AIChE

Transformation of Ferulic Acid to Vanillin Using a Fed-Batch Solid–Liquid Two-Phase Partitioning Bioreactor

Xiao-kui Ma
Key Laboratory of Medicinal Resources and Natural Pharmaceutical Chemistry, Ministry of Education, National Engineering Laboratory for Resource Developing of Endangered Chinese Crude Drugs in Northwest of China, College of Life Sciences, Shaanxi Normal University, Xi'an 710062, Shaanxi, China

Andrew J. Daugulis
Dept. of Chemical Engineering, Queen's University, Kingston, Ontario K7L3N6, Canada

DOI 10.1002/btpr.1830
Published online October 26, 2013 in Wiley Online Library (wileyonlinelibrary.com)

Amycolatopsis sp. ATCC 39116 (formerly Streptomyces setonii) has shown promising results in converting ferulic acid (trans-4-hydroxy-3-methoxycinnamic acid; substrate), which can be derived from natural plant wastes, to vanillin (4-hydroxy-3-methoxybenzaldehyde). After exploring the influence of adding vanillin at different times during the growth cycle on cell growth and transformation performance of this strain and demonstrating the inhibitory effect of vanillin, a solid–liquid two-phase partitioning bioreactor (TPPB) system was used as an in situ product removal technique to enhance transformation productivity by this strain. The thermoplastic polymer Hytrel® G4078W was found to have superior partitioning capacity for vanillin with a partition coefficient of 12 and a low affinity for the substrate. A 3-L working volume solid–liquid fed-batch TPPB mode, using 300 g Hytrel G4078W as the sequestering phase, produced a final vanillin concentration of 19.5 g/L. The overall productivity of this reactor system was 450 mg/L. h, among the highest reported in literature. Vanillin was easily and quantitatively recovered from the polymers mostly by single stage extraction into methanol or other organic solvents used in food industry, simultaneously regenerating polymer beads for reuse. A polymer–liquid two phase bioreactor was again confirmed to easily outperform single phase systems that feature inhibitory or easily further degraded substrates/products. This enhancement strategy might reasonably be expected in the production of other flavor and fragrance compounds obtained by biotransformations.
© 2013 American Institute of Chemical Engineers *Biotechnol. Prog.*, 30:207–214, 2014
Keywords: Amycolatopsis *sp. ATCC 39116, bioconversion, ferulic acid, vanillin, two-phase partitioning bioreactor, Hytrel G4078W*

Introduction

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is one of the most widely used flavor chemicals in the food, cosmetics, and pharmaceutical industries and is responsible for the well-known vanilla aroma. Two commercial types exist: pure vanillin obtained by chemical synthesis from guaiacol and natural vanillin from vanilla beans, whose cost is approximately US$3200 per kg to US$4000 per kg.[1] The higher price of natural vanillin, combined with the increasing customer-led demand for natural flavors, has stimulated a growing interest to produce natural vanillin by biotransformations from natural substrates.[2,3] The microbial transformation of natural precursors by living microbial cells or their enzymes can generate the desired bio-vanillin and is considered to be a "natural" product by international standards such as is found in European legislation.

Among studies related to the microbial bioproduction of vanillin, transformations by the *Actinomycetes*, and in particular several species of this genera such as *Streptomyces* and *Amycolatopsis* sp. ATCC 39116 (formerly *Streptomyces setonii*),[4,5] have been shown to be particularly efficient in utilizing hydroxycinnamic acids, especially ferulic acid (*trans*-4-hydroxy-3-methoxycinnamic acid), which can be naturally derived from agro-industrial waste,[6] with relatively high yield of product and minimal by-products.[4,5,7] However, the yield of vanillin in batch,[5] or even in fed-batch[4] culture at flask scale, is still far below the final product concentration that is required for a commercially competitive processes.[8] The low yield of vanillin may arise from the inhibitory effect of vanillin on cell growth and metabolic activity during the transformation, but there is a lack of direct evidence for this, even though the metabolic pathway of vanillin and the genome sequence of *Amycolatopsis* such as *Amycolatopsis* sp. ATCC 39116 have been shown previously,[5,9]

There are at least two molecules in the transformation including vanillin and guaiacol resulting from vanillin transformation by *Streptomyces*[5] that may be toxic to the cells, as

Correspondence concerning this article should be addressed to A. J. Daugulis at andrew.daugulis@chee.queensu.ca.

© 2013 American Institute of Chemical Engineers

Table 1. Polymer Properties and Partition Coefficients for Vanillin and Ferulic Acid

Polymer	Supplier	Glass Transition Temperature, T_g (°C)	Specific Gravity	Description	Partition Coefficient for Vanillin	Partition Coefficient for Ferulic acid
Hytrel 8238	DuPont	−50	1.28	Poly(butylene terephthalate) and polyether block copolymer	3.6	1.4
Hytrel G3548L	DuPont	−40	1.16		8.3	0.1
Hytrel 6108	DuPont	20	1.25		1.2	0.3
Hytrel G4078	DuPont	−37	1.18		12	0.1
PEBAX MH1657	Arkema	−40	1	Polyether block amide	0.6	0.6
PDC 1274	Basell	N/A	N/A	Homopolymer of propylene	0.9	0.5

N/A: not available.

vanillin and guaiacol possess very low log P (octanol–water partition coefficients) values, that is, 1.28 and 1.32, respectively, which are far below 4, a value that has been considered to be cytotoxically critical.[10,11] In situ product removal (ISPR) has been undertaken using auxiliary phases such as highly crystalline polymeric resins[4] to ameliorate this possible limitation. In these systems, the presence of salts in the aqueous solutions interfered with the adsorption ability of these macroporous resins which consisted of a cross-linked-polystyrene framework.[12] Moreover, the performance of these relatively nonselective resins in the sorption of vanillin may be limited, as these biotransformation systems contain a great diversity of molecules including some structurally similar metabolites.[5] Finally, except for the fungal production of vanillin in a mechanically agitated, air-lift bioreactor by *Pycnoporus cinnabarinus*, which was reported to exhibit a yield of 1.58 g/L vanillin when using Amberlite XAD-2 resin to reduce the vanillin concentration,[13] there are no reports of ISPR processes using resins or other polymers as the sequestering phase to reduce possible inhibition at a bioreactor scale.

Use of solid–liquid two-phase partitioning bioreactors (TPPBs) is an effective technique for ISPR, in which amorphous, soft polymers are used as a sequestering phase, usually in the form of polymer beads, to absorb the desired product into the polymeric matrix, not only targeting product inhibition, but also acting as the initial concentrating step for downstream processing.[8,14] The successful application of TPPBs in the bioproduction of flavor/fragrance compounds such as carvone[15] and 2-phenylethanol[16] additionally showed that polymers offer advantages over organic solvents in ISPR applications by not imparting any flavor or fragrance to the desired product, thus maintaining organoleptic quality. These improvements in the performance of the process can lead to a high-desired final product concentration that is significant for developing an industrial-scale biotransformation process.[8]

The production of vanillin from ferulic acid by this strain was strongly influenced by medium pH[17] and in this work, the influence of the presence of vanillin in the broth on cell growth and metabolism of *Amycolatopsis* sp. ATCC 39116 was first confirmed and examined in detail. The biotransformation of ferulic acid to vanillin by this strain was then undertaken at a bioreactor scale in a single phase to provide benchmark performance. A variety of polymers were explored for potential use in TPPBs based on their partition coefficients for the biotransformation substrate and product to select the best sequestering phase. A fed-batch reactor operating in TPPB mode using a single polymer was then used at bioreactor scale with the aim of improving the yield of vanillin and of taking a first step toward ultimate product recovery.

Materials and Methods

Chemicals and polymers

Ferulic acid (≥99%, product number: 128708), vanillin (99%, solubility in water: 10 g/L, product number: W310727), vanillic acid (4-hydroxy-3-methoxybenzoic acid, ≥97.0%, solubility in water: partly miscible, product number: W398802), and guaiacol (solubility in water: 17 g/L at 15 °C, product number: W253200) were purchased from Sigma–Aldrich. Glucose, yeast extract, and other chemicals were obtained from Fisher Scientific (Oakville, ON, Canada). Solvents used in analysis were high-performance liquid chromatography (HPLC) grade. The source and composition of the polymers that were tested for use as the sequestering phase are shown in Table 1.

Microorganism, medium, and inoculum preparation

Amycolatosis sp. ATCC 39116 was used for the biotransformation of ferulic acid. This strain had been confirmed by us to be able to transform ferulic acid to vanillin with relatively high yield of vanillin as also seen by others.[5] The medium formulation used was an optimized version of that reported previously[4,5] with a composition of (g/L): yeast extract 8, glucose 10, NaCl 0.5, Na_2HPO_4 4, KH_2PO_4 1, $MgSO_4 \cdot 7H_2O$ 0.2, $CaCl_2 \cdot 2H_2O$ 0.05, pH 8 (For transformation, 0.1 g/L vanillic acid was supplemented along with substrate based on our previous study). The medium was steam sterilized before use. For the vanillin toxicity experiments, the inoculum was obtained by adding 1 mL stock culture to 50 mL growth medium in 125 mL Erlenmeyer flasks. For shake flask biotransformation experiments, the inoculum was prepared by adding 4–5 mL stock culture to 100 mL growth medium in 250 mL Erlenmeyer flasks. The inoculum flasks were incubated at 28°C for 18 h at 220 rpm. For transformation in a 5-L bioreactor, 300 mL of inoculum was added to the reactor, which contained 3 L sterile medium.

Influence of vanillin on cell growth and metabolism of **Amycolatopsis** *sp. ATCC 39116*

To investigate the possible inhibition of the presence of vanillin on the cell growth (OD_{600}) and metabolism of *Amycolatopsis* sp. ATCC 39116, cells were cultivated in the above medium. At various times during the growth cycle, aliquots of the broths produced under same conditions were separately provided with 3 g/L substrate (ferulic acid) and synchronously supplemented with various concentrations of vanillin (with no vanillin addition in the control culture). The addition of 3 g/L ferulic acid had been confirmed to have minimal negative effects on cell growth and the

metabolism of *Amycolatopsis* sp. ATCC 39116 because ferulic acid at this level was shown to disappear quickly in the broth upon supplementation (data not shown). The impact was measured by the change in ferulic acid and by-product concentrations including guaiacol and vanillic acid as well as OD_{600}.

Polymer screening and partitioning coefficient tests

Based on preliminary screening involving single concentration uptake tests (data not shown), a short list of six polymers was examined in more detail via partition coefficient tests. A solution of vanillin and ferulic acid (10 g/L) was added to 15 mL of the above medium without glucose and yeast extract in a sealed 20 mL vial and various masses of polymers were then added to each vial. After equilibrium was established, the aqueous phase vanillin and ferulic acid concentrations were analyzed by HPLC, and the amount remaining in the polymer phase was calculated using mass balance. The partition coefficients were calculated as the ratio of concentration of solute in the polymer phase divided by the concentration of the solute in the aqueous phase at equilibrium. The control (no polymer) showed no detectable change in vanillin and ferulic acid concentration. The slope of the straight line of polymer phase concentrations vs. aqueous phase concentrations of vanillin or ferulic acid was the partition coefficient of that polymer toward vanillin or ferulic acid.

Transformation operation

A 5-L bioreactor (3 L sterile medium) equipped with pH, temperature, and dissolved oxygen monitoring was used for all reactor runs (New Brunswick Scientific, Edison, NJ, Bio-Flo III). The temperature was maintained at 28°C and the agitation was controlled to ensure that the system was never oxygen limited, with aeration at 3 L/min and the percent saturation of dissolved oxygen at 60–100%. After inoculation, once the biomass concentration in the bioreactor had reached approximately 3.5–4.0 g wet weight/100 mL, the biotransformation was initiated by the addition of substrate.

For the 5-L bioreactor fed-batch TPPB transformation, 0.26 L of the selected polymer (300 g) was used as the partitioning phase for a total working volume of 3.26 L, and the system was operated in a manner similar to single phase operation. During transformation, when the ferulic acid fell below 4 g/L, more substrate solution, also supplemented with glucose (60 g/L), was added such that ferulic acid was always available for biotransformation. When the aqueous phase vanillin concentration approached a level that did not increase even with substrate supplementation, the transformation was terminated.

Vanillin recovery and polymer regeneration

To demonstrate vanillin recovery from the polymer beads and to regenerate the polymers, several organic solvents, generally utilized in the flavor industry, methanol, ethanol, and butyl acetate were used as extractants. It is generally the case that isolation of flavor compounds from fermentation broth is typically undertaken via extraction into organic solvents, distillation, or extractive distillation.[18,19] For polymer regeneration, it was assumed that the polymer could be reused when there is no remaining solute to extract from the used polymer. For polymer extraction experiments, 10 mL of solvent was added to a 20-mL vial together with approximately 1 g of polymer randomly selected from the recovered polymers from the bioreactor after TPPB operation. The mixture was stirred overnight to achieve equilibrium between the phases, and the solvents were then transferred to a new vial. An amount of 10 mL of fresh solvents were then added to the vial that contained the polymers for a second round of overnight extraction. The procedure was repeated several times until there was no detectable vanillin remaining in the solvents. The total amount of vanillin contained in the polymer was calculated by mass balance by adding the amount of vanillin extracted into solvents from all rounds of organic solution extractions.

Analytical methods

Biomass Quantification. Growth was monitored by optical density measurements at 600 nm using a UV–Vis Spectrophotometer; alternatively, aqueous samples of known volume were centrifuged at 5000 rpm for 20 min, washed, and weighed to obtain the cell wet weight per liter or 100 mL.

Concentration Measurements. The transformation species were analyzed by reverse phase HPLC on a C18 column (Polaris C18-A 150 $\times$ 4.6 mm, VARIAN, Inc.) using a Varian HPLC, equipped with dual pumps and a UV–Vis detector (320 nm). The mobile phase consisted of water, methanol, and formic acid (80:20:1, v/v/v), and the flow rate used was 1 mL/min. The samples were prepared by centrifuging at 5000 rpm for 5 min and then filtered through 0.22 μm PTFE (Polytetrafluoroethylene) membrane, centrifuged, and filtered again before analyzing with HPLC. The standard deviations of the analyses were less than 5% using external standards. The compounds were eluted in the following order: vanillic acid (retention time, 6.1 min), vanillin (6.8 min), *trans*-ferulic acid (8.4 min), and guaiacol (10.2 min). Vanillin yield (g/L) was calculated based on the volume of the fermentation broth, while molar yield (percent) was calculated as (mole vanillin accumulated $\times$ 100)/(mole substrate supplemented).

Results and Discussion

Influence of vanillin on growth and metabolism of Amycolatopsis sp. ATCC 39116

When *Amycolatopsis* sp. was cultivated with vanillin initially added in the medium over the range 2–10 g/L, the cells were observed to have no growth (shown by OD_{600}), suggesting that vanillin has an almost completely inhibitory effect on cell growth when present at the lag phase (data not shown). When vanillin was provided 12 h after inoculation, that is, at the exponential phase, cell growth was significantly repressed subsequently. However, the cell density of the control culture was observed to have increased during the initial 20 h (Figure 1A). These results indicate that the presence of vanillin may also have a significant inhibitory effect on cell growth during the exponential phase. When vanillin was added to the broth of *Amycolatopsis* sp. at the stationary phase, the cell density was observed not to decrease over several concentrations (Figure 1B). Interestingly, growth inhibition due to the presence of vanillin at the stationary phase was observed to not be dependent on the concentration of vanillin added and in the case of 6 g/L supplementation seems to show a higher increase in cell density compared with other cases. An analysis of the broth samples

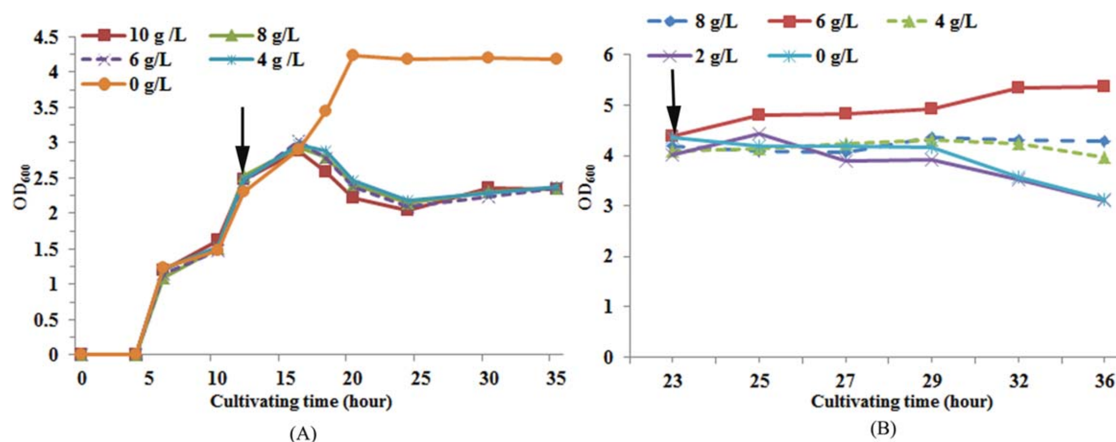

Figure 1. The effect of added vanillin in the medium on cell growth of *Amycolatopsis* sp. ATCC 39116.

A. The inhibition of vanillin added at the exponential phase. B. The influence of vanillin added at the stationary phase. The arrows represent the time point of vanillin addition to the broth.

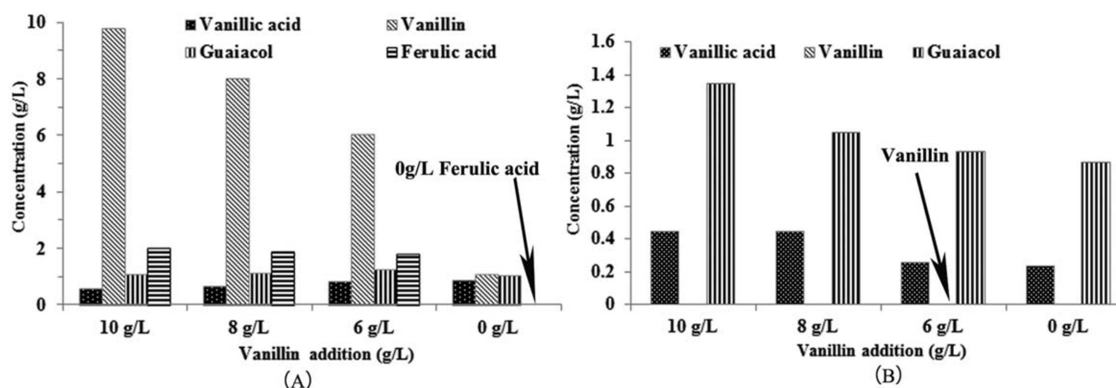

Figure 2. Metabolic analysis of transformation in the broth with different concentrations of vanillin added at the stationary phase.

A. An analysis of results of transformation at 24th h. B. An analysis of results of transformation at 30th h. Arrow in (A) represents that no ferulic acid is supplemented in broth. Arrow in (B) shows that no vanillin is detected at 30th h.

during stationary phase revealed that the ferulic acid in the control culture (without vanillin addition) was completely consumed by 24 h; however, the broth with different separate additions of vanillin showed a very low consumption of substrate (Figure 2A) by 24 h. By 30 h, both ferulic acid and vanillin added in the broth were observed to disappear and different concentrations of vanillic acid and guaiacol were detected (Figure 2B). These results suggest that the presence of vanillin in the stationary phase (i.e. added at this phase) would have unfavorable effects on vanillin production, as it can be utilized as a substrate for cell growth thereby reducing its concentration, which was also observed previously.[20]

Several studies have investigated the toxicity of vanillin on the cell growth of recombinant *E. coli*[12] and *Pycnoporus cinnabarinus*,[13] but there have been no reports related to *Amycolatopsis* sp. ATCC 39116 and the influence of the presence of vanillin on the metabolism of producers has not been reported to date. The present results suggest that vanillin may have a significant inhibitory effect on cell growth during the lag and exponential phases and less of an effect at the stationary phase, and therefore vanillin transformations are often performed during or after the stationary phase. Nevertheless, even here, the presence of or produced vanillin may result in vanillin utilization, which is a significant drawback for the establishment of vanillin bioproduction. This work has shown for the first time the consumption of vanillin during the biotransformation by *Amycolatopsis* sp. ATCC 39116, and therefore the potential advantage of producing vanillin and simultaneously extracting it from the bioconversion broth during the stationary phase to avoid its further degradation.

Determination of polymer bead partition coefficients

The partition coefficients of candidate polymers are summarized in Table 1, which shows that Hytrel®G4078W and Hytrel G3548L have a superior partitioning capacity for vanillin but have a low affinity for ferulic acid, a desirable result. Both these polymers are block copolymers of poly (butylene terephthalate) and polyether, with differing amounts of hard and soft segments. Hytrel polymers are block copolymers of poly(butylene terephthalate) as the hard segment and polyether as the soft segment, with harder grades containing a larger proportion of poly (butylene terephthalate). Importantly, Hytrel is also an Food and Drug Administration compliant polymer for use in the food and fragrance industry, is odorless, biocompatible, and nonbioavailable, and is thermally resistant to sterilization even at 121 °C. Hytrel C4078W was thus a good option for this biotransformation and was used in the TPPB transformation by *Amycolatopsis* sp.

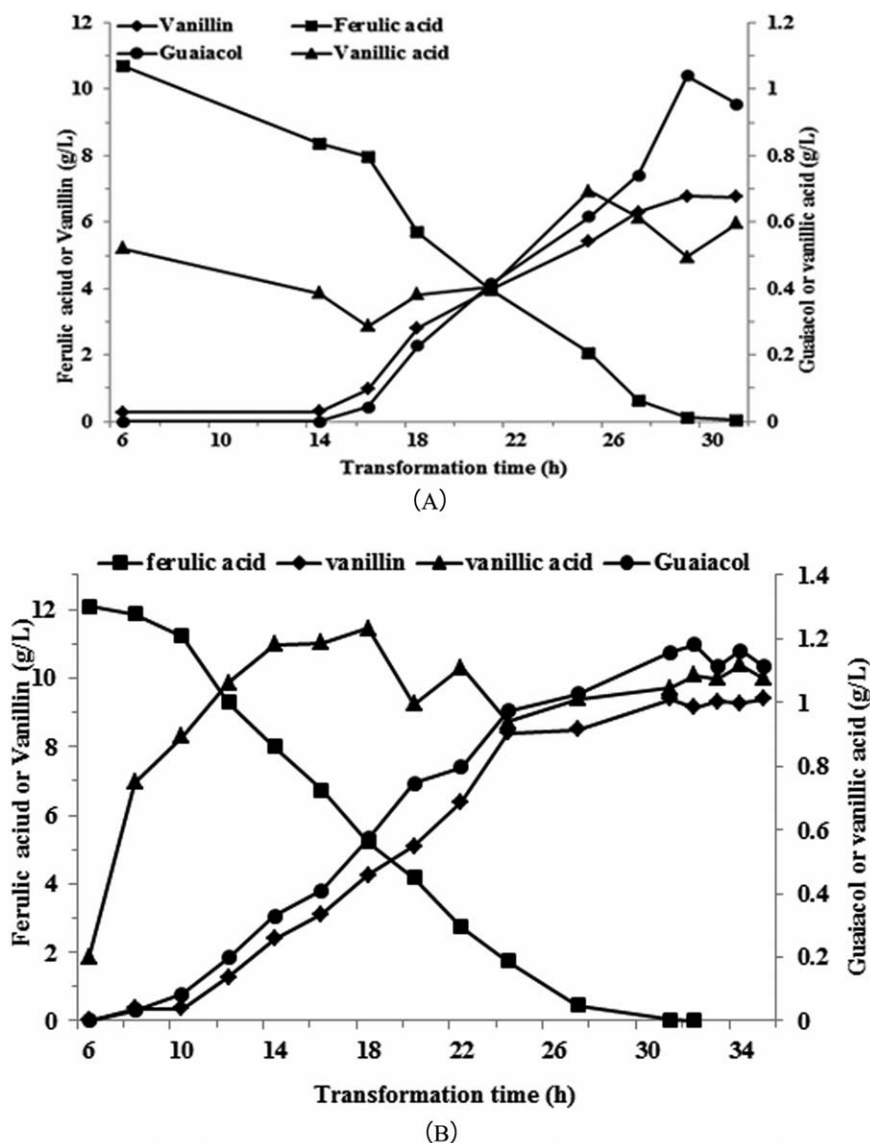

Figure 3. Transformation of vanillin in single aqueous phase.

A. The transformation at 400 rpm. B. The transformation at 280–370 rpm.

The difference in the uptake performance of polymers such as Hytrel G4078 W in absorbing vanillin and ferulic acid support a different mechanism than that of hard, resin polymers. Previously, macroporous adsorbent resins such as DM11[4] and XAD-2 resin[12] showed relatively good uptake results for vanillin, although there is some confusion in the article as to whether the uptake of vanillin by these polymers is via absorption or adsorption. Generally speaking, adsorption by these resins is based on the interaction between the outer surface of the polymers and the target molecules and thereby their adsorption is limited by the polymer's surface area, whereas absorption by soft polymers such as Hytrel G4078W is by dissolution of the molecule into the physical structure of the polymers themselves.[21]

Transformation at bioreactor scale

As there are no reports concerning the bioproduction of vanillin by *Amycolatopsis* sp. ATCC 39116 at bioreactor scale, transformations by this strain were performed at 280 and 400 rpm in a 5-L reactor, to provide single phase benchmark performance and establish appropriate conditions for further transformations. The time course of substrate consumption, relevant metabolites, and product accumulation are illustrated in Figure 3A,B. A maximum vanillin concentration of 9.4 g/L was reached in the 280 rpm case with a molecular yield of 89.2% by the time the substrate was depleted (Figure 3A), while the transformation at 400 rpm accumulated a concentration of only 6.8 g/L vanillin with a yield of 62% (Figure 3B). During the transformation at 280 rpm, the percentage saturation of dissolved oxygen was shown to decline quickly to 36.5% and the rpm was then adjusted to 370 to avoid possible oxygen limitation. This suggests that low stirring rate may be inadequate for the transformation. However, with a higher stirring speed of 400 rpm, the molecular yield and maximum concentration of vanillin were lower than that of the transformation at low stirring speed, which may be in part ascribed to the deleterious effect of stirring on cell viability and transformation performance in transformation as *Amycolatopsis* sp. is a filamentous cell and excessive stirring could destroy its morphology and thereby may decrease cell viability. It was indeed observed that the filaments of this strain operating at 400 rpm were shorter and more dispersed than those in the

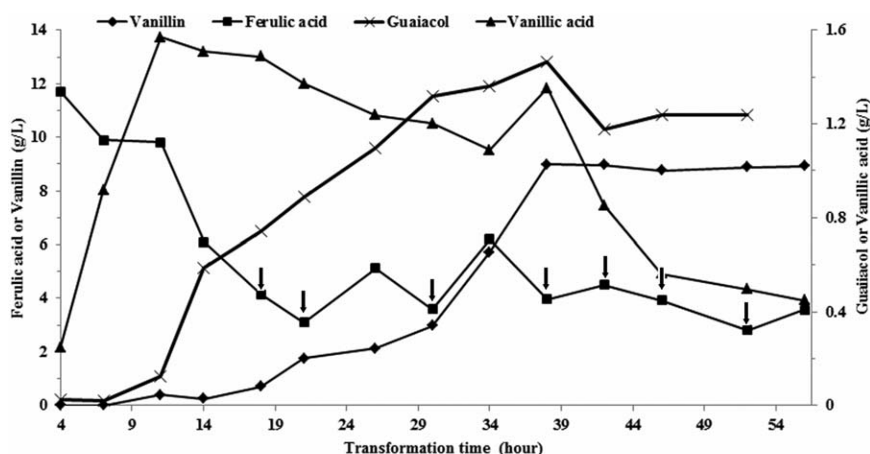

Figure 4. TPPB transformation of vanillin using a fed-batch strategy.

The arrows represent the time of substrate addition to the broth.

Table 2. Summary and Comparison of TPPB Bioreactor Performance

Mode	Scale	Aqueous Vanillin	Polymer Vanillin	Polymer (w/v)	Volumetric Productivity (mg/L. h)
Single phase	3 L working volume reactor	9.4 g/L	NA	NA	268
Fed batch TPPB	3 L working volume reactor	Total 19.5 g/L*		Hytrel® (10%)	450
Fed-batch[4]	500 mL flask	Total 19.2 g/L		DM11(8%)	349
Batch[12]	100 mL flask	Total 2.9 g/L		XAD-2 (50%)	60.4

*An amount of 106 g/kg vanillin in polymer (total polymer is 300 g).

transformation at 280 rpm. In light of these, and the earlier results, ISPR was implemented in a 5-L TPPB at a stirring speed not exceeding 400 rpm.

Fed-batch transformation of vanillin in a TPPB

Figure 4 shows the time course for substrate, vanillin, and the main by-products including vanillic acid and guaiacol throughout the fed-batch biotransformation. Compared with the previous single phase result (Figure 3B), it is clear that operating in TPPB mode increased the transformation time and the amount of substrate addition. A similar quantity of substrate was consumed more quickly and more substrate was added into the reaction system than in the transformation in single phase (Figure 3B). It is also clear that the TPPB operating strategy was highly successful in improving reactor performance as a total supplementation of 48 g/L ferulic acid to the reactor was achieved over 56 h before the reaction rate decreased significantly. This run obtained a vanillin volumetric productivity of 450 mg/L h with a vanillin concentration of 8.9 g/L in the aqueous phase and 106 g/kg in the polymer phase (Figure 4 and Table 2). As reported previously,[15] the volumetric productivity was calculated by dividing the final system vanillin mass by the total system working volume (aqueous plus polymer) and the biotransformation time. The final aqueous concentrations of by-products including vanillic acid and guaiacol in this operating reached 0.5 and 1.1 g/L, respectively. The reactor performance criteria (volume of substrate added before transformation termination, obtainable biotransformation time, and vanillin volumetric productivity) were greatly improved compared with those achieved in the single aqueous phase reactors as is summarized in Table 2, along with the two other reported solid–liquid phase reactors, which used adsorptive resin as the immiscible solid phase (Table 2).[4,12] Additionally the "online" separation of vanillin from the broth (i.e. by absorption into the polymer) decreased the possibility of vanillin being consumed as an energy source or carbon source and thereby increased the total production of vanillin in the process. This investigation showed that by selecting an appropriate polymer, a solid–liquid TPPB system can greatly enhance the biotransformation process and achieve high final vanillin concentration and high productivity, although further polymer screening may identify an even more effective sequestering phase than Hytrel. Overall, the solid–liquid TPPB system showed promising results in removing a toxic and easily consumed product via an ISPR technique and is also an efficient initial concentrating step for downstream processing.

A closer examination of the vanillin curve in Figure 4 suggests that the aqueous concentration of vanillin before 30 h had been relatively invariable although the concentration of ferulic acid decreased rapidly. Also, the concentration of vanillic acid, a main by-product of vanillin by *Amycolatopsis* sp., was observed to decline with time after 38 h and these results suggest that the substrate was transformed to vanillin but did not accumulate in the aqueous solution; rather it must be taken up by the polymer beads. In addition, with each substrate addition after 38 h, the aqueous concentration of vanillin was shown not to increase in accordance with the consumption of substrate. These results also suggest that the application of the TPPB had reduced the concentration of vanillin in the aqueous phase thereby reducing its toxicity to cell growth as well as preventing biodegradation of vanillin in the aqueous phase.

Compared with the instantaneous equilibrium that occurs in a liquid–liquid TPPB system,[22] the equilibrium between a

Table 3. Vanillin Recovery Capabilities Using Organic Solvent Extraction and Performance of Polymer Regeneration

Organic Solvent	Total Vanillin Extraction Ratio (%)*	Total Ferulic Acid Extraction
Methanol	64.6	0
Ethanol	62.6	0
Butyl acetate	64.2	0

*The total extraction ratio of vanillin is based on the product content in polymer and the complete desorption of products from polymer beads through three extraction cycles.

solid and liquid phase requires more time due to the diffusion of the solute into the solid polymer matrix.[23] The diffusion rates of vanillin into the polymer was not the focus of this study; however, there are some reports involving the diffusivities of similarly sized-molecules (benzene and phenol) into Hytrel polymers and ethylene–vinyl acetate.[24]

As reported before, aside from the improved performance in volumetric productivity, increase in substrate addition and biotransformation time, there are several operational advantages over the two liquid–liquid phase bioreactor.[15] In the case of vanillin, it is interesting to note that as a typical filamentous bacterium, the filaments of *Amycolatopsis* sp. ATCC 39116 are fragile and susceptible to mixing shear forces and that too strong a shear force could destroy the filaments. However, the cells in the TPPB transformation at 400 rpm still displayed branched-rich filaments, a morphology of this strain itself in liquid culture, and there were no any ndications of the destructive effects of strong shear force on cells as had been seen in the single phase bioreactor configuration (data not shown). The incorporation of the polymer beads in the bioreactor may decrease or buffer the destructive effects of strong shear force on the cells, which is significant for the performance of such strains.[25]

In previous reports of two phase biotransformations with resin beads as the sequestering phase such as XAD-2,[13] HZ802,[3] and DM11,[4] no information was provided concerning the adsorption of the by-products of vanillin by the resins; however, it is known that adsorption by such resins is nonselective.[26] Therefore, such adsorptive resins may inadvertently bind essential nutrients or adsorb metabolites in the system potentially affecting performance. In case of vanillin bioproduction by *Amycolatopsis* sp., the accumulation of vanillic acid to 200 mg/L was thought to be a prerequisite for the initialization of vanillin production.[5] Since vanillic acid is dissociated in the medium (pH 8.0), Hytrel polymers would not absorb such molecules.

Using polymer fractions of 0.1 kg/L yielded vanillin production enhancements of 19.5 g/L relative to the single-phase control. However, polymer fractions above 0.1 kg/L were not examined here. In other studies involving the adsorption of butanol by hard resins, excessive binding of medium components was reported to be the cause of decreased performance at high polymer fraction of resin.[26] Increasing the proportion of these polymer resins in the aqueous phase may possibly result in further production enhancement as was addressed previously in the bioproduction of 2-phenylethanol in a TPPB.[16] From this limited data set combined with the limitation of reactor volume, there appears to be an optimum polymer fraction in a reactor whose specification is a critical design element and will require further investigation.

Vanillin recovery and polymer regeneration

The ability to recover and purify vanillin from the polymer phase, thereby regenerating the polymer for subsequent reuse, was investigated using different organic solvents. The results of three extraction–regeneration cycles are shown in Table 3. No significant difference in extraction performance was seen among extractions by the three solvents, achieving a total desorption of 64.6% from the recovered polymer in the bioreactor by methanol. The decreased desorption of vanillin may be ascribed to volatilization during the drying of polymer beads. In addition, some guaiacol was desorbed from the polymer, although it is not predominant in the desorbed mixture. However, there was no detectable ferulic acid, vanillic acid, and other by-products present in the desorbed mixture. These results demonstrated that the application of TPPB did not unfavorably affect the metabolic pathway of vanillin as the accumulation of vanillic acid with 200 mg/L has been confirmed to be a necessary condition for vanillin production by *Amycolatopsis* sp.[5] The above results also demonstrated that it is possible to efficiently recover the products from the beads using a small volume of methanol or other organic solvent. In addition to product recovery, the results also suggest that this downstream step allows for the regeneration of the polymer beads for future reuse using organic solvents generally used in the food industry. Product extraction from polymer beads in this manner may be much easier than the removal of products from a liquid organic extracting phase where distillation of the solvent or back extraction using another immiscible solvent is required. The use of polymers in TPPBs is confirmed again to be advantageous in the microbial bioproduction of flavor/fragrance compounds, as the polymers in medium do not produce any fugitive off-aromas or chemicals that could affect product quality or other organoleptic properties.

In addition, after extraction of blank polymers (without solute absorbed) using methanol for 24 h, it was observed that there were no chemical species in the methanol as detected using gas chromatography (100–300 °C of oven scanning), suggesting no inherent contaminants within the polymer itself. Vanillin was easily and quantitatively recovered from the polymers mostly by single stage extraction into methanol or other organic solvents used in food industry, simultaneously regenerating polymer beads for reuse.

Conclusion

In summary, vanillin had a significant influence on cell growth and vanillin production in the aqueous phase at different phases of cell and the added vanillin in the medium at the stationary phase can be utilized or degraded for cell growth. The use of Hytrel C4078W polymer beads as the partitioning phase in TPPBs resulted in enhanced operability in the bioproduction of vanillin at bioreactor level by *Amycolatopsis* sp. relative to the single aqueous phase configurations at the same scale. This enhancement might reasonably be expected in the production of other flavor and fragrance compounds obtained by biotransformations.

Acknowledgments

This study was in part supported financially by the Fundamental Research Funds for the Central Universities (grant

no. GK 201003002). The authors thank Julian Dafoe and Eric Peterson for their support during this study.

Literature Cited

1. Sciubba L, Di Gioia D, Fava F, Gostoli C. Membrane-based solvent extraction of vanillin in hollow fiber contactors. *Desalination*. 2009;241:357–364.
2. Wangrangsimagul N, Klinsakul K, Vangnai AS, Wongkongkatep J, Inprakhon P, Honda K, Ohtake H, Kato J, Pongtharangkul T. Bioproduction of vanillin using an organic solvent-tolerant *Brevibacillus agri* 13. *Appl Microbiol Biotechnol*. 2012;93:555–563.
3. Zheng L, Zheng P, Sun Z, Bai Y, Wang J, Guo X. Production of vanillin from waste residue of rice bran oil by *Aspergillus niger* and *Pycnoporus cinnabarinus*. *Bioresour Technol*. 2007; 98:1115–1119.
4. Hua DL, Ma CQ, Song LF, Lin S, Zhang ZB, Deng ZX, Xu P. Enhanced vanillin production from ferulic acid using adsorbent resin. *Appl Microbiol Biotechnol*. 2007;74:783–790.
5. Muheim A, Lerch K. Towards a high-yield bioconversion of ferulic acid to vanillin. *Appl Microbiol Biotechnol*. 1999;51: 456–461.
6. Cheng F, Sheng J, Dong R, Men Y, Gan L, Shen L. Novel xylanase from a holstein cattle rumen metagenomic library and its application in xylooligosaccharide and ferulic acid production from wheat straw. *J Agric Food Chem*. 2012;60:12516–12524.
7. Narbad A, Gasson MJ. Metabolism of ferulic acid via vanillin using a novel CoA-dependent pathway in a newly-isolated strain of *Pseudomonas fluorescens*. *Microbiology*. 1998;144:1397–1405.
8. Straathof AJ. Auxiliary phase guidelines for microbial biotransformations of toxic substrate into toxic product. *Biotechnol Prog*. 2003;19:755–62.
9. Davis JR, Goodwin LA, Woyke T, Teshima H, Bruce D, Detter C, Tapia R, Han S, Han J, Pitluck S, Nolan M, Mikhailova N, Land ML, Sello JK. Genome sequence of *Amycolatopsis* sp. strain ATCC 39116, a plant biomass-degrading actinomycete. *J Bacteriol*. 2012;194:2396–2397.
10. Murakami Y, Hirata A, Ito S, Shoji M, Tanaka S, Yasui T, Machino M, Fujisawa S. Re-evaluation of cyclooxygenase-2-inhibiting activity of vanillin and guaiacol in macrophages stimulated with lipopolysaccharide. *Anticancer Res*. 2007;27: 801–807.
11. Laane C, Boeren S, Vos K, Veeger C. Rules for optimization of biocatalysis in organic solvents. *Biotechnol Bioeng*. 1987;30:81–87.
12. Yoon SH, Lee EG, Das A, Lee SH, Li C, Ryu HK, Choi MS, Seo WT, Kim SW. Enhanced vanillin production from recombinant *E. coli* using NTG mutagenesis and adsorbent resin. *Biotechnol Prog*. 2007;23:1143–1148.
13. Stentelaire C, Lesage-Meessen L, Oddou J, Bernard O, Bastin G, Ceccaldi BC, Asther M. Design of a fungal bioprocess for vanillin production from vanillic acid at scalable level by *Pycnoporus cinnabarinus*. *J Biosci Bioeng*. 2000;89:223–230.
14. Tomei MC, Annesini MC, Daugulis AJ. Solid-liquid two-phase partitioning bioreactors (TPPBs) operated with waste polymers. Case study: 2,4-dichlorophenol biodegradation with used automobile tires as the partitioning phase. *Biotechnol Lett*. 2012;34: 2037–2042.
15. Morrish JL, Daugulis AJ. Improved reactor performance and operability in the biotransformation of carveol to carvone using a solid-liquid two-phase partitioning bioreactor. *Biotechnol Bioeng*. 2008;101:946–956.
16. Gao F, Daugulis AJ. Bioproduction of the aroma compound 2-phenylethanol in a solid-liquid two-phase partitioning bioreactor system by *Kluyveromyces marxianus*. *Biotechnol Bioeng*. 2009; 104:332–339.
17. Gunnarsson N, Palmqvis, EA. Influence of pH and carbon source on the production of vanillin from ferulic acid by *Streptomyces setonii* ATCC 39116. *Dev Food Sci*. 2006;43, 73–76.
18. Krings U, Berger RG. Biotechnological production of flavours and fragrances. *Appl Microbiol Biotechnol*. 1998;49:1–8.
19. Eikani MH, Golmohammad F, Rowshanzamir S, Mirza M. Recovery of water-soluble constituents of rose oil using simultaneous distillation–extraction. *Flav Fragr J*. 2005;20:555–558.
20. Sutherland JB, Crawford DL, Pometto AL 3rd. Metabolism of cinnamic, p-coumaric, and ferulic acids by *Streptomyces setonii*. *Can J Microbiol*. 1983;29:1253–1257.
21. Craig T, Daugulis AJ. Polymer characterization and optimization of conditions for the enhanced bioproduction of benzaldehyde by *Pichia pastoris* in a two-phase partitioning bioreactor. *Biotechnol Bioeng*. 2013;110:1098–1105.
22. Fam H, Daugulis AJ. Substrate mass transport in two-phase partitioning bioreactors employing liquid and solid non-aqueous phases. *Bioprocess Biosyst Eng*. 2012;35:1367–1374.
23. Rehmann L, Sun B, Daugulis AJ. Polymer selection for biphenyl degradation in a solid-liquid two-phase partitioning bioreactor. *Biotechnol Prog*. 2007;23:814–819.
24. Prpich GP, Daugulis AJ. Polymer development for enhanced delivery of phenol in a solid-liquid two-phase partitioning bioreactor. *Biotechnol Prog*. 2004;20:1725–1732.
25. Littlejohns JV, Daugulis AJ. Oxygen mass transfer and hydrodynamics in a multi-phase airlift bioscrubber system. *Chem Eng Sci*. 2009;64, 4171–4177.
26. Nielsen DR, Prather KJ. In situ product recovery of n-butanol using polymeric resins. *Biotechnol Bioeng*. 2009;102:811–821.

Manuscript received Jun. 11, 2013, and revision received Oct. 8, 2013.