Integrated Bioprocessing for the pH-Dependent Production of 4-Valerolactone from Levulinate in *Pseudomonas putida* KT2440<sup>7</sup>

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Enzymes are powerful biocatalysts capable of performing specific chemical transformations under mild conditions, yet as catalysts they remain subject to the laws of thermodynamics, namely, that they cannot catalyze chemical reactions beyond equilibrium. Here we report the phenomenon and application of using extractosolic enzymes and medium conditions, such as pH, to catalyze metabolic pathways beyond their intracellular catalytic limitations. This methodology, termed “integrated bioprocessing” because it integrates intracellular and extracellulosic catalysis, was applied to a lactonization reaction in *Pseudomonas putida* for the economical and high-titer biosynthesis of 4-valerolactone from the inexpensive and renewable source levulinic acid. Mutant paraoxonase I (PON1) was expressed in *P. putida*, shown to export from the cytosol in *Escherichia coli* and *P. putida* using an N-terminal sequence, and demonstrated to catalyze the extracellulosic and pH-dependent lactonization of 4-hydroxyvalerate to 4-valerolactone. With this production system, the titer of 4-valerolactone was enhanced substantially in acidic medium using extractosolisically expressed lactonase versus an intracellular lactonase: from <0.2 g liter<sup>−1</sup> to 2.1 ± 0.4 g liter<sup>−1</sup> at the shake flask scale. Based on these results, the production of 4-hydroxyvalerate and 4-valerolactone was examined in a 2-liter bioreactor, and titers of 27.1 g liter<sup>−1</sup> and 8.2 g liter<sup>−1</sup> for the two respective compounds were achieved. These results illustrate the utility of integrated bioprocessing as a strategy for enabling production from novel metabolic pathways and enhancing product titers.

In nature, metabolism is not restricted to the cytosol. Metabolic activity can occur in the periplasm, on cell surfaces, or even extracellularly in the environment. When extractosolisic enzyme expression occurs, it is often to convey an advantage to the cell that is not possible with the enzyme in the cytosol. For instance, *Escherichia coli* alkaline phosphatase is expressed in the periplasm to detoxify compounds before they can enter the cell and to allow the enzyme better access to the extracellular environment for scavenging of phosphate (8). *Penicillium decumbens* secretes cellulases, presumably to break down extracellular substrates into a form amenable to uptake by the cell for further metabolism (18). Each of these enzymes exists as a component of larger metabolic pathways (phosphate and celululosic material metabolism), and each has evolved for noncytosolic expression to facilitate the physiological goals of the respective pathways.

In microbial biotechnology, the objective is typically not physiological but commercial in nature: to increase the titer of a small-molecule metabolic product. Though the goal has changed, the lessons we can learn from natural systems remain. The cytoplasm is not always the best choice for enzyme expression because cytoplasmic conditions are not necessarily optimal for enzyme productivity. One such system is the intramolecular lactonization of hydroxycids, such as 4-hydroxyvalerate (4HV), to lactones, such as 4-valerolactone (4VL). This reaction, catalyzed in this work by the G3C9 variant of human paraoxonase I (PON1) (1), is known to be highly pH dependent. Because lactonization is acid catalyzed and because hydroxycids and lactones exist in pH-dependent equilibrium with each other, control of the pH at which lactonization occurs is critical to achieving high titers of lactones (19).

The cytoplasmic pH, typically about 7.5, is too high to achieve good titers of lactones at equilibrium (20). This limitation in the lactone titer is thermodynamic in nature, meaning that overexpressing the lactonase or most other traditional metabolic engineering techniques aimed at increasing flux toward the product would be largely ineffective at improving lactone production. However, having the lactonase perform catalysis outside the cytosol, where the pH can be lowered, would alter the equilibrium in favor of lactone production, thereby increasing the product titer.

To implement such a system, we used extractosolisically expressed PON1 to catalyze the lactonization reaction in acidic medium (pH ~6). Recently we found that *Pseudomonas putida* was capable of producing high titers of 4HV from the renewable carbon source levulinic acid (15). This process is achieved through coenzyme A (CoA) carriers, and the secretion of 4HV into the extracellular medium was enhanced by the expression of an intracellular thioesterase. Thus, *P. putida* can serve as an intracellular source of 4HV. This 4HV can then be lactonized by extractosolisically expressed PON1 in acidic medium to yield 4VL (Fig. 1). This general strategy is termed “integrated bioprocessing” for its integration of cytosolic and extractosolisic biocatalysis to enhance production.
4-VL has been reported to be an ideal compound for use as a fuel and in the production of carbon-based chemicals (9). It has also seen extensive use as a component of block copolymers for drug delivery (2, 6) and as a precursor for acrylic polymers (10). Current synthetic methods for 4-VL also utilize levulinate as the starting material but require supercritical solvents (14) or carbon dioxide (3) with hydrogen gas under harsh conditions (14) or with ruthenium-based catalysts. In contrast, our biocatalytic method of production is done under mild conditions (200°C) and ruthenium-based catalysts. This integrated bioprocessing system allows for 4-VL production without the need for harsh solvents, hydrogen, rare metal catalysts, or supercritical fluids. This integrated bioprocessing system to produce 4-VL from levulinate (Fig. 1) was compared to an entirely intracellular 4-VL production pathway utilizing cytologically expressed PON1 to demonstrate the effectiveness of integrated bioprocessing to improve product titers at different pH values. The system was tested in a 2.0-liter bioreactor to further increase product titers and take advantage of automated pH control. To our knowledge, this work represents the first report of 4-VL synthesis in a biological system.

MATERIALS AND METHODS

Chemicals. All chemicals purchased were of the highest grade or purity available unless otherwise indicated. LB broth, glucose, isopropyl-β-D-1-thiogalactopyranoside (IPTG), and antibiotics were purchased from Becton Dickinson and Company (Sparks, MD), Mallinckrodt (Hazelwood, MO), Teknova (Hollister, CA), and Calbiochem (San Diego, CA), respectively. The sources for other chemicals for 4-VL production, glucose, isopropyl-1-thiogalactopyranoside (IPTG), and antibiotics were purchased from Becton Dickinson and Company (Sparks, MD), Mallinckrodt (Hazelwood, MO), Teknova (Hollister, CA), and Calbiochem (San Diego, CA), respectively. The sources for other chemicals are described in the relevant methods below.

Strains and plasmids. Escherichia coli DH10B was used for all E. coli studies and molecular cloning in this work and was purchased from Invitrogen (Carlsbad, CA). Pseudomonas putida KT2440 was obtained from the American Type Culture Collection (ATCC 47054). pRK415 (TesB) was used to express the thioredoxin II (TesB) gene from E. coli MG1655, while a gentamicin-resistant variant of the plasmid pMMB206, called pMMB206G, was used for the expression of PON1. pRK415 was a generous gift from Keith Poole, while pMMB206 was obtained from ATCC (ATCC 37808). pMMB206G was produced by cloning pRK415 by PCR into pMMB206G to make pMMB206G-PON1. All alkaline phosphatase (PhoA) protein fusion vectors were constructed by first cloning phoA by PCR from E. coli genomic DNA into pRK415 using the primers XbaI-phoA-FP (5'-GACATCTAGAATGAAACAAAGCACTATTGC-3') and XbaI-phoA-RP (5'-GACAGGTACCTTATTTCAGCCCCAGAGC-3'). HotStar HiFidelity DNA polymerase was purchased from Qiagen (Valencia, CA) and used according to the manufacturer’s instructions. The G309 PON1 gene derived from Homo sapiens (human) PON1 (1) was similarly cloned by PCR into pMMB206G using the primers Psbl-PON1-FP (5'-AGACACGTCAGATGGCTAAACTGACAGCG-3') and KpnI-phoA-RP (5'-GACACTGCAGATGTCTTCTTTCCAAACAGCAT-3') and cloned by PCR into pMMB206G using the primers PstI-PON1-FP (5'-GACACTGCAGATGTCTTCTTTCCAAACAGCAT-3') and XbaI-phoA-RP (5'-GACACTGCAGATGTCTTCTTTCCAAACAGCAT-3') and XbaI-phoA-RP (5'-GACACTGCAGATGTCTTCTTTCCAAACAGCAT-3') and ligating into similarly digested pMMB206G. All molecular biology manipulations were performed using standard cloning protocols (16).

The tesB gene was cloned by PCR into pRK415. Primers were purchased from Sigma-Genosys (St. Louis, MO) and were as follows (restriction sites used for cloning are underlined): EcoR1-tesB-FP (5'-GAATTCCTACTGAGAGTTATATGAGTCAGG-3') and Sall-tesB-RP (5'-GTCGACTTTATTTGTAGACTCCAT-3'). HotStar HiFidelity DNA polymerase was purchased from Qiagen (Valencia, CA) and used according to the manufacturer’s instructions. The P309 PON1 gene derived from Homo sapiens (human) PON1 (1) was similarly cloned by PCR into pMMB206G using the primers Psbl-PON1-FP (5'-AGACACGTCAGATGGCTAAACTGACAGCG-3') and XbaI-PON1-RP (5'-GACACTGCAGATGTCTTCTTTCCAAACAGCAT-3') and KpnI-phoA-RP (5'-GACACTGCAGATGTCTTCTTTCCAAACAGCAT-3') and PhoA-FP (5'-GACACTGCAGATGTCTTCTTTCCAAACAGCAT-3') to make pRK415-phoA. Truncated PhoA (PhoA') was cloned in a similar manner into pRK415 using the primers XbaI-phoA-FP (5'-GACACTGCAGATGTCTTCTTTCCAAACAGCAT-3') and XbaI-phoA-RP (5'-GACACTGCAGATGTCTTCTTTCCAAACAGCAT-3'). 4-Hydroxyvalerolactone (4VL) has been reported to be an ideal compound for use as a fuel and in the production of carbon-based chemicals (9). It has also seen extensive use as a component of block copolymers for drug delivery (2, 6) and as a precursor for acrylic polymers (10). Current synthetic methods for 4-VL also utilize levulinate as the starting material but require supercritical solvents (14) or carbon dioxide (3) with hydrogen gas under harsh conditions (14) or with ruthenium-based catalysts. In contrast, our biocatalytic method of production is done under mild conditions (200°C) and ruthenium-based catalysts. This integrated bioprocessing system allows for 4-VL production without the need for harsh solvents, hydrogen, rare metal catalysts, or supercritical fluids. This integrated bioprocessing system to produce 4-VL from levulinate (Fig. 1) was compared to an entirely intracellular 4-VL production pathway utilizing cytologically expressed PON1 to demonstrate the effectiveness of integrated bioprocessing to improve product titers at different pH values. The system was tested in a 2.0-liter bioreactor to further increase product titers and take advantage of automated pH control. To our knowledge, this work represents the first report of 4-VL synthesis in a biological system.

FIG. 1. Integrated bioprocessing system for the production of 4-VL from levulinate. Production of 4HV from levulinate occurs intracellularly, while the lactonization reaction takes place extracytosolically in acidic medium. The enzyme(s) responsible reaction step(s) from levulinate to 4-hydroxyvaleryl-CoA occur in P. putida and are currently not known.
exponential-phase recombinant _P. putida_ harboring the plasmids pRK415-tesB and pMMP26G-PON1. The working volume of the bioreactor was 1.0 liters, and it was operated at 32°C. Air (0.5 to 1.0 reactor volumes per minute) was sparged into the bioreactor, and the stirrer speed was varied between 300 and 850 rpm to maintain dissolved O₂ levels between 10% and 40% of air saturation. The pH of the reactor was set to 7.0 and was controlled with the automatic addition of 28% ammonium hydroxide and 4.0 M phosphoric acid. The medium in the reactor consisted of Terrific Broth (TB) (12 g liter⁻¹ tryptone, 24 g liter⁻¹ yeast extract, 9.4 g liter⁻¹ potassium dihydrogen phosphate, and 2.2 g liter⁻¹ potassium dihydrogen phosphate) with 1.0 mM IPTG, 2.0% glucose, 20 mg liter⁻¹ gentamicin, 10 mg liter⁻¹ tetracycline, 4 mM magnesium sulfate, 0.2 mM calcium chloride, and 0.1 mg liter⁻¹ ferric ammonium citrate. After 22 h of initial growth, levulinic acid was continuously fed to the reactor from a 400 g liter⁻¹ feedstock to maintain the concentration of levulinate between 2 and 20 g liter⁻¹.

The concentrations of levulinate, 4HV, and 4VL were detected on an instrument equipped with a Zorbax SB-Aq column (0.46 cm by 15 cm, 3.5 μm), monitored by measuring the OD₆₀₀ and converted into g dry cell weight (DCW) liter⁻¹ using a conversion factor of 0.42 g DCW OD₆₀₀⁻¹ liter⁻¹ (15). After 67 h, the pH of the culture was adjusted to 6.0, and levulinate feeding was discontinued. The culture was continued at 32°C for an additional 50 h to allow for 4VL production.

### Lactonase assays
All whole-cell and lysate samples were tested for lactonase activity in 1X M9 salts (16) with the nitrogen source ammonium chloride removed and exchanged for an equal molarity of sodium chloride to prevent the growth of whole-cell samples. Unless otherwise noted, this medium was supplemented with 0.1 M CaCl₂ to supply a divalent ion to PON1 (19), 50 mM 4-hydroxyvalerate, and 40 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer. The pH of this medium was adjusted with 10 N NaOH or 6 N HCl to a desired value in the range of 5.0 to 7.0. When testing whole cells, cells were centrifuged at 2,500 × g for 5 min, their original medium was removed, and the cells were resuspended in 0.9% (wt/vol) sterile sodium chloride to an OD₆₀₀ of 25. This suspension was then used to supply cells to whole-cell lactonase assay experiments to an OD₆₀₀ of 0.5. For the analysis of cell lysates (prepared by repeated freezing and thawing of lysozyme-treated cells), 1 mg of total protein (bovine serum albumin equivalent as assayed by the Bradford method [4]) was added to the lactonase assay mixture. All samples were then incubated with shaking at 37°C, and samples were periodically withdrawn for HPLC analysis to determine the amount of 4VL produced.

### Alkaline phosphatase assays
Alkaline phosphatase (PhoA) activity was qualitatively assessed on agar plates using bromo-4-chloro-3-indolyl phosphate (XP) purchased from Amresco (Solon, OH) as an indicator. Recombinant _E. coli_ E. coli P. putida to be tested for PhoA activity was streaked on LB plates supplemented with 10 mg liter⁻¹ tetracycline, 1 mM IPTG, 100 mg liter⁻¹ XP, and 75 mM phosphatase (to suppress endogenous phosphatase expression). These plates were incubated at 37°C (E. coli) or 30°C (E. putida) for 24 to 48 h, and their color was assessed by eye. Blue colonies indicated active, periplasmic PhoA, while white colonies indicated that the PhoA construct was inactive.

PhoA activity was quantitatively assessed in cell lysates using _p_-nitrophenyl phosphate (PNP) purchased from Amresco (Solon, OH). The assay mixture consisted of 15 mM PNP and 2.0 mM MgSO₄ in 1.0 M Tris-HCl, pH 8.0. To this mixture, crude protein lysate (30 μg of total protein bovine serum albumin equivalent as assayed by the Bradford method [4]) was added, and the solution was briefly vortexed to mix. The liberation of _p_-nitrophenol was monitored by measuring the absorbance of the mixture at 405 nm at room temperature. One unit of PhoA activity is defined as 1 μmol of _p_-nitrophenol liberated per minute at room temperature.

### HPLC analysis
All HPLC samples were prepared by taking 1 ml of culture broth, centrifuging for 5 min at 16,000 × g to pellet cells, and taking the supernatant for analysis. HPLC analysis was performed on an Agilent 1100 series instrument equipped with a Zorbax SB-Aq column (0.46 cm by 15 cm, 3.5 μm) purchased from Agilent Technologies (Santa Clara, CA). The column temperature was maintained at 65°C. Levulinic acid, 4HV, and 4VL were detected on a refractive index detector and had retention times of approximately 3.43, 3.28, and 5.11 min, respectively. The mobile phase was 25 mM ammonium formate in water (pH 2.0), and the flow rate through the column was 1.0 ml min⁻¹. Levulinic acid and 4VL purchased from Alfa Aesar (Ward Hill, MA) were used as standards, while the 4HV standard was prepared by saponification of 4VL with 10 N sodium hydroxide at 4°C. HPLC data were used to calculate product yields (product produced divided by levulinate consumed), lactonization conversion (4VL concentration divided by the sum of 4HV and 4VL concentrations), and productivity (grams of product formed per hour per liter of culture volume).

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FIG. 2. Comparison of the first 25 N-terminal residues of native human PON1, the G3C9 variant of PON1 created by Aharoni and coworkers (1), and _E. coli_ PhoA (a protein known to export into the periplasm). Aliphatic residues (which bury within the membrane) are bold, while cationic residues (presumed to bind to the negatively charged heads of phospholipids) are underlined. In this work, it is hypothesized that the G3C9 variant of PON1 possesses an N-terminal signal sequence similar enough to bacterial signal sequences to allow PON1 export from the cytosol in both _E. coli_ and _P. putida_.

### RESULTS

#### Determination of PON1 localization using PhoA protein fusions
To demonstrate the integrated bioprocessing system for the production of 4VL, it was first necessary to establish the localization of the PON1 lactonase. We suspected that the G3C9 PON1 variant (1) might export from the cytosol using an N-terminal sequence (Fig. 2). In particular, we identified key similarities between the first 23 amino acids of G3C9 PON1 and _E. coli_ alkaline phosphatase, a protein known to export from the cytoplasm. First, the spacing between the two lysines in the N termini of these two proteins was identical. These lysines are cationic at physiological pH and can associate with negatively charged phospholipid heads in a cell membrane. Second, both N termini have several hydrophobic residues between these two lysines, which would help anchor that part of the protein inside a cell membrane. Comparing the N termini of native human PON1 and G3C9 PON1 shows that the spacing between the two cationic residues differs substantially between the two proteins. We suspected that this difference would allow G3C9 to export from the cytosol. Considering that G3C9 PON1 was evolved from human PON1 with the goal of functional expression in _E. coli_ (1), this key difference in the N-terminal signal sequences of the two lactonases may be what allows G3C9 to be functional in _E. coli_.

To test G3C9 PON1 (hereinafter referred to as PON1) for any ability to export from the cytosol, fusions of PON1 with _E. coli_ alkaline phosphatase (PhoA), an enzyme active only in the periplasm (8), were constructed. PON1 fusions that successfully export from the cytoplasm enable PhoA activity. To construct the protein fusions, PON1 and a truncated version of PON1 with residues 2 to 23 removed, tPON1, were fused to a truncated version of phoA (tPhoA) with residues 1 to 23 removed. The fusions constructed were N-PON1-tPhoA-C and N-tPON1-tPhoA-C, with an XbaI restriction site used as the linker between PON1 or tPON1 and tPhoA. The stop codons of PON1 and tPON1 were removed to allow translation of the entire fusion. Additionally, PhoA and tPhoA were tested as controls. Cells to be tested were streaked on LB
plates supplemented with phosphate (to suppress endogenous phosphatase expression), IPTG, and the indicator bromo-4-chloro-3-indolyl phosphate (XP). Colony color was detected by eye: blue or bluish colonies indicated active, periplasmic PhoA, while completely white colonies indicated that the PhoA construct was inactive. The qualitative results of this assay are shown in Table 1 for constructs in E. coli and P. putida, along with data from a quantitative PNP-based PhoA assay of E. coli construct lysates. These data show that in both organisms, PON1-tPhoA is exported from the cytosol while tPON1-tPhoA is not, indicating that PON1 is capable of export from the cytosol and that the first 23 N-terminal residues of PON1 are essential for this process. Interestingly, the PON1-tPhoA construct had approximately twice the activity of PhoA alone. This may be due to differences between the export signal sequences between PON1 and PhoA (Fig. 2).

<table>
<thead>
<tr>
<th>Construct</th>
<th>E. coli colony color on XP plates</th>
<th>P. putida colony color on XP plates</th>
<th>PNP PhoA activity in E. coli (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PhoA</td>
<td>Blue</td>
<td>Blue</td>
<td>1.79 ± 0.06</td>
</tr>
<tr>
<td>tPhoA</td>
<td>White</td>
<td>White</td>
<td>0.25 ± 0.05</td>
</tr>
<tr>
<td>PON1-tPhoA</td>
<td>Blue</td>
<td>Blue</td>
<td>3.88 ± 0.13</td>
</tr>
<tr>
<td>tPON1-tPhoA</td>
<td>White</td>
<td>White</td>
<td>0.26 ± 0.05</td>
</tr>
</tbody>
</table>

*Qualitative results of an XP plate-based assay for PhoA activity for various protein constructs in E. coli and P. putida are listed, along with quantitative data from a PNP-based PhoA assay of E. coli construct lysates. Protein fusions are written from N to C terminus (e.g., N-PON1-tPhoA-C). A “t” in front of a protein name indicates that the protein has had its N-terminal signal sequence removed. PNP assay data are given as the averages and standard deviations of results from three independent experiments.

**Confirmation of extracytosolic PON1 expression using lactonase assays.** To provide additional verification of extracytosolic PON1 expression, whole cells and lysates of E. coli expressing PON1 and tPON1 were assayed for lactonase activity at low pH (6.2) and high pH (7.2) (Fig. 3). Based on the results of the PhoA fusion studies in the previous section, PON1 should be an extracytosolic lactonase while tPON1 should be a cytosolic lactonase. Thus, in whole cells, PON1 should be exposed to the extracellular pH while tPON1 should be exposed...

![Figure 3](http://aem.asm.org/)

**FIG. 3.** Conversion of 4HV to 4VL by E. coli whole cells and lysates with expressed PON1 or tPON1 versus time at a pH of 6.2 (a) or 7.2 (b). Solid squares and triangles represent whole-cell data, while empty squares and triangles represent lysate data. Squares are data obtained using PON1, while triangles are data obtained using tPON1. The data points shown are the averages and standard deviations of results from three independent experiments.
to only the intracellular pH of approximately 7.5. In lysates, however, both PON1 and tPON1 should be exposed to the medium pH, since there is no cytosolic membrane to shield them. Since the degree of PON1-catalyzed lactonization is known to be pH dependent (19), PON1 exposed to lower pH values produces more 4VL from 4HV. Thus, lactone production can be used to estimate the pH that PON1 is exposed to and consequently to identify whether PON1 is localized intracellularly or extracytosolically. At an extracellular pH of 6.2, both whole cells and lysates containing PON1 are highly active, while only the lysate from tPON1 cells is highly active at this pH (Fig. 3a). Whole cells expressing tPON1 are only minimally active, achieving only 1.5 to 2.0% conversion of 4HV into 4VL. At an extracellular pH of 7.2, the results were essentially the same (Fig. 3b), the only difference being that the more active samples were limited to 4% conversion by the higher pH. The tPON1 sample here again achieves approximately 1.5 to 2.0% conversion, presumably because the tPON1 in whole cells is exposed to the same higher intracellular pH (7.5) irrespective of the medium pH value.

Effect of medium pH on 4-valerolactone production. To better understand how medium pH affects lactone production, we assayed whole E. coli cells expressing PON1 for their ability to convert 75 mM 4HV into 4VL at pH values of 5.9, 6.2, 6.4, 6.7, and 7.2. E. coli cells expressing tPON1 and no PON1 (empty plasmid control) were also tested at a pH of 6.2, and the conversion of 4HV into 4VL was monitored over time. Both the rate and amount of conversion were strongly dependent on the pH, with lower pHs allowing the highest conversions but at lower production rates (Fig. 4). Higher pH values allowed more rapid conversion, but the conversion leveled out at a much lower level. This behavior is consistent with a pH-dependent equilibrium being established between 4HV and 4VL, a phenomenon that has previously been observed (19). The pH effect on lactone production is significant—a decrease of a single pH unit (from 7.2 to 6.2) creates a 7-fold improvement in lactone production (Fig. 4). The extracytosolic version of PON1 produced 11-fold more 4VL than tPON1 at a medium pH of 6.2.

Production of 4-valerolactone from levulinate in shake flasks. The above observations of PON1 localization outside of the cytosol and the lactonization reaction being strongly pH dependent were combined to create an integrated bioprocessing approach to producing 4VL (Fig. 1). Recently we found that P. putida is capable of producing high concentrations of 4HV from levulinate when E. coli thioesterase II (encoded by tesB) is expressed (15). Thus, by supplying levulinate to recombinant P. putida expressing tesB and PON1, first 4HV and then 4VL is produced. Because low pH values can inhibit P. putida growth, 4VL production from levulinate was done in two phases. In the first phase, recombinant P. putida cells expressing tesB and either PON1 or tPON1 were grown in LB medium supplemented with levulinate, and the pH was unregulated. During this time, 10.9 ± 1.1 g liter⁻¹ and 12.0 ± 0.9 g liter⁻¹ of 4HV were produced in the PON1 and tPON1 cultures, respectively, and the pH of the cultures rose to approximately 8.0 to 8.5 (data not shown). No 4VL was detected during the first phase. After 96 h, the cultures were split into two halves and the pH of the medium in each half was adjusted downward to either 6.3 or 7.3. During this phase, lactone production was monitored for an additional 96 h. While all cultures had similarly high concentrations of 4HV at the beginning of the second phase, the 4VL titer was significantly enhanced only by extracytosolic PON1 at pH 6.3 (Fig. 5). The enhancement of the 4VL titer was 11-fold for PON1 at pH 6.3 versus 7.3 (2.1 ± 0.4 g liter⁻¹ 4VL versus 0.19 ± 0.02 g liter⁻¹) and 13-fold for PON1 versus tPON1 at pH 6.3 (2.1 ± 0.4 g liter⁻¹ 4VL versus 0.15 ± 0.02 g liter⁻¹).

Bioreactor-scale production of 4-valerolactone. Production of 4VL was examined in a 2.0-liter fed-batch reactor to take...
advantage of automated pH control and to further improve the 4VL titer. A 1.0-liter volume of TB medium was inoculated with \textit{P. putida} KT2440 expressing \textit{tesB} and \textit{PON1}, and the production of 4HV and 4VL was monitored over time (Fig. 6). Feeding of levulinate to the reactor did not begin until the cells were in stationary phase (22 h after inoculation). Levulinate concentrations were maintained between 2 and 20 g liter$^{-1}$ throughout the experiment to allow for 4HV production without inhibiting cellular metabolism with excessive levulinate. The pH was maintained at 7.0 during the 4HV accumulation phase. After 67 h of cultivation, the pH was shifted to 6.0 to allow for 4VL production and levulinate feeding was stopped.

During the 4HV accumulation phase, the 4HV titer reached 27.1 g liter$^{-1}$ (Fig. 6), which corresponded to a 26.2% yield from levulinate. The 4HV productivity of the reactor during this phase was 0.81 g liter$^{-1}$ h$^{-1}$. Prior to the pH shift, the titer of 4VL reached 1.6 g liter$^{-1}$, corresponding to 6.5% lactonization of the 4HV produced. Significant 4VL accumulation did not occur until after the pH was shifted downward from 7.0 to 6.0. After the pH shift, the titer of 4VL improved 5-fold to 8.2 g liter$^{-1}$ and the fraction of 4HV converted to 4VL increased to 33.1%.

**DISCUSSION**

The integrated bioprocessing scheme described here to produce 4VL was designed to allow for activity of the \textit{PON1} enzyme under more optimal conditions than the cytosolic environment would allow. The G3C9 variant of the human \textit{PON1} enzyme had been reported to be expressed in the cytosol of \textit{E. coli} (1), while the native variant is known to associate with the cell membrane. The combination of the G3C9 variant and the cell type used in the study allowed for the efficient production of 4VL from levulinate.

![FIG. 5. Production of 4VL from levulinate by recombinant \textit{P. putida} expressing \textit{tesB} and either \textit{PON1} (filled symbols) or \textit{tPON1} (open symbols) grown in shake flasks. Cultures were grown in LB medium supplemented with glucose and levulinate for 96 h prior to time 0, during which time the pH was unregulated. 4HV, 10.9 \pm 1.3 g liter$^{-1}$ and 12.0 \pm 0.9 g liter$^{-1}$, was produced in the \textit{PON1} and \textit{tPON1} cultures, respectively. At $t = 0$ h, the pH of the medium was adjusted to either 6.3 (squares) or 7.3 (circles). The data points shown are the averages and standard deviations of results from three independent experiments.](http://aem.asm.org/)

![FIG. 6. Production of 4HV (gray-filled squares) and 4VL (black squares) from levulinate (empty squares) by recombinant \textit{P. putida} expressing \textit{tesB} and \textit{PON1} grown in a 2.0-liter bioreactor. Cell density is indicated by cross-shaped symbols. Levulinate feeding began after 23 h (first dashed line), and at $t = 67$ h, the pH of the medium was set to 6.0 to allow for 4VL accumulation (second dashed line).](http://aem.asm.org/)
lipid membrane (7). We first attempted to express G3C9 with protein tags designed to export the enzyme from the cytosol; however, these fusions did not show the expected pH dependence of activity (data not shown). We thus proceeded to determine whether the G3C9 variant was intra- or extracytosolic. First, qualitative screening of protein fusions of PON1 with truncated PhoA (tPhoA) was performed with E. coli and P. putida by streaking these cells on plates containing the chromogenic PhoA substrate XP. Second, lactonase assays were carried out with recombinant E. coli cells expressing PON1 and tPON1 to corroborate PON1 export, since lactone production is known to be highly pH dependent (Fig. 4) (19). The results of these experiments (Table 1 and Fig. 3), taken together, strongly support the hypothesis that the G3C9 PON1 variant is capable of export from the cytosol. The primary N-terminal sequence of G3C9 PON1 contains a stretch of hydrophobic residues bracketed by appropriately positioned lysine residues that are similar to the N-terminal sequence of E. coli PhoA and presumably direct PON1 to the cellular membrane (Fig. 2). This primary sequence is characteristic of Sec-dependent protein transport (12), though additional studies would be needed to verify this.

Lactone production using PON1 is highly pH dependent (19); however, the intracellular pH is maintained at a relatively high and unfavorable level for lactone production—approximately 7.5 for E. coli (20). By employing extracytosolic PON1 in media with lower pH values (relative to the cytosolic pH), the titers of lactone produced can be increased. The pH effect on lactonization was found to be quite potent: a decrease in a single pH unit increased the equilibrium amount of lactone 7-fold (23.6% conversion at pH 6.2 versus 3.3% conversion at pH 7.2). Expressing PON1 extracytosolically conveyed a full order of magnitude difference in the lactone titer (23.6% conversion at pH 6.2 versus 2.1% for the tPON1 sample at a pH of 6.2).

To complete the integrated bioprocessing lactone production system, the 4HV should be produced by the cell rather than supplied directly to the medium. This establishes that the increase in 4VL production with use of an extracytosolic lactonase is due to the integrated bioprocessing effect rather than being an artifact of substrate transport across the cytosolic membrane. Recently we found that recombinant P. putida expressing thioesterase II (tesB) from E. coli was capable of producing high titers of 4HV from levulinate (15). Thus, the full integrated bioprocessing system, one which combines both cytosolic hydroxacyl production with extracytosolic lactonization (Fig. 1), can be tested in recombinant P. putida expressing both tesB and PON1. Using this system, 4VL was produced directly from levulinic acid, and a 13-fold improvement in the lactone titer was realized by employing an extracytosolic lactonase for the lactonization reaction versus an intracellular lactonase control (Fig. 5). The benefits of using an extracytosolic lactonase over an intracellular one in this system disappear when the medium pH approaches that of the cytosol (0.19 ± 0.02 g liter⁻¹ 4VL with PON1 at pH 7.3 versus 0.15 ± 0.02 g liter⁻¹ for tPON1). To further increase the 4VL titer and allow for automated pH control, the 4VL integrated bioprocessing system was tested at the bioreactor scale, and 8.2 g liter⁻¹ of 4VL was produced (Fig. 6). As expected, 4VL production in the bioreactor was highly dependent on medium pH, and a 5-fold improvement in the equilibrium 4VL titer was observed when the pH of the medium was decreased to 6.0 from 7.0. While a significant amount of levulanate was left unreacted in both the shake flask and bioreactor experiments, we have found that the excess levulanate inhibits the catabolism of 4HV by P. putida (15). Identification and characterization of the enzymes responsible for the conversion of levulanate to 4HV could facilitate host engineering to address this limitation. The yield of 4VL could potentially be further improved by the removal of 4VL from the culture, driving the equilibrium toward the additional lactonization of 4HV.

Integrated bioprocessing is a viable strategy for enabling and improving product production in a broad array of biological systems. In general, integrated bioprocessing is applicable to any enzyme that performs suboptimally under cytosolic conditions. Such enzymes would exhibit poor activity and consequently may become bottlenecks in production from desired metabolic pathways. By placing these enzymes outside of the cytosol, the conditions under which the enzymes operate can be easily manipulated by altering the properties of the culture medium.

The integrated bioprocessing approach employed here can be generalized to other enzymes which function better at lower or higher pH values than those found in the cytosol. Examples of such enzymes are E. coli glutamate decarboxylase, which has a pH optimum of about 4.5 (5), and Pseudomonas pseudocaligenes alkaline lipase, which has a pH optimum of 8 to 10 (11). By localizing such enzymes outside of the cytosol and manipulating the medium pH, the activity from these enzymes can be improved. Yet another opportunity to use integrated bioprocessing is for overcoming substrate transport issues with the cell membrane. A classic example of this strategy is the use of extracellular cellulosomes in both natural (18) and engineered (17) systems to degrade cellulosic matter for cellular uptake and metabolism. This concept can be expanded, for instance, to include extracytosolic expression of oxygen-requiring enzymes (such as oxygenases) in a metabolic pathway to give them better access to molecular oxygen. When enzymes are placed outside of the cytosolic membrane, where significant oxygen consumption takes place due to oxidative phosphorylation, these enzymes would be exposed to higher concentrations of oxygen.

However, integrated bioprocessing has several limitations, chief among them being the infeasibility of using enzymes that require expensive cofactors. Cofactors such as NAD(H), NADP(H), and ATP are cytosolic; enzymes requiring these molecules cannot function extracytosolically without them and therefore do not practically lend themselves to integrated bioprocessing. Also, while integrated bioprocessing allows one to manipulate the reaction conditions for a given enzyme, one still cannot use conditions that would kill the cell expressing the enzyme or denature the enzyme itself. For instance in this work, we could not obtain even larger amounts of 4VL by performing integrated bioprocessing with P. putida cells in pH 2 medium because neither the cells nor the enzyme would tolerate a pH value that low. While integrated bioprocessing allows one to “bend” the conditions under which biocatalysis occurs, one still cannot “break” the enzyme or its host cell.

Despite these exceptions, integrated bioprocessing remains a valuable option for enhancing the activity of enzymes that
underperform in the cytosol. This methodology has been successfully applied to lactonization, a reaction that is difficult to carry out in aqueous and cellular systems due to the significant rate of lactone hydrolysis that occurs at neutral pH. Through the use of integrated bioprocessing in this work, the lactone titer increased by more than an order of magnitude. Though in this work a pH difference across the cytosolic membrane was exploited to enhance the product titer, other differences across this membrane, such as the oxygen concentration or the redox state, can be exploited as well. All one needs is a suboptimal state, an N-terminal signal sequence to export the cytosolic enzyme, and a parameter (pH, dissolved oxygen, etc.) that can be manipulated in the medium to enhance the exported enzyme’s activity.

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