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Integrated Bioprocessing for the pH-Dependent Production of 4-Valerolactone from Levulinate in *Pseudomonas putida* KT2440

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Abstract

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 extracytosolic 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 extracytosolic 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 extracytosolic and pH-dependent lactonization of 4-hydroxyvalerate to 4-valerolactone. With this production system, the titer of 4-valerolactone was enhanced substantially in acidic media using extracytosolically-expressed lactonase vs. an intracellular lactonase: from <0.2 g L^{-1} to 2.1 ± 0.4 g L^{-1} at the shake flask scale. Based on these results, the production of 4-hydroxyvalerate and 4-valerolactone was examined in a 2 L bioreactor, and titers of 27.1 g L^{-1} and 8.2 g L^{-1} 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.

Introduction

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 extracytosolic 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, *E. 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 phosphate (7). *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 cellulosic material metabolism) and each has evolved for non-cytosolic expression to facilitate the physiological goals of their 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 hydroxyacids 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 hydroxyacids 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 lactone titer is thermodynamic in nature, meaning that overexpressing the lactonase or most other traditional metabolic engineering techniques aimed at increasing flux towards 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 product titer.

To implement such a system, we used extracytosolically-expressed PON1 to catalyze the lactonization reaction in acidic media (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 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 extracytosolically-expressed PON1 in acidic media to yield 4VL (Figure 1). This general strategy is termed "integrated bioprocessing" for its integration of cytosolic and extracytosolic biocatalysis to enhance production.

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), as a precursor for acrylic compounds (13), and can be used as a precursor for the production of "bio-nylon" polymers (10). Current synthetic methods for 4VL also utilize levulinate as the starting material but require

92 supercritical solvents (14) or carbon dioxide (3) with hydrogen gas under harsh
93 conditions (~10-40 MPa and ~200^{o}C) and ruthenium-based catalysts. In contrast, our
94 biological method of production is done under mild conditions without the need for
95 harsh solvents, hydrogen, rare metal catalysts, or supercritical fluids. This integrated
96 bioprocessing system to produce 4VL from levulinate (Figure 1) was compared to an
97 entirely intracellular 4VL production pathway using cytosolically-expressed PON1 to
98 demonstrate the effectiveness of integrated bioprocessing to improve product titers at
99 different pH values. The system was tested in a 2.0 L bioreactor to further increase
100 product titers as well as take advantage of automated pH control. To our knowledge,
101 this work represents the first report of 4VL synthesis in a biological system.
102

Materials and Methods

Chemicals

All chemicals were purchased at 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 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 (Tet^R) was used to express the thioesterase II (*tes*B) gene from *E. coli* MG1655, while a gentamycin-resistant variant of the plasmid pMMB206, called pMMB206G, was used for the expression of PON1. pRK415 was a generous gift from Prof. Keith Poole while pMMB206 was obtained from ATCC (ATCC # 37808). pMMB206G was produced from pMMB206 by the introduction of a gentamycin resistance cassette excised with *Xmn*I from the plasmid pBSL141 (ATCC #87146) at an *Xmn*I restriction site in pMMB206. All molecular biology manipulations were performed using standard cloning protocols (16).

The *tes*B gene was cloned by PCR into pRK415. Primers used were purchased from Sigma-Genosys (St. Louis, MO, USA) and were as follows (restriction sites used for cloning are underlined): EcoRI-tesB-FP (5'-GAATTCTACTGGAGAGTTATATGAGTCAGG-3') and SalI-tesB-RP (5'-GTCGACTTAATTGTGATTACGCATC-3'). HotStar HiFidelity DNA polymerase was purchased from Qiagen (Valencia, CA, USA) and used according to the manufacturer's instructions. The G3C9 PON1 gene derived from *Homo sapiens* (human) PON1 (1) was similarly cloned by PCR into pMMB206G using the primers PstI-PON1-FP (5'-GACACTGCAGATGGCTAAACTGACAGCG-3') and XbaI-PON1-RP (5'-CATATCTAGATTACAGCTCACAGTAAAGAGCTTTG-3'). Truncated PON1 (tPON1) was made by eliminating the second through the 23rd amino acids from the N-terminus of PON1. tPON1 was amplified from pMMB206G-PON1 by PCR using PstI-tPON1-FP (5'-GACACTGCAGATGTCTTCTTTCCAAACACGAT-3') and XbaI-PON1-RP and inserted into pMMB206G to make pMMB206G-tPON1.

Alkaline phosphatase (PhoA) protein fusion vectors were constructed by first cloning *pho*A by PCR from DH10B *E. coli* genomic DNA into pRK415 using the primers XbaI-phoA-FP (5'-GACATCTAGAATGAAACAAAGCACTATTGCAC-3') and KpnI-phoA-RP (5'-GACAGGTACCTTATTTCAGCCCCAGAGC-3') to make pRK415-phoA. Truncated phoA (tPhoA) was cloned in a similar manner into pRK415 using the primers XbaI-tphoA-FP (5'-GACATCTAGACGGACACCAGAAATGCCT-3') and KpnI-phoA-RP). pRK415-PON1-tphoA and pRK415-tPON1-tphoA vectors were made by digesting

147 pMMB206G-PON1 and pMMB206G-tPON1 with *Pst*I and *Xba*I and ligating into a
148 similarly digested pRK415-tphoA.
149
150 **Culture Conditions**
151 For shake-flask scale 4VL production experiments, recombinant *P. putida* harboring the
152 plasmids pRK415-tesB and pMMB206G-PON1 or pMMB206G-tPON1 was cultured in
153 50 mL LB in a 250 mL shake flask at 32^{o}C. Gentamycin (20 mg L^{-1}) and tetracycline (10
154 mg L^{-1}) were added to provide selective pressure for plasmid maintenance. Cultures were
155 inoculated to an initial optical density at 600 nm (OD_{600}) of 0.05, and 50 μL of 1 M IPTG
156 was added at the beginning of the culture for induction of gene expression. 200 μL of a 5
157 mg L^{-1} aqueous solution of bromothymol blue was added to the cultures as an indicator of
158 culture pH. 1 mL of 20% glucose was added at approximately t = 0, 24, 48, and 72
159 hours. Similarly 2.0 M levulinate was supplied to the cultures as follows: 0.875 mL at t =
160 0 hours, 2.5 mL at t = 24 hours, and 3.75 mL at t = 48 and 72 hours. At t = 96 hours, the
161 pH of the cultures was adjusted to the desired value using 6 N HCl. Additional 6 N HCl
162 was added as needed to maintain the desired pH values. Cultures were then incubated at
163 32^{o}C at the desired pH for an additional 96 hours to allow for 4VL production.
164
165 Bioreactor experiments were performed in a 2.0 L Biostat B bioreactor from Sartorius
166 AG (Goettingen, Germany) equipped with two six-blade disk impellers. The bioreactor
167 was inoculated with 50 mL of LB containing late-exponential phase recombinant *P.*
168 *putida* harboring the plasmids pRK415-tesB and pMMB206G-PON1. The working
169 volume of the bioreactor was 1.0 L, and it was operated at 32^{o}C. Air (0.5-1.0 vvm) was

sparged into the bioreactor and the stirrer speed was varied between 300 and 850 rpm to maintain dissolved O_2 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 L^{-1} tryptone, 24 g L^{-1} yeast extract, 9.4 g L^{-1} potassium hydrogen phosphate and 2.2 g L^{-1} potassium dihydrogen phosphate) with 1.0 mM IPTG, 2.0% glucose, 20 mg L^{-1} gentamycin, 10 mg L^{-1} tetracycline, 4 mM magnesium sulfate, 0.2 mM calcium chloride, and 0.1 mg/L ferric ammonium citrate. After 22 hours of initial growth, levulinic acid was continuously fed to the reactor from a 400 g L^{-1} feedstock to maintain the concentration of levulinate between 2 and 20 g L^{-1}. The concentrations of levulinate, 4HV, and 4VL in the bioreactor were monitored by HPLC, while cell density was monitored by measuring optical density at OD_{600} and converted into g DCW L^{-1} using a conversion factor of 0.42 g DCW OD_{600}^{-1} L^{-1} (15). After 67 hours, the pH of the culture was adjusted to 6.0 and levulinate feeding was discontinued. The culture was continued at 32^{o}C for an additional 50 hours to allow for 4VL production.

Lactonase Assays

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 $CaCl_2$ 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 5.0-7.0. When testing whole-cells, cells were centrifuged at 2,500 x g for 5 minutes, their original medium removed, and the cells were resuspended in 0.9% (w/v) sterile sodium chloride to an OD_{600} of 25. This suspension was then used to supply cells to whole-cell lactonase assay experiments to an OD_{600} of 0.5. For the analysis of cell lysates (prepared by repeateded 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^{o}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* or *P. putida* to be tested for PhoA activity were streaked onto LB plates supplemented with 10 mg L^{-1} tetracycline, 1 mM IPTG, 100 mg L^{-1} XP, and 75 mM phosphate (to suppress endogenous phosphatase expression). These plates were incubated at 37^{o}C (*E. coli*) or 30^{o}C (*P. putida*) for 24-48 hours 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 $MgSO_4$ 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 minutes at 16,000 x *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 x 15 cm, 3.5 μm) purchased from Agilent Technologies (Santa Clara, CA). The column temperature was maintained at 65^{o}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 minutes 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^{o}C. HPLC data was 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).

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 (Figure 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 with 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 (hereinto referred to as PON1) for any ability to export from the cytosol, fusions of PON1 with *E. coli* alkaline phosphatase (PhoA), an enzyme only active in the periplasm (7), 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 2-23 removed, tPON1, were fused to a truncated version of phoA (tPhoA) with residues 1-23 removed. The fusions were constructed as N-PON1-tPhoA-C and N-tPON1-tPhoA-C, with an *Xba*I restriction site used as the linker between PON1 or tPON1 and tPhoA. The stop codon of PON1 and tPON1 was 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 phophatase 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 (Figure 2).

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) (Figure 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 to only the intracellular pH of approximately 7.5. In lysates, however, both PON1 and tPON1 should be exposed to the medium pH, as there is no cytosolic membrane to shield them. As 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 (Figure 3a). Whole-cells expressing tPON1 are only minimally active, achieving only 1.5-2.0% conversion of 4HV into 4VL. At an extracellular pH of 7.2, the results were essentially the same (Figure 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 1.5-2.0% 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 pH's allowing for the highest conversions but at lower production

rates (Figure 4). Higher pH values allowed for 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 (Figure 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 (Figure 1). Recently, we found that *P. putida* is capable of producing high concentrations of 4HV from levulinate when *E. coli* thioesterase II (*tes*B) is expressed (15). Thus by supplying levulinate to recombinant *P. putida* expressing *tes*B 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 *tes*B and either PON1 or tPON1 were grown in LB medium supplemented with levulinate and the pH was unregulated. During this time 10.9 ± 1.3 g L^{-1} and 12.0 ± 0.9 g L^{-1} of 4HV were produced in the PON1 and tPON1 cultures respectively, and the pH of the cultures rose to approximately 8.0-8.5 (data not shown). No 4VL was detected during the first phase. After 96 hours, 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 second phase, lactone

production was monitored for an additional 96 hours. 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 (Figure 5). The enhancement of 4VL titer was 11-fold for PON1 at pH 6.3 vs. 7.3 (2.1 ± 0.4 g L^{-1} 4VL vs. 0.19 ± 0.02 g L^{-1}) and 13-fold for PON1 vs. tPON1 at pH 6.3 (2.1 ± 0.4 g L^{-1} 4VL vs. 0.15 ± 0.02 g L^{-1}).

Bioreactor-Scale Production of 4-Valerolactone

Production of 4VL was examined in a 2.0 L fed-batch reactor to take advantage of automated pH control as well as to further improve 4VL titer. 1.0 L of TB medium was inoculated with *P. putida* KT2440 expressing *tes*B and PON1 and the production of 4HV and 4VL was monitored over time (Figure 6). Levulinate feeding to the reactor did not begin until the cells were in stationary phase (22 hours after inoculation). Levulinate concentrations were maintained between 2 and 20 g L^{-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 hours 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 L^{-1} (Figure 6), which corresponded to a 26.2% yield from levulinate. The 4HV productivity of the reactor during this phase was 0.81 g L^{-1} hr^{-1}. Prior to the pH shift, the titer of 4VL reached 1.6 g L^{-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 L^{-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 PON1 enzyme under more optimal conditions than the cytosolic environment would allow. The G3C9 variant of the human PON1 enzyme had been reported to be expressed in the cytosol of *E. coli* (1) while the native variant is known to associate with the lipid membrane (8). 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 extra-cytosolic. First, qualitative screening of protein fusions of PON1 with truncated PhoA (tPhoA) was performed in *E. coli* and *P. putida* by streaking these cells on plates containing the chromogenic PhoA substrate XP. Second, lactonase assays were done with recombinant *E. coli* cells expressing PON1 and tPON1 to corroborate PON1 export, since lactone production is known to be highly pH-dependent (Figure 4; (19)). The results of these experiments (Table 1, Figure 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 (Figure 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 seven-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 lactone titer (23.6% conversion at a pH of 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 by using an extracytosolic lactonase is due to the integrated bioprocessing effect rather than an artifact of substrate transport across the cytosolic membrane. Recently we found that recombinant *P. putida* expressing thioesterase II (*tes*B) 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 hydroxyacid production with extracytosolic lactonization (Figure 1), can be tested in recombinant *P. putida* expressing both *tes*B and PON1. Using this system, 4VL was produced directly from levulinic acid, and a 13-fold improvement in lactone titer was realized by employing an extracytosolic lactonase for the lactonization reaction versus an intracellular lactonase control (Figure 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 L^{-1} 4VL with PON1 at pH 7.3 versus 0.15 ± 0.02 g L^{-1} for tPON1). To further increase 4VL titer and allow for automated pH control, the 4VL integrated bioprocessing system was tested at the bioreactor scale, and 8.2 g L^{-1} of 4VL was produced (Figure 6). As expected, 4VL production in the bioreactor was highly dependent on medium pH, and a 5-fold improvement in equilibrium 4VL titer was observed when the pH of the medium was decreased to 6.0 from 7.0. While a significant amount of levulinate was left unreacted in both the shake flask and bioreactor experiments, we have found that the excess levulinate inhibits the catabolism of 4HV by *P. putida* (15). Identification and characterization of the enzymes responsible for the conversion of levulinate 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 towards 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 sub-optimally 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 who better function 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 pseudoalcaligenes* alkaline lipase, which has a pH optimum of 8-10 (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 to overcome substrate transport issues with the cell membrane. A classic example of this strategy is the use of extracellular cellulases 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. By placing enzymes 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 is 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 perform 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, lactone titer increased by over an order of magnitude. Though in this work a pH difference across the cytosolic membrane was exploited to enhance product titer, other differences across this membrane such as oxygen concentration or redox state can be exploited as well. All one needs is a suboptimal cytosolic enzyme, an N-terminal signal sequence to export the 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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Tables

Table 1: PhoA Assay results for PON1-PhoA protein fusions. Qualitative results of a 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 given as the averages and standard deviations of three independent experiments.

Table 1

Construct	*E. coli* Colony Color on XP Plates	*P. putida* Colony Color on XP Plates	PNP PhoA Activity in *E. coli* (U/mg)
PhoA	Blue	Blue	1.79 ± 0.06
tPhoA	White	White	0.25 ± 0.05
PON1-tPhoA	Blue	Blue	3.88 ± 0.13
tPON1-tPhoA	White	White	0.26 ± 0.05

Figure Captions

Figure 1: Integrated bioprocessing system for the production of 4VL from levulinate. Production of 4HV from levulinate occurs intracellularly, while the lactonization reaction takes place extracytosolically in acidic media. The enzyme(s) responsible reaction step(s) from levulinate to 4-hydroxyvaleryl-CoA occur in *P. putida* and are currently not known.

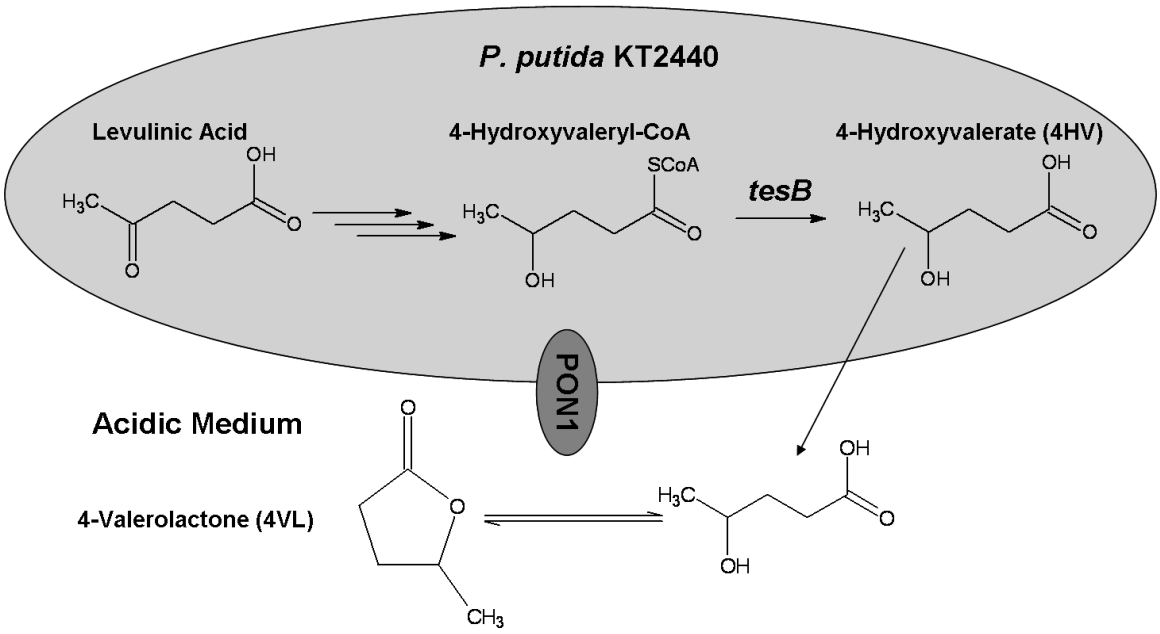
Figure 2: Comparison of the first 25 N-terminal residues of native human PON1, the G3C9 variant of PON1 created by Aharoni and coworkers (2004), and *E. coli* PhoA (a protein known to export into the periplasm). Aliphatic residues (which bury within the membrane) are bolded while cationic residues (presumed 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*.

Figure 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) and 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 three independent experiments.

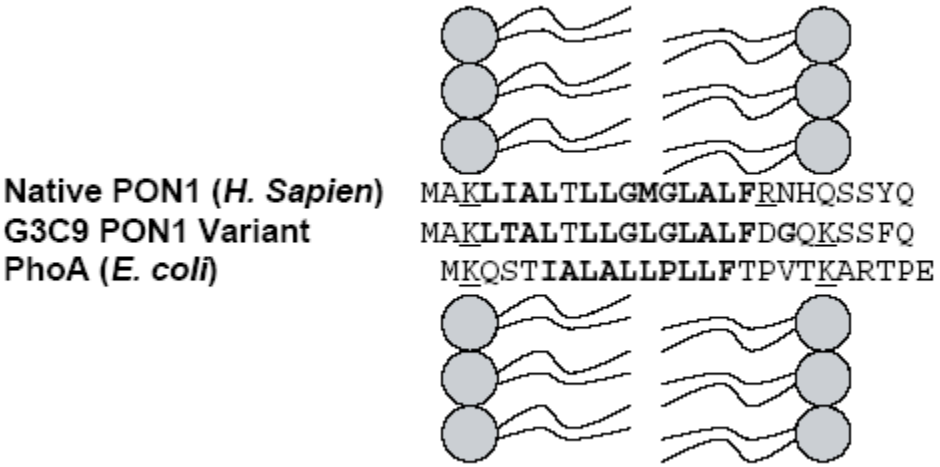
585 **Figure 4:** Conversion of 4HV to 4VL at various pH values by whole-cell *E. coli*
586 expressing PON1 or tPON1. The solid squares, gray-filled squares, empty squares, solid
587 triangles, and empty triangles represent samples with pH values of 5.9, 6.2, 6.4, 6.7 and
588 7.2 respectively for cells expressing PON1. The filled and empty circles represent
589 lactonization by tPON1 and a no PON1 control, respectively, both at a pH value of 6.2.
590 The data points shown are the averages and standard deviations of three independent
591 experiments.
592
593 **Figure 5:** Production of 4VL from levulinate by recombinant *P. putida* expressing tesB
594 and either PON1 (filled symbols) or tPON1 (open symbols) grown in shake flasks.
595 Cultures were grown in LB medium supplemented with glucose and levulinate for 96
596 hours prior to t = 0, during which time the pH was unregulated. 10.9 ± 1.3 g L^{-1} and 12.0
597 $\pm$ 0.9 g L^{-1} of 4HV was produced in the PON1 and tPON1 cultures, respectively. At t = 0
598 hours the pH of the medium was adjusted to either 6.3 (squares) or 7.3 (circles). The data
599 points shown are the averages and standard deviations of three independent experiments.
600
601 **Figure 6:** Production of 4HV (gray-filled squares) and 4VL (black squares) from
602 levulinate (empty squares) by recombinant *P. putida* expressing *tes*B and PON1 grown in
603 a 2.0 L bioreactor. Cell density is indicated by cross-shaped symbols. Levulinate
604 feeding began after 23 hours (first dashed line), and at t = 67 hours the pH of the medium
605 was set to 6.0 to allow for 4VL accumulation (second dashed line). Cell density is
606 indicated by crosses.
607

Figures

Figure 1



611 **Figure 2**



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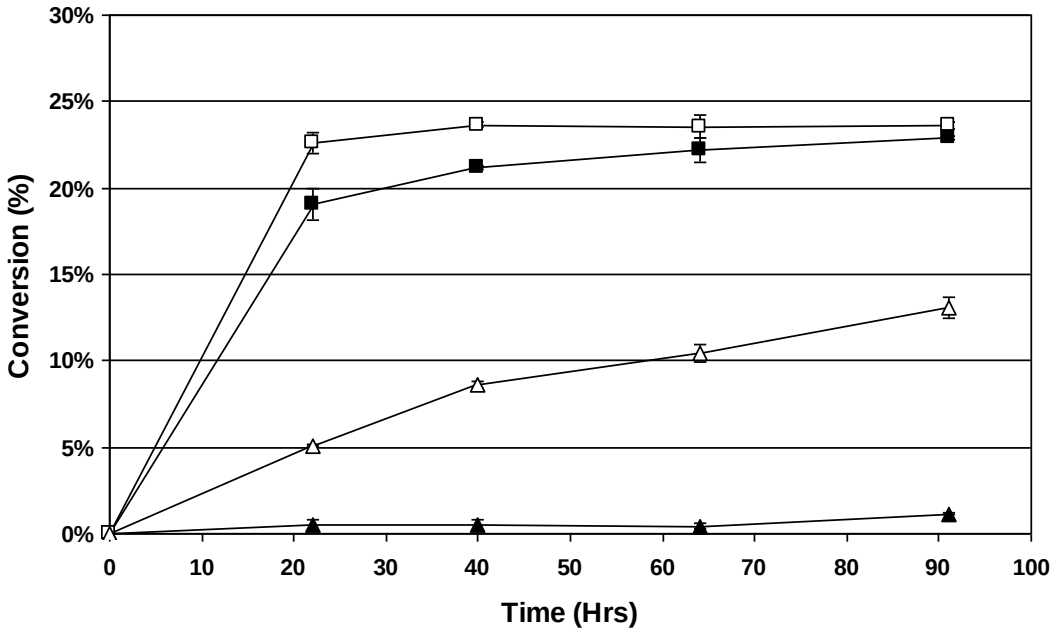
614 **Figure 3a**



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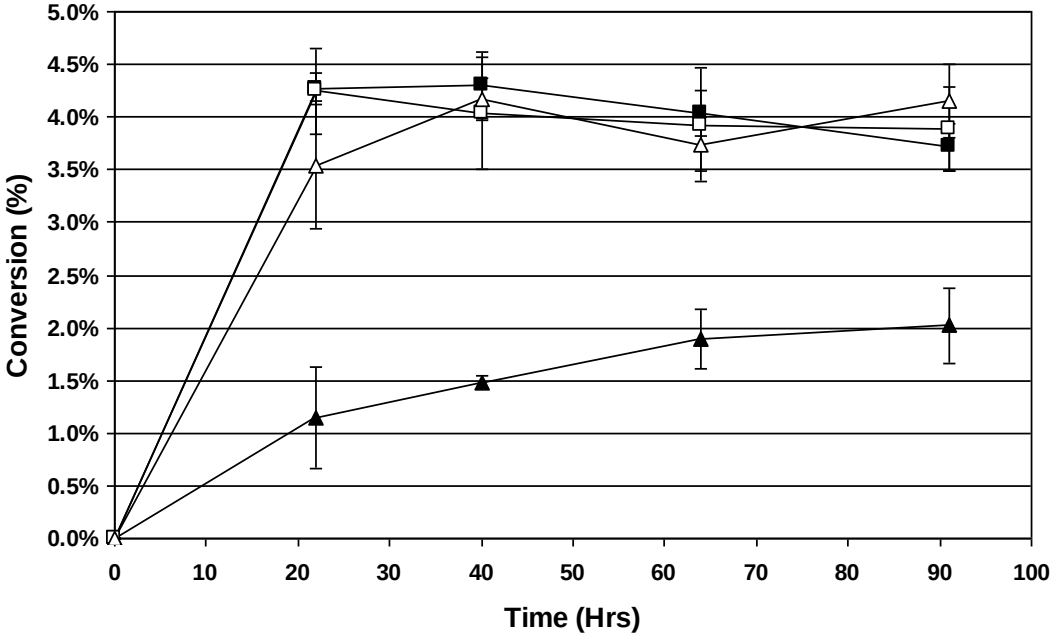
616 **Figure 3b**



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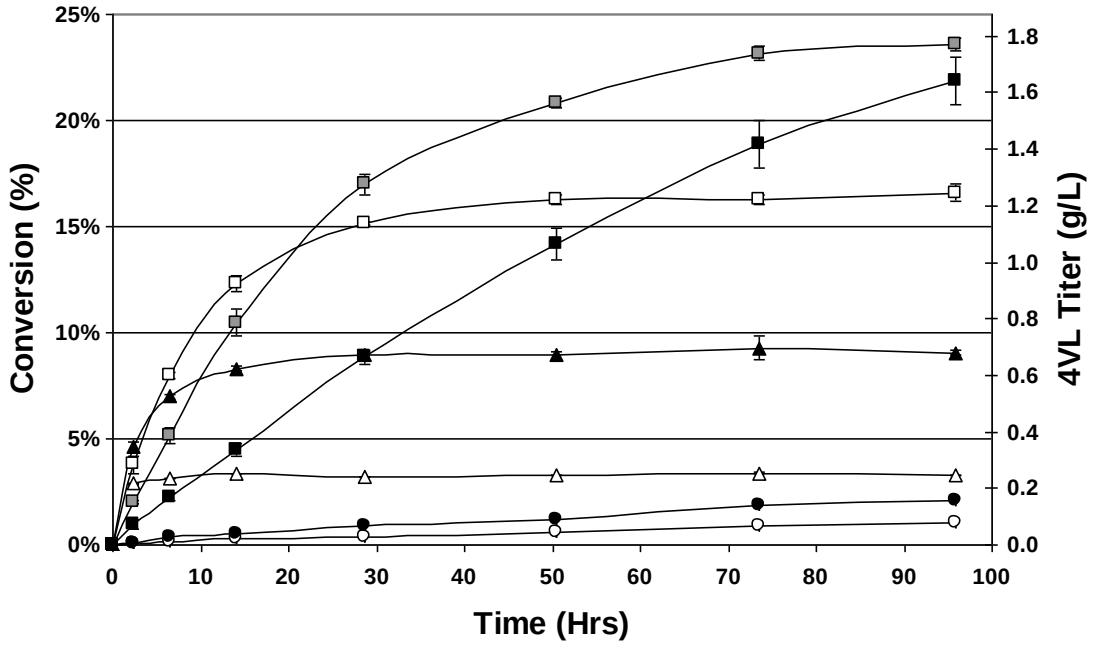
619 **Figure 4**



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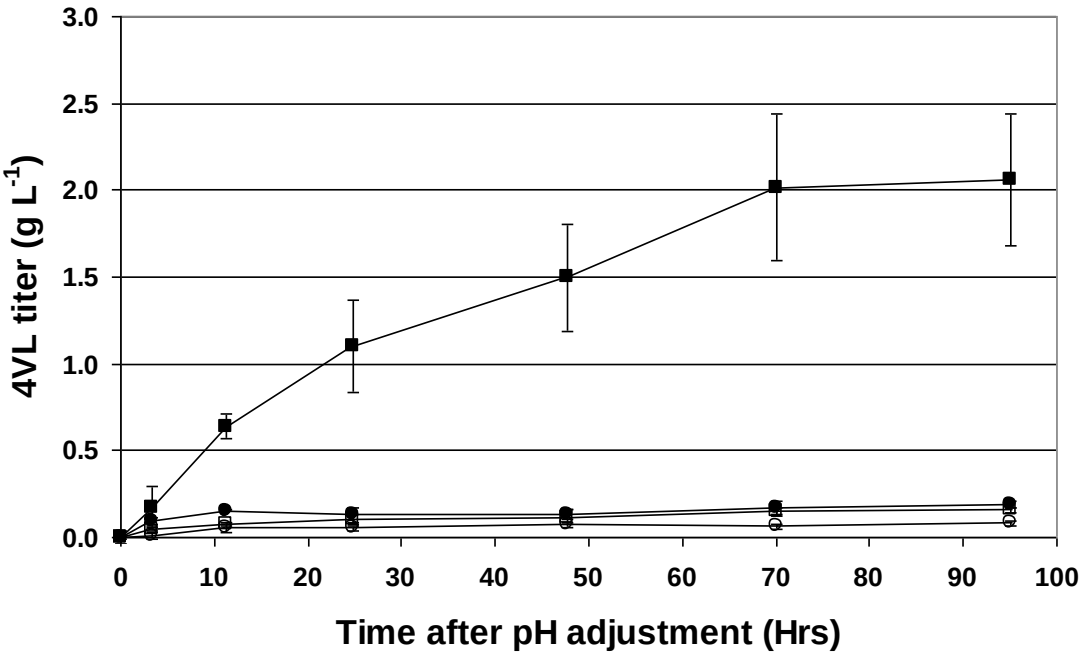
622 **Figure 5**

623



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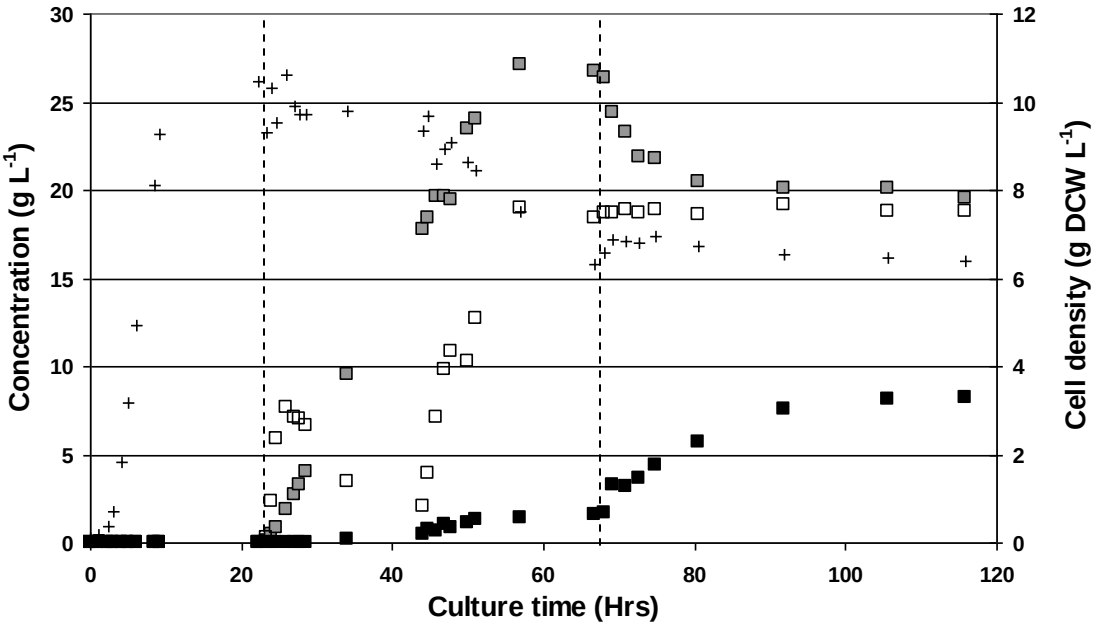
626 **Figure 6**



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