. 3A). Again, the result confirmed the positive effect of *n*-butanol in that the overall 3MC concentration was enhanced in both the OM-1 and MSB medium, but a significantly higher (1.5-fold) overall 3MC production level was obtained in the OM-1 than in the MSB medium. Therefore, OM-1 had a potential use for larger scale 3MC production.

Two-step cell preparation of batch production of 3MC

In order to produce 3MC on a larger scale, two important criteria were taken into consideration, namely 1) the growth period of the starting cells and the preparation as a high-density cell suspension, and 2) 3MC production and cell viability during the production. Although the optimized medium OM-1 had been successfully applied for the small-scale production of 3MC by TODE1 in a two-phase system (as shown above), it was found that cells grown in glycerol-containing OM-1 medium had a 12-h lag period and the growth rate was as slow as 0.12 or 0.16 OD_{600}/h in the presence of 4 mM or 11 mM glycerol, respectively. On the other hand, cell growth in the non-optimized medium (MSBG) had a 4-h lag period with up to 3 times faster growth rate (0.35 OD_{600}/h). This result suggested that glucose was still preferred for a shorter cell cultivation time during the initial cell preparation step. Our test result later revealed that glucose was completely consumed for cell growth within 8 h of incubation. Accordingly, the cell preparation protocol and medium were modified for batch production of 3MC and named OM-2 (Fig. 3B). In brief, TODE1 cells were initially grown in 11-mM glucose containing MSB medium up to 8 h. Then, to start the 3MC production step, glycerol and Fe^{2+} were subsequently provided to the final concentration of 4 mM and 0.4 mM, respectively, while toluene and *n*-butanol were simultaneously supplied. With the two-step method with two sequential media (OM-2), not only the problems regarding slow growth rate and catabolite repression were prevented, but TDO activity could be promoted resulting in 30% enhancement of the 3MC production level from 25.0 mM to 31.8 mM within a 20-h production period (Fig. 3B). This work has successfully optimized various parameters affecting the performance of the TODE1 whole-cell biocatalyst to obtain a greater concentration of 3MC in a small-scale production compared to the previous reports (Table 2).

Fed-batch production of 3MC in a 2-L bioreactor

3MC production on a larger scale was further investigated in a 2-L bioreactor with a 0.8-L working volume. In the larger scale production, it would be more controllable if toluene and *n*-butanol were provided in a liquid form in a two-phase system. Concerning the toxicity of toluene and *n*-butanol to cells, the effect of various initial concentrations of liquid toluene (30−70 mM) and *n*-butanol (1−40 mM) on 3MC production was examined. The result showed that

Table 2. Comparison of 3MC production from toluene by various *Pseudomonas* strains and production systems.

Microorganism	Production system (Medium)[a]	3MC production (mM)			References
		Aqueous phase	Organic phase	Overall	
Small scale: 10 ml					
P. putida MC2	Single phase (MM)	14.0	—	—	Husken et al. (2001)
P. putida MC2	Single phase (MM)	14.2	—	—	Husken et al. (2002)
P. putida DS10	Two-phase (LB/*n*-octanol)	7.0	11.5	8.3	Wery et al. (2000)
P. putida MC2	Two-phase (LB/*n*-octanol)	3.8	83.0	19.6	Husken et al. (2001)
P. putida TODE1	Two-phase (MSB/oleyl alcohol)	16.1	107.3	24.4	Faizal et al. (2007)
P. putida TODE1	Two-phase (OM-2/*n*-decanol)	28.3	166.5	31.8	This study
Bioreactor scale: 800 ml					
P. putida TODE1	Two-phase (OM-2/*n*-decanol)	142.9	333.2	160.5	This study

[a]MM: Minimal medium supplemented with 80 mM glucose (Husken et al., 2001); LB: Luria-Bertani medium; MSB: Minimal salt basal medium (Faizal et al., 2007); OM-2: the optimized medium with two-step production (This study).

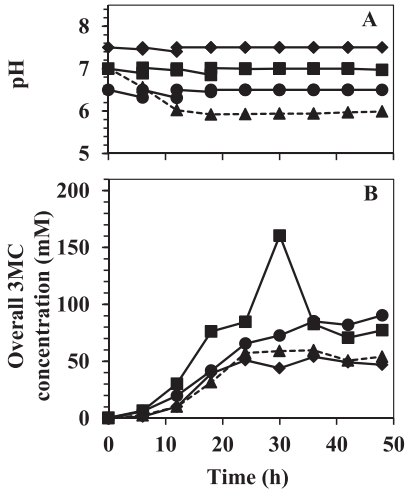

Fig. 4. Time course of fed-batch production of 3MC by *P. putida* strain TODE1 using a two-phase system in a 2-L bioreactor.

The production was conducted under pH control at: pH 6.5 (●), pH 7.0 (■), pH 7.5 (◆), and without pH control (▲, dashed line) at 30°C, 1 vvm and 400 rpm. The system pH (A) and overall 3MC production level (B) were monitored.

supplementation of toluene at an initial concentration at 60 mM and *n*-butanol at 10 mM yielded the highest 3MC production rate of 2.24 ± 0.89 mM/h, and 1.40 ± 0.16 mM/h, respectively.

The main production conditions were maintained similarly to the small-scale production, i.e. the volume ratio of 10 : 1 between an aqueous phase and *n*-decanol as an organic phase, the two-step cell preparation (OM-2) and the initial cell density (initial OD_{600} of 1.0), and the production temperature of 30°C. Nevertheless, the production conditions were further optimized to enhance the 3MC production level including the oxygen level, pH, and toluene feeding.

Since the oxygen level (as the culture medium: headspace (v/v) ratio) had a strong influence on the 3MC production level obtained in the small-scale (10 ml) reaction, the effect of aeration and agitation in the 2-L bioreactor to improve the oxygen supply was also investigated. The highest 3MC concentration after a 30 h cultivation was obtained in the presence of 1 vvm aeration and 400 rpm agitation (data not shown), and therefore these conditions were adopted for the rest of the study.

The production of 3MC has been reported to be pH-dependent (Husken et al., 2001). Therefore, the effect of the pH on the fed-batch production of 3MC by TODE1 was evaluated at three controlled pH values and compared to that of an uncontrolled pH (Fig. 4). Without any buffering, the pH decreased from the initial pH 6.9 to a stable value at pH 5.9 within 18 h, having a 3MC production rate of 1.96 mM/h. Of the three controlled pH conditions, that at pH 7.0 provided the highest 3MC production rate of 5.4 mM/h and the highest overall 3MC production level at 160.5 mM after a 30-h production period.

During the bioproduction of 3MC, toluene was constantly consumed and partly evaporated due to aeration and agitation in the bioreactor. Thus, a fed-batch production system with a regular supply of toluene at 60 mM was adopted to increase the final concentration and yield of 3MC. Under a 1-vvm aeration rate and agitation at 400 rpm, the regular supplementation of toluene every 6 h resulted in a 7.9- and 2.4-fold

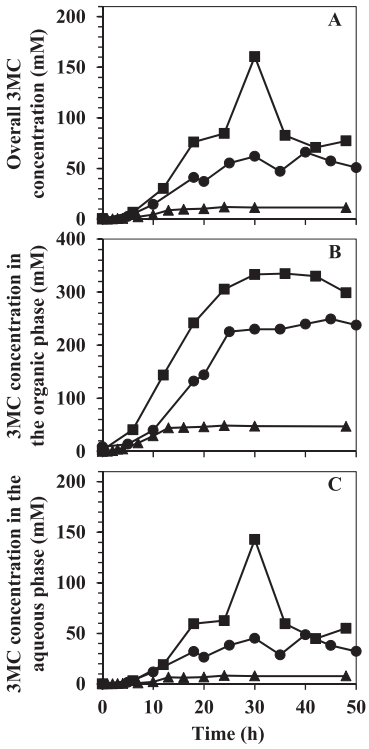

Fig. 5. Time course results of fed-batch production of 3MC by *P. putida* strain TODE1 using a two-phase system in a 2-L bioreactor.

The fed-batch fermentation was performed at 30°C. The initial concentration of toluene was 60 mM. Toluene was fed at the same concentration at various times (▲, 3 h; ■, 6 h; and ●, 10 h). The 3MC concentrations were monitored as overall 3MC (A), 3MC in the organic phase (B), and 3MC in the aqueous phase (C). The process was carried out in OM-2 medium at 1 vvm aeration, 400 rpm agitation, at pH 7.0.

higher 3MC production than that obtained with toluene supplementation at 3 and 10 h, respectively (Fig. 5A–C). Thus, the optimum conditions for the fed-batch production of 3MC by TODE1 were 60 mM of toluene supplied every 6 h, 30°C, pH 7.0, 1.0 vvm of aeration and agitation at 400 rpm.

However, during the production, the color of the medium changed from white to dark reddish-brown after 30 h of cultivation. Increase of the color intensity corresponded to the decrease of 3MC level in the aqueous phase (Fig. 5C), and thus overall 3MC level (Fig. 5A), but not in the organic phase (Fig. 5B). This is in agreement with previous reports that 3MC is unstable in aqueous phase at a relatively basic pH and either undergoes oxidation or chemical degradation (Husken et al., 2001, 2002) or polymerization to form *o*-benzoquinone (Borraccino et al., 2001; Hüsken et al., 2001; Mijangos et al., 2006; Wery et al., 2000), while it was more stable in organic phase (Fig. 5B). This fact strengthens the advantage of a two liquid-phase (aqueous-organic) fermentation for the production of 3MC. The product accumulated in the organic phase is more stable and can be further recovered by downstream recovery process.

Conclusion

In summary, this study demonstrated how a two liquid-phase (aqueous-organic) fermentation can improve the bioproduction of water-insoluble products using 3MC as a model product. The bioproduction efficiency was based on

toluene dioxygenase activity of *P. putida* strain TODE1; therefore appropriate nutrients and metal ions were identified for the optimal fermentation medium, while the two-step cell preparation protocol/medium was developed to minimize some limitations and to enhance bioproduction efficiency. A suitable organic solvent and a co-solvent as well as the bioconversion process for a two-phase system were then optimized. Our approach and findings are applicable to similar target compounds and host systems, although they would need to be modified according to the individual reaction and experiment goals.

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References

Borraccino, R., Kharoune, M., Giot, R., Agathos, S. N., Nyns, E. et al. (2001) Abiotic transformation of catechol and 1-nephthol in aqueous solution—Influence of environmental factors. *Water Res*., **35**, 3729−3737.

de Smet, M. J., Kingma, J., and Witholt, B. (1978) The effect of toluene on the structure and permeability of the outer and cytoplasmic membranes of *Escherichia coli*. *Biochim*. *Biophys*. *Acta — Biomembranes*, **506**, 64−80.

Ebert, B. E., Kurth, F., Grund, M., Blank, L. M., and Schmid, A. (2011) Response of *Pseudomonas putida* KT2440 to increased NADH and ATP demand. *Appl*. *Environ*. *Microbiol*., **77**, 6597−6605.

Faizal, I., Dozen, K., Hong, C. S., Kuroda, A., Takiguchi, N. et al. (2005) Isolation and characterization of solvent-tolerant *Pseudomonas putida* strain T-57, and its application to biotransformation of toluene to cresol in a two-phase (organic-aqueous) system. *J*. *Ind*. *Microbiol*. *Biotechnol*., **32**, 542−547.

Faizal, I., Ohba, M., Kuroda, A., Takiguchi, N., Ohtake, H. et al. (2007) Bioproduction of 3-methylcatechol from toluene in a two-phase (organic-aqueous) system by a genetically modified solvent-toleant *Pseudomonas putida* strain T-57. *J*. *Environ*. *Biotechnol*., **7**, 39−44.

Fillet, S., Daniels, C., Pini, C., Krell, T., Duque, E. et al. (2012) Transcriptional control of the main aromatic hydrocarbon efflux pump in *Pseudomonas*. *Environ*. *Microbiol*. *Rep*., **4**, 158−167.

Heipieper, H. J., Neumann, G., Cornelissen, S., and Meinhardt, F. (2007) Solvent-tolerant bacteria for biotransformations in two-phase fermentation systems. *Appl*. *Microbiol*. *Biotechnol*., **74**, 961−973.

Held, M., Schmid, A., Kohler, H. P., Suske, W., Witholt, B. et al. (1999) An integrated process for the production of toxic catechols from toxic phenols based on a designer biocatalyst. *Biotechnol*. *Bioeng*., **62**, 641−648.

Hummel, W. (1997) New alcohol dehydrogenases for the synthesis of chiral compounds. *Adv*. *Biochem*. *Eng*. *Biotechnol*., **58**, 145−184.

Hüsken, L. E., Beeftink, R., Bont, J. A. M. d., and Wery, J. (2001) High-rate 3-methylcatechol production in *Pseudomonas putida* strains by means of a novel expression system. *Appl*. *Microbiol*. *Biotechnol*., **55**, 571−577.

Husken, L. E., Bont, J. A. M. d., Beeftink, R., and Wery, J. (2002) Optimisation of microbial 3-methylcatechol production as ffected by culture conditions. *Biocatal*. *Biotransform*., **20**, 57−61.

Husken, L. E., Dalm, M. C. F., Tramper, J., Wery, J., Bont, J. A. M. d. et al. (2001) Integrated bioproduction and extraction of 3-methylcatechol. *J*. *Biotechnol*., **88**, 11−19.

Isken, S. and Bont, J. A. M. d. (1998) Bacteria tolerant to organic solvents. *Extremophiles*, **2**, 229−238.

Kehres, D. G. and Maguire, M. E. (2003) Emerging themes in manganese transport, biochemistry and pathogenesis in bacteria. *FEMS Microbiol*. *Rev*., **27**, 263−290.

Kuhn, D., Blank, L. M., Schmid, A., and Bühler, B. (2010) Systems biotechnology — Rational whole-cell biocatalyst and bioprocess design. *Eng*. *Life Sci*., **10**, 384−397.

Lee, K., Friemann, R., Parales, J. V., Gibson, D. T., and Ramaswamy, S. (2005) Purification, crystallization and preliminary X-ray diffraction studies of the three components of the toluene 2,3-dioxygenase enzyme system. *Acta Crystallogr*. *Sect*. *F Struct*. *Biol*. *Cryst*. *Commun*., **61**, 669−672.

Lee, K. Y. and Peters, C.A. (2004) UNIFAC modeling of cosolvent phase partitioning in nonaqueous phase liquid-water systems. *J*. *Environ*. *Eng*., **130**, 478−483.

Mijangos, F., Varona, F., and Villota, N. (2006) Changes in solution color during phenol oxidation by fenton reagent. *Environ*. *Sci*. *Technol*., **40**, 5538−5543.

Prpich, G. P. and Daugulis, A. J. (2007) Solvent selection for enhanced bioproduction of 3-methylcatechol in a two-phase partitioning bioreactor. *Biotech*. *Bioeng*., **97**, 536−543.

Shirai, K. (1986) Screening of microorganisms for catechols production from benzene. *Agric*. *Biol*.*Chem*., **50**, 2875−2880.

Wackett, L. P. and Gibson, D. T. (1988) Degradation of trichloroethylene by toluene dioxygenase in whole-cell studies with *Pseudomonas putida* F1. *Appl*. *Environ*. *Microbiol*., **54**, 1703−1708.

Weckbecker, A., Groger, H., and Hummel, W. (2010) Regeneration of nicotinamide coenzymes: Principles and applications for the synthesis of chiral compounds. *Adv*. *Biochem*. *Eng*. *Biotechnol*., **120**, 195−242.

Wery, J., Mendes da Silva, D. I., and Bont, J. A. M. d. (2000) A genetically modified solvent-tolerant bacterium for opimized production of a toxic fine chemical. *Appl*. *Microbiol*. *Biotechnol*., **54**, 180−185.

Wichmann, R. and Vasic-Racki, D. (2005) Cofactor regeneration at the lab scale. *Adv*. *Biochem*. *Eng*. *Biotechnol*., **92**, 225−260.

Zylstra, G. J. and Gibson, D. T. (1989) Toluene degradation by *Pseudomonas putida* F1. Nucleotide sequence of the *todC1C2BADE* genes and their expression in *Escherichia coli*. *J*. *Biol*. *Chem*., **264**, 14940−14946.