

Metabolic engineering of 3-hydroxypropionic acid biosynthesis in *Escherichia coli*[†]

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Running title: Screening and engineering of a novel aldehyde dehydrogenase for the biosynthesis of 3-hydroxypropionic acid

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[†]This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: [10.1002/bit.25444]

Additional Supporting Information may be found in the online version of this article.

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Received March 12, 2014; Revision Received August 3, 2014; Accepted August 18, 2014

Abstract

3-Hydroxypropionic acid (3-HP) can be produced in microorganisms as a versatile platform chemical. However, owing to the toxicity of the intermediate product 3-hydroxypropionaldehyde (3-HPA), the minimization of 3-HPA accumulation is critical for enhancing the productivity of 3-HP. In this study, we identified a novel aldehyde dehydrogenase, GabD4 from *Cupriavidus necator*, and found that it possessed the highest enzyme activity towards 3-HPA reported to date. To augment the activity of GabD4, several variants were obtained by site-directed and saturation mutagenesis based on homology modeling. *Escherichia coli* transformed with the mutant GabD4_E209Q/E269Q showed the highest enzyme activity, which was 1.4-fold higher than that of wild type GabD4, and produced up to 71.9 g/L of 3-HP with a productivity of 1.8 g/L/h. To the best of our knowledge, these are the highest 3-HP titer and productivity values among those reported in the literature. Additionally, our study demonstrates that GabD4 can be a key enzyme for the development of industrial 3-HP-producing microbial strains, and provides further insight into the mechanism of aldehyde dehydrogenase activity.

Keywords: 3-hydroxypropionic acid (3-HP), aldehyde dehydrogenase, GabD4, glycerol

Introduction

The production of biodiesel is becoming crucial in light of diminishing petroleum reserves and enhanced environmental concerns due to global warming. The rapid growth of the biodiesel industry has led to the generation of vast amounts of crude glycerol as a biodiesel by-product, resulting in a dramatic decrease in crude glycerol prices (Forrest et al., 2010). Significant research efforts have been made to produce high value-added products from biodiesel-derived crude glycerol; thus, the biological conversion of crude glycerol into high-value biochemicals has become a highly active field of study (Nitayavardhana and Khanal, 2011). The utilization of several renewable materials has been proposed; these include succinic acid, lactic acid, and 3-hydroxypropionic acid (3-HP), which advantageously can be produced via biological methods. 3-HP is a 3-hydroxy isomer of lactic acid and is an important building block in the synthesis of many industrially valuable chemicals. This platform chemical is one of the top 12 renewable building blocks (Werpy and Petersen, 2004); thus, great attention has been paid to the microbial conversion of glycerol to 3-HP (True and Lindquist, 2000). The bifunctionality of 3-HP allows its conversion to many chemical intermediates, including malonic acid, 1,3-propandiol, acrylic acid, propiolactone, 3-hydroxymethyl-propionate, and several polymers (Fig. 1). Acrylic acid is a commercially valuable chemical with a \$6 billion global market, and can be derived from 3-HP by catalytic reactions. However, the thermal instability of 3-HP makes its synthesis via chemical reactions difficult (Vollenweider and Lacroix, 2004). The biological conversion of glycerol to 3-HP can be a good alternative to chemical conversion, as glycerol is nontoxic to many microorganisms including *E. coli* even at high concentrations. 3-HP can be biologically produced by a two-step enzyme reaction: the conversion of glycerol into 3-hydroxypropionaldehyde (3-HPA) by glycerol dehydratase and the oxidation of 3-HPA into 3-HP by aldehyde dehydrogenase. Many reports have demonstrated that imbalanced activity between dehydratase and dehydrogenase is a major limiting factor in 3-HP production and 3-HPA accumulation above 30mM caused by the imbalance is harmful to host cells, eventually inhibiting 3-HP production (Slininger et al., 1983). For this reason, the discovery of a novel aldehyde dehydrogenase with high enzyme activity will be necessary to reinforce the flux from 3-HPA to 3-HP and to adjust balance between dehydratase and dehydrogenase.

In this work, we successfully identified a novel aldehyde dehydrogenase, GabD4 derived from *Cupriavidus necator*, by screening 17 candidate aldehyde dehydrogenases from several microorganisms. In

addition, structural analyses based on the predicted structure model of GabD4 were carried out to determine the active site and key amino acid residues related to coenzyme binding. To improve the specific activity of the novel aldehyde dehydrogenase, *gabD4* variants were obtained by site-directed and saturation mutagenesis based on homology modeling, and the enzymatic activities and 3-HP production levels of the variants were analyzed. This is the first report to show the feasibility of the biological production of 3-HP from glycerol at an industrial level, with a titer of 71.9 g/L and a productivity of 1.8 g/L/h.

Materials and Methods

Materials

A list of bacterial strains and plasmids used in this study is provided in Table 1. *E. coli* strain DH5 α and the pETDuet-1 plasmid was purchased from Novagen (Madison, WI, USA). T4 DNA ligase and restriction endonucleases were obtained from New England Biolabs (Beverly, MA, USA). Plasmid DNA was purified using Spin Miniprep kits from Promega (Madison, WI, USA). A gel extraction kit was purchased from Qiagen Inc. (Valencia, CA, USA). 3-HP was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan) and 3-HPA was synthesized chemically from acrolein (Hall and Stern, 1950). Genetic manipulations were carried out according to standard methods (Sambrook et al., 2001). All other reagents were purchased from Sigma (St. Louis, MO, USA) and used without additional purification.

Identification and cloning of aldehyde dehydrogenase genes in *E. coli*

Several candidate aldehyde dehydrogenases were selected by comparison with the reference amino acid sequence of *E. coli aldH* using the online Basic Local Alignment Search Tool (BLAST). The alignment was then analyzed using ProtTest to determine which protein evolution model had the best fit for aldehyde dehydrogenase sequence alignment, and the corresponding crystal structures were downloaded from the PDB database. DNA segments encoding aldehyde dehydrogenase were amplified by PCR using *pfu* DNA polymerase (Stratagene, LaJolla, CA, USA) and pairs of specific primers. The amplified genes (Table 2) were digested with *Bam*HI/*Pst*I (pSPC1), *Nhe*I/*Pst*I (pSPC11), *Nhe*I/*Xho*I (pSPC12), or *Nhe*I/*Kpn*I (all others). Each of the digested fragments (960–1560 bp) was ligated with a pTrcHis vector containing the coding sequences for glycerol dehydratase (*dhaB*) and regeneration factor (*gdrAB*), which is a molecular chaperone that participates in

reactivation of the inactivated coenzyme B₁₂ (Rathnasingh et al. 2009), from *Klebsiella pneumoniae* DSM2026. The resulting expression plasmids, each containing *dhaB*, *gdrAB*, and one of the candidate aldehyde dehydrogenase genes, were transformed into *E. coli* W3110.

Construction of *E.coli* Δ *ackA-pta* Δ *yqhD* mutant strains

Mutants lacking *ackA-pta* and *yqhD* were generated using the Red/ET recombination system (Datsenko and Wanner, 2000). To construct the *ackA-pta* knockout, PCR was performed with two primers: 5'-CGTAGTGATCGATGAGTCTGTTATTCAGGGTATC AAAGGTGTAGGCTGGAGCTGCTTC-3' (sense) and 5'-CAATCCCTGCACCCAGTTCTA CACCCTGAGACGCTGATTCCGGGGATCCGTCGACC-3' (antisense). The oligonucleotide primers used to introduce the *yqhD* mutation were 5'-CGCCATCATGGCGGTGCGGCGCTGCCTTCCAGTTCGGTTAACACGGTGTAGGCTGGAGCTGCTTC-3' (sense) and 5'-GCGCGAGTTCTCAATAATGGCGCGTTTGGTGCGAACTTCGTGGTAATTCCGGGG ATCCGTCGACC-3' (antisense). In both cases, plasmid pKD4 containing an FRT-flanked kanamycin resistance element was used as a template. Each PCR product carrying a 45-bp homologous region at each end was recombined into the chromosome of *E. coli* W3110 by using the Red plasmid pKD46 and selecting kanamycin resistant transformants. The plasmid pCP20, which contains the thermally induced *flp* recombinase gene (Cherepanov and Wackernagel, 1995), was transformed into the *ackA-pta::Km* strain to eliminate the kanamycin resistance gene, and the double crossover mutant Δ *ackA-pta* was identified by PCR on the basis of its genotype. The PCR fragment containing the *yqhD* mutation was transformed into *E. coli* Δ *ackA-pta* by electroporation and double crossover mutants were selected to yield the Δ *ackA-pta* Δ *yqhD* strain. Strains SH004, SH401, and SH501 were then generated by transforming the Δ *ackA-pta* Δ *yqhD* strain with pET-*dhaB-gdrAB-aldH*, pET-*dhaB-gdrAB-gabD4*, and pET-*dhaB-gdrAB*-mutant *gabD4*, respectively.

Site-directed and saturation mutagenesis

Mutations were introduced into a pET-duet plasmid containing aldehyde dehydrogenase (*gabD4*) using the QuikChange™ XL site-directed mutagenesis kit (Stratagene) according to the manufacturer's protocol. Two overlapping complementary primers containing the desired nucleotide changes were designed for each mutation, and were as follows: E209Q sense 5'-GTCGAGACCTGGCTGGG-3' and antisense 5'-

CCCAGCCAGGTCTCGAC-3'; E269Q sense 5'-GCGCGCCAGCATCTGGGCGGC-3' and antisense 5'-GCCGCCCAGATGCTGGCGCGC-3'; E335Q sense 5'-CCAGGAACTGCTG CATTGAC-3' and antisense 5'-GTCAATGCAGCAGTTCCTGG-3'. The PCR reaction mixture consisted of 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM (NH₄)₂SO₄, 2 mM MgSO₄, 0.1% Triton X100, 0.1 mg/mL nuclease-free bovine serum albumin (BSA), 20 ng of template DNA, 0.2 mM dNTPs, 1 µM each of the complementary primers, and 1.5 units of *Pfu* DNA polymerase. The PCRs were performed on an Applied Biosystems (Forster City, CA, USA) thermal cycler with a thermal cycling program consisting of 2 min at 95°C and 16 cycles of 30 sec at 95°C, 60 sec at 55°C, and 12 min at 68°C. All PCR products were amplified a second time with two primers: 5'-CATATGGAGAAAAAATCTACCAGG-3' and 5'-AGATCTTCAGGCCTGGGTGATG-3'. The amplified products were digested with 10 units of *DpnI* at 37°C for 1 h prior to their use for transformation into *E. coli* W3110 cells. The mutations were confirmed by an automatic DNA sequencer (Applied Biosystems).

Saturation mutagenesis was applied to the nucleotides corresponding to amino acid positions 209 and 269 using primer pairs 5'-CAGGTAGGTCGAGACNNN GCTGGGCACGCCCCA-3' (sense) with 5'-TGGGGCGTGCCCAGCENNNGTCTCGACCTA CCTG-3' (antisense), and 5'-GAAGCGCGCCAGCATNNNGGCGGCGGGGTCGAT-3' (sense) with 5'-ATCGACCCCGCCGCCNNNATGCTGGCGCGCTTC-3' (antisense), respectively, where NNN indicates the codon to be mutated. Following enzymatic characterization of the mutants, their DNA was recovered from *E. coli* cells and sequenced to confirm the positions of the mutations.

Determination of enzyme activity

The enzyme activities of wild type and mutant GabD4 were analyzed using 3-HPA in the presence of the coenzyme NAD⁺, and the specific activity was measured (Leal et al., 1995; Jo et al., 2008). The reaction mixture, containing 50 mM potassium phosphate buffer (pH 7.0), 1 mM 2-mercaptoethanol, and 0.02 µM enzyme, was incubated at 37°C for 5 min. The reaction was initiated by adding 2 mM of GabD4, 2 mM 3-HPA and 4 mM NAD⁺. The enzyme activity was determined by measuring the reduction of NAD⁺ to NADH at 340 nm. The amount of NADH formed was determined using a molar extinction coefficient ($\Delta\epsilon_{340}$) of 6.22×10^3 M⁻¹ cm⁻¹. One unit of GabD4 activity was defined as the amount of enzyme required to reduce 1 µmol of NAD⁺ to NADH in 1 min. All enzyme activities were determined in duplicate. The pH effects were determined

in the range of pH 5 to 9 at 45°C using 50 mM sodium acetate (pH 4~5), potassium phosphate (pH 6~8), and glycine-NaOH buffers (pH 9~10) and temperature effects were studied at pH 8.0 in the range of 20°C to 45°C using a spectrophotometer.

Flask-scale production of 3-HP by recombinant *E. coli*

The recombinant strains were cultured in a modified M9 medium containing the following components per liter of deionized water: glycerol, 40 g; MgSO₄•7H₂O, 0.6 g; NaCl, 1.5 g; Na₂HPO₄•7H₂O, 12.8 g; K₂HPO₄, 17.4 g; NH₄Cl, 2 g; yeast extract, 0.5 g; KH₂PO₄, 3 g; and CaCl₂, 0.11 g. The cells were grown aerobically in 250 mL Erlenmeyer flasks containing 100 mL of the medium at 33°C and 250 rpm in an orbital incubator shaker. The cells were induced at ~0.6 OD₆₀₀ with 0.05 mM isopropyl-beta-D-thiogalactopyranoside (IPTG), and 50 µM of filter-sterilized vitamin B₁₂ was added.

Production of 3-HP by recombinant *E. coli* in a 5 L bioreactor

All recombinant strains were cultured in a 5 L bioreactor with a 2 L working volume. The strains were cultured in M9 medium containing the following components per liter of deionized water: glycerol, 80 g; glucose 40 g; MgSO₄•7H₂O, 0.6 g; NaCl, 1.5 g; Na₂HPO₄•7H₂O, 12.8 g; K₂HPO₄, 17.4 g; NH₄Cl, 2 g; yeast extract, 0.5 g; KH₂PO₄, 3 g; CaCl₂, 0.11 g; and ampicillin, 50 mg L⁻¹. Strains grown overnight were inoculated to an OD₆₀₀ of 0.2 in the medium. Aerobic fermentation was carried out in a fed-batch mode by feeding with concentrated glycerol (700 g/L) intermittently. The temperature and agitation were maintained at 35°C and 500 rpm, respectively. A constant flow of sterile air at 1 vvm was maintained throughout fermentation and the pH was kept at 7.0 with 5 N NH₄OH. The recombinant cells were induced at ~0.8 OD₆₀₀ with 0.05 mM IPTG, and sterilized vitamin B₁₂ was added. The glycerol and 3-HP concentrations were monitored periodically using high-performance liquid chromatography (HPLC), and fermentation was continued for 48 h.

Analytical method

The concentrations of 3-HP, glycerol, and other metabolites in the culture broth were determined by HPLC using refractive index (RI) and photodiode array (PDA) detectors (Waters, MILFORD, MA, USA) equipped with an Aminex HPX-87H column (300 mm × 7.8 mm). Operating conditions (0.5 mM H₂SO₄ in the

mobile phase, column temperature 35°C, flow rate 0.4 mL/min) were determined to optimize peak separation. The glycerol and 3-HP were eluted at 16.2 min and 18.3 min, respectively.

Results and discussion

Identification of a novel aldehyde dehydrogenase for 3-HP production

In order to examine the feasibility of the production of 3-HP from glycerol, we constructed 17 different plasmids (pSPC1–17) containing glycerol dehydratase (*dhaB*) and regeneration factor (*gdrAB*) from *Klebsiella pneumoniae* DSM2026 and aldehyde dehydrogenases from *Pseudomonas fluorescens* DSM50090, *Cupriavidus necator* ATCC 17699, *Escherichia coli* K12 DSM18039, *Klebsiella pneumoniae* DSM2026, *Rhodobacter sphaeroides* ATCC17025, *Saccharomyces cerevisiae* ATCC7754, *Pseudomonas aeruginosa* ATCC27853, and *Gluconobacter oxydans* ATCC19357 (Table 1). Each plasmid was transformed into *E. coli* W3110. The resulting pSPC1–17 strains were cultivated in modified M9 medium supplemented with 0.05 mM IPTG and 50 μ M vitamin B₁₂ at 33°C for 48 h. 3-HP production in *E. Coli* transformed with pSPC2, which harbored *gabD4* from *Cupriavidus necator*, was the highest among the 17 candidate aldehyde dehydrogenase genes, including the *aldH* control from *E. coli* (Table 2).

Characterization of enzymatic properties and the specific activity of GabD4

The key enzymatic properties of GabD4 were characterized, including optimum pH and optimum temperature. GabD4 exhibited maximum activity at pH 8 (Fig. 2A), while activity was lower in both acidic and alkaline conditions. This implies that both acidic and alkaline broth conditions have a negative effect on the production of 3-HP in recombinant *E. coli*. With respect to temperature, GabD4 had the highest enzyme activity (55.5 U/mg protein) at 37°C (Fig. 2A). This activity was higher than that reported for AldH from *E. coli* in a previous study (33.2 U/mg protein; Jo et al., 2008).

Site-directed and saturation mutagenesis of *gabD4* based on 3D structure

To improve both the specific activity of GabD4 towards 3-HPA and the 3-HP conversion yield, several *gabD4* mutant strains were constructed by applying 3D structure modeling and mutagenesis techniques. The 3D structure of wild type GabD4 was constructed based on the template retrieved from PDB (3JZ4) by SWISS-

MODEL homology modeling (Fig. 3). The active site of aldehyde dehydrogenases contains conserved cysteine residues, which directly interact with the aldehyde moiety of the substrate (Tu and Weiner, 1998). As shown in Fig. 4, the substrate binds to an enzyme-NAD⁺ complex to form the thiohemiacetal intermediate, which is then oxidized to an acyl intermediate (Craig and Henry, 1999). To determine which cysteine residue is involved in substrate binding, four cysteine coding positions (117, 144, 169, and 283) of GabD4 were altered to alanine by site-directed mutagenesis. Alanines were used for the substitutions because the methyl functional group of alanine cannot efficiently make a nucleophilic attack on the carbonyl carbon of the aldehyde. The dehydrogenase activities of the C117A, C144A, and C169A mutants were not significantly changed compared with native GabD4. In contrast, the C283A mutant lost its enzyme activity towards 3-HPA and produced a low concentration of 3-HP (Fig. 5A); thus, as the toxic intermediate (3-HPA) accumulated, the cell growth for C283A was significantly lower than that for other mutants (Fig 5B). These results indicate that C283 comprises the active site of GabD4.

Based on the crucial role of C283 for the conversion of aldehyde to carboxylic acid, six amino acid residues (E209, V210, E269, M270, L271, and E335) involved in substrate recognition were rationally redesigned around C283, while preserving all remaining positions as wild type. The specific activity of the GabD4 mutants was determined at pH 8 using the method reported by Jo et al. (2008) (Table 3, Supplementary Table 1). Of these mutants, E209Q and E269Q showed the most remarkable increases in catalytic efficiency and substrate-dependent activity. V210L, M270R, L271Q, and E335Q showed the highest specific activity observed at their respective positions; however, their activities were lower than those of E209Q and E269Q. To further investigate positions 209 and 269, we generated a saturation mutagenesis library for residues E209 and E269. Position 269 was observed to be more sensitive to amino acid replacement than position 209, since only three out of 19 mutants of position 269 had activity, while the rest were completely inactivated (Table 4). Moreover, only one of the three active mutants (E269Q) had a higher activity than the wild type (Table 4, Supplementary Table 2). In contrast, enzymatic activity was detected for all 19 mutants of position 209, indicating that this position is not as critical as position 269. The most active mutant assessed was the double mutant E209Q/E269Q, which showed a 1.4-fold increase in catalytic activity when 3-HPA was used as a substrate. Together, these results show that mutations at positions 209 and 269, located near the active site residue (C283), can have great effects on the catalytic activity of GabD4.

Interestingly, the only mutation that was beneficial to enzyme activity was the substitution of glutamic acid with glutamine. Previous works (Wang and Weiner, 1995; Feldman and Weiner, 1972) suggested that deacylation was the rate-limiting step for horse liver aldehyde dehydrogenase and human ALDH2. In these studies, C302 was identified as the active site cysteine and E268 was identified as the general base that interacts with the active site cysteine or water during enzyme catalysis. More recently, additional conserved residues have been mutated (Li et al., 1997; Sheikh et al., 1997). These studies revealed that mutation of E399, a residue that interacts with the ribose moiety of NAD⁺, to glutamine converted the rate limiting step from deacylation to hydride transfer. With respect to our results, it is possible that the active site cysteine residue (position 283) is positioned in a manner that is spatially similar to the conserved glutamic acid residues. The rate-limiting step of the abovementioned AldH-mediated reaction may be related to the conformational difference of bound NAD⁺ and the relative flexibility of the nicotinamide ring. In this scenario, the substitution of glutamic acid for glutamine at position 209 and 269 of GabD4 would result in proper positioning for NAD⁺ or substrate binding, leading to increased activity towards 3-HPA (Supplementary Fig. 1).

Cell growth and flask-scale 3-HP production by recombinant *E. coli* containing engineered *gabD4*

To evaluate the potential of the recombinants SH004 (*E. coli* W3110 harboring *dhaB*, *gdrAB* and *aldH*), SH401 (*E. coli* W3110 harboring *dhaB*, *gdrAB* and *gabD4*), and SH501 (*E. coli* W3110 harboring *dhaB*, *gdrAB* and mutant *gabD4*) for 3-HP production, each strain was cultivated aerobically in a 100 mL of a modified M9 medium in a 250 mL Erlenmeyer flask. To reduce the accumulation of the major by-products of 3-HP production, two genes that are essential for their formation, *ackA-pta* and *yqhD*, were eliminated in the chromosomal DNA of *E. coli* W3110. *AckA-pta* is responsible for acetic acid formation from acetyl-CoA (Yun et al., 2005), while *yqhD* is involved in 1,3-propanediol production from 3-HPA (Jarboe, 2011). As to 3-HP titer, 10.2 g/L of 3-HP was detected in the flask culture broth of *E. coli* W3110 with Δ *ackA-pta* and Δ *yqhD* (SH004), which is 38% increase compared to the 3-HP production (7.4 g/L) of wild type *E. coli* W3110 containing *dhaB*, *gdrAB* and *aldH* (SH003). Concretely, *E. coli* W3110 containing *dhaB*, *gdrAB* and *aldH* with Δ *ackA-pta* produced 8.2 g/L of 3-HP and *E. coli* W3110 containing *dhaB*, *gdrAB* and *aldH* with Δ *yqhD* produced 9.2 g/L of 3-HP, respectively. The flask-scale experiments showed that SH501 had higher 3-HP production efficiency than SH004 and SH401. The cell growth of all mutants showed no difference at an OD₆₀₀ of 2.5–3 (Fig. 6A). Under

shake-flask culture conditions, SH004 produced 10.2 g/L of 3-HP and SH401 produced 11.4 g/L. Most of the strains harboring the GabD4 mutants produced 3-HP more efficiently than SH004 and SH401, particularly GabD4_E209Q/E269Q, which produced 16.7 g/L of 3-HP (Fig. 6B). This is 1.6-fold higher than the 3-HP production by strain SH401, suggesting that the activity of GabD4_E209Q/E269Q is more efficient than those of AldH or wild type GabD4, and that its balance with DhaB is more suitable for improving the titer of 3-HP production from glycerol.

Cultivation of the SH501_E209Q/E269Q in a 5 L bioreactor

In the flask-scale experiments, strain SH501_E209Q/E269Q exhibited the highest 3-HP production, at 16.7 g/L. In order to further investigate the potential of SH501_E209Q/E269Q, the strain was cultured at pH 7.0 in a 5 L bioreactor with a working volume of 2 L (Fig. 6C). The initial glycerol concentration was 80 g/L and concentrated glycerol (700 g/L) was added to the culture broth as a feeding substrate to maintain the glycerol concentration. After 40 h of culture, the titers of SH004 and SH401 were 50.8 g/L and 60.1 g/L, respectively. The titer of SH501_E209Q/E269Q was 71.9 g/L, which is approximately 4.3-fold higher than the titer obtained from the flask culture, and the productivity was 1.8 g/L/h. This titer is higher than the corresponding values previously reported by other researchers (Table 5) (Kumar et al., 2013). Although our approach to produce 3-HP from glycerol and glucose is similar to those used by other researchers, the present study employed different aldehyde dehydrogenases (*gabD4*) and engineered the enzyme to enhance its activity. High level of 3-HP productivity (2.7 g/L/h) was observed at 16–37 h. A decrease in the specific rate of 3-HP production at 37–40 h may be linked to the decrease in both DhaB and the GabD4_E209Q/E269Q activities. This decrease could also be related to a redox imbalance resulting from inadequate NAD⁺ regeneration, which is essential for GabD4 activity. However, the specific rate of 3-HP production was maintained at high levels for up to 37 h, even though the activities of both DhaB and the GabD4_E209Q/E269Q had deteriorated significantly at this time.

Conclusions

In this study, we report two parallel methods for improving 3-HP production from glycerol in *E. coli*. The utilization of a novel glycerol dehydrogenase, GabD4 from *Cupriavidus necator*, resulted in an 18.3%

increase in 3-HP production, yielding final 3-HP titers of 60.1 g/L. Systematic engineering of GabD4, on the other hand, resulted in a 41.5% increase in 3-HP production, yielding final titers of 71.9 g/L. Furthermore, a combination of these strategies led to an increase in 3-HP productivity. Further work, such as fine-tuning of enzyme expression, scale-up, and process engineering, will be necessary to further increase the titer and productivity to levels that are sufficient for commercialization. However, our successful enhancement of 3-HP production bodes well for continued engineering efforts, as our engineering approach is directly applicable to the high-level production of many commercially important compounds using microbial biotechnology.

References

- Ashok, S., Sankaranarayanan, M., Ko, Y., Jae, K.E., Ainala, S.K., Kumar, V., Park, S.H., 2012. Production of 3-hydroxypropionic acid from glycerol by recombinant *Klebsiella pneumoniae* $\Delta dhA T \Delta yqhD$ which can produce vitamin B₁₂ naturally. *Biotechnol. Bioeng.* 110, 511–524.
- Cherepanov, P.P., Wackernagel, W., 1995. Gene disruption in *Escherichia coli*: Tc^R and Km^R cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. *Gene* 158, 9-14.
- Craig, J.M., Henry, W., 1999. Differences in the roles of conserved glutamic acid residues in the active site of human class 3 and class 2 aldehyde dehydrogenases. *Protein Science* 8, 1922–1929.
- Datsenko, K. A. and Wanner, B.L., 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *PNAS* 97, 6640–6645.
- Feldman, R.I., Weiner, H., 1972. Horse liver aldehyde dehydrogenase. II: kinetics and mechanistic implications of the dehydrogenase and esterase activity. *J. Biol. Chem.* 247, 267–272.
- Forrest, A.K., Sierra, R., Holtzapple, M.T., 2010. Effect of biodiesel glycerol type and fermentor configuration on mixed-acid fermentations. *Bioresour. Technol.* 101, 9185–9189.
- Garai-Ibabe, G., Ibarburu, I., Berregi, A., Claisse, O., Lonvaud-Funel, A., Irastorza, A., Dueñas, M.T., 2008. Glycerol metabolism and bitterness producing lactic acid bacteria in cidermaking. *Int. J. Food Microbiol.* 121, 253-261.
- Hall, R.H., Stern, E.S., 1950. Acid-catalysed hydration of acrylaldehyde: kinetics of the reaction and isolation of β -hydroxypropionaldehyde. *J. Chem. Soc.* 490–498.
- Huang, Y., Li, Z., Shimizu, K., Ye, Q., 2012. Simultaneous production of 3-hydroxypropionic acid and 1,3-propanediol from glycerol by a recombinant strain of *Klebsiella pneumoniae*. *Bioresour. Technol.* 103, 351–359.
- Huang, Y., Li, Z., Shimizu, K., Ye, Q., 2013. Co-production of 3-hydroxypropionic acid and 1,3-propanediol by *Klebsiella pneumoniae* expressing aldH under micro-aerobic conditions. *Bioresour. Technol.* 128, 505–512.
- Jarboe, L.R., 2011. *yqhD*: a broad-substrate range aldehyde reductase with various applications in production of biorenewable fuels and chemicals. *Appl. Microbiol. Biotechnol.* 89(2), 249-257.
- Jo, J. E., Mohanraj, S., Rathnasingh, C., Selvakumar, E., Jung, W.C., Park, S.H., 2008. Cloning, expression, and

- characterization of an aldehyde dehydrogenase from *Escherichia coli* K-12 that utilizes 3-Hydroxypropionaldehyde as a substrate. Appl. Microbiol. Biotechnol. 81, 51-60.
- Ko, Y., Ashok, S., Zhou, S., Kumar, V., Park, S.H., 2012. Aldehyde dehydrogenase activity is important to the production of 3-hydroxypropionic acid from glycerol by recombinant *Klebsiella pneumonia*. Process Biochem. 47, 1135–1143.
- Kumar, V. Ashok, S. Park, S.H., 2013. Recent advances in biological production of 3-hydroxypropionic acid. Biotechnology Advances.
- Kumar, V., Mugesh, S., Durgapal, M., Zhou, M., Ko, Y., Ashok, S., Sarkar, R., Park, S.H., 2012. Simultaneous production of 3-hydroxypropionic acid and 1,3-propanediol from glycerol using resting cell system of D-lactic acid deficient mutant of *Klebsiella pneumoniae* with overexpression of KGSADH. Bioresour. Technol. 103, 351-359.
- Kwak, S., Park, Y.C., Seo, J.H., 2012. Biosynthesis of 3-hydroxypropionic acid from glycerol in recombinant *Escherichia coli* expressing *Lactobacillus brevis dhaB* and *dhaR* gene clusters and *E. coli* K-12 *aldH*. Bioresour. Technol. 135, 432-439.
- Leal, N.A., Havemann, G.D., Bobik, T.A., 2003. *PduP* is a coenzyme-A-acylating propionaldehyde dehydrogenase associated with the polyhedral bodies involved in B12-dependent 1,2-propanediol degradation by *Salmonella enterica* serovar *Typhimurium* LT2. Arch. Microbiol. 180, 353–361.
- Li, N., Sheikh, S., Weiner, H., 1997. Involvement of glutamate 399 and lysine 192 in the mechanism of human liver mitochondrial aldehyde dehydrogenase. J. Biol. Chem. 272, 18823–18826.
- Luo, L.H., Kim, C.H., Heo, S.Y., Oh, B.R., Hong, W.K., Kim, S., Kim, D.H., Seo, J.W., 2012. Production of 3-hydroxypropionic acid through propionaldehyde dehydrogenase PduP mediated biosynthetic pathway in *Klebsiella pneumonia*. Bioresour. Technol. 103, 1–6.
- Luo, L.H., Seo, J.W., Oh, B.R., Seo, P.S., Heo, S.Y., Hong, W.K., Kim, D.H., Kim, C.H., 2011. Stimulation of reductive glycerol metabolism by overexpression of an aldehyde dehydrogenase in a recombinant *Klebsiella pneumoniae* strain defective in the oxidative pathway. J. Ind. Microbiol. Biotechnol. 38, 991–999.
- Lynch, M.D., Gill, R.T., Warnecke, L.T., 2011. Method for producing 3-hydroxypropionic acid and other products. PCT Patent 038,364 A1.
- Nitayavardhana, S., Khanal, S.K., 2011. Biodiesel-derived crude glycerol bioconversion to animal feed: a

- sustainable option for a biodiesel refinery. *Bioresour. Technol.* 102, 5808–5814.
- Raj, S.M., Rathnasingh, C., Jo, J.E., Park, S.H., 2008. Production of 3-hydroxypropionic acid from glycerol by a novel recombinant *Escherichia coli* BL21 strain. *Process Biochem.* 43, 1440-1446.
- Raj, S.M., Rathnasingh, C., Jung, W.C., Park, S.H., 2009. Effect of process parameters on 3-hydroxypropionic acid production from glycerol using a recombinant *Escherichia coli*. *Appl. Microbiol. Biotechnol.* 84, 649–657.
- Rathnasingh, C., Raj, S.M., Jo, J.E., Park, S.H., 2009. Development and evaluation of efficient recombinant *Escherichia coli* strains for the production of 3-hydroxypropionic acid from glycerol. *Biotechnol. Bioeng.* 104, 729–739.
- Sambrook, J., Fritsch, E. F., Maniatis, T., 2001. *Molecular cloning: a laboratory manual*. 3rd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Sheikh, S., Ni, L., Hurley, T.D., Weiner, H., 1997. The potential roles of the conserved amino acids in human liver mitochondrial aldehyde dehydrogenase. *J. Biol. Chem.* 272, 18817–18822.
- Slininger, P.J., Bothast, R.J., Smiley, K.L., 1983. Production of 3-hydroxypropionaldehyde from glycerol. *Appl. Environ. Microbiol.* 46, 62–67.
- True, H.L., Lindquist, S.L., 2000. A yeast prion provides a mechanism for genetic variation and phenotypic diversity. *Nature* 407, 477–483.
- Tu, G., Weiner, H. 1998. Identification of the cysteine residue in the active site of horse liver mitochondrial aldehyde dehydrogenase. *J. Biol. Chem.* 273, 1212-1217.
- Vollenweider, S., Lacroix, C., 2004. 3-Hydroxypropion-aldehyde: applications and perspective. *Appl. Microbiol. Biotechnol.* 64, 16–27.
- Wang, X., Weiner, H., 1995. Involvement of glutamate 268 in the active site of human liver mitochondrial (class 2) aldehyde dehydrogenase as probed by site-directed mutagenesis. *Biochemistry* 34, 237–243.
- Werpy, T., Petersen, G., 2004. ‘Top Value Added Chemicals from Biomass’, US Department of Energy.
- Yun, N.R. San, K.Y. Bennett, G.N., 2005. Enhancement of lactate and succinate formation in *adhE* or *pta-ackA* mutants of NADH dehydrogenase-deficient *Escherichia coli*. *J. Appl. Microbiol.* 99, 1404-1412.

Tables

Table 1. Bacterial strains and plasmids used in this study

Plasmid or strain	Relevant characteristics	Reference
Plasmid		
pETDuet-1	Expression vector	Novagen
pTrcHis	Expression vector	Novagen
pSPC1	pTrcHis:: <i>dhaB</i> , <i>gdrAB</i> (<i>K. pneumonia</i>) and <i>hapE</i> (AAK54073.1) from <i>Pseudomonas fluorescens</i>	This study
pSPC2	pTrcHis:: <i>dhaB</i> , <i>gdrAB</i> (<i>K. pneumonia</i>) and <i>gabD4</i> (CAJ96839.1) from <i>Cupriavidus necator</i>	This study
pSPC3	pTrcHis:: <i>dhaB</i> , <i>gdrAB</i> (<i>K. pneumonia</i>) and <i>gabD3</i> (CAJ96324.1) from <i>Cupriavidus necator</i>	This study
pSPC4	pTrcHis:: <i>dhaB</i> , <i>gdrAB</i> (<i>K. pneumonia</i>) and <i>gabD2</i> (CAJ95970.1) from <i>Cupriavidus necator</i>	This study
pSPC5	pTrcHis:: <i>dhaB</i> , <i>gdrAB</i> (<i>K. pneumonia</i>) and <i>gabD1</i> (CAJ95773.1) from <i>Cupriavidus necator</i>	This study
pSPC6	pTrcHis:: <i>dhaB</i> , <i>gdrAB</i> (<i>K. pneumonia</i>) and <i>gabD</i> (AAC75708.1) from <i>E. coli</i> K12 MG1655	This study
pSPC7	pTrcHis:: <i>dhaB</i> , <i>gdrAB</i> (<i>K. pneumonia</i>) and <i>sad</i> (CDH65097.1) from <i>E. coli</i>	This study
pSPC8	pTrcHis:: <i>dhaB</i> , <i>gdrAB</i> (<i>K. pneumonia</i>) and <i>gabD3</i> (EEW40128.1) from <i>Klebsiella pneumoniae</i>	This study
pSPC9	pTrcHis:: <i>dhaB</i> , <i>gdrAB</i> (<i>K. pneumonia</i>) and <i>rsph17025</i> (ABP68928.1) from <i>Rhodobacter sphaeroides</i>	This study
pSPC10	pTrcHis:: <i>dhaB</i> , <i>gdrAB</i> (<i>K. pneumonia</i>) and <i>uga2</i> (CAA84945.1) from <i>Saccharomyces cerevisiae</i>	This study
pSPC11	pTrcHis:: <i>dhaB</i> , <i>gdrAB</i> (<i>K. pneumonia</i>) and <i>gabD</i> (CDH68601.1) from <i>Pseudomonas aeruginosa</i>	This study
pSPC12	pTrcHis:: <i>dhaB</i> , <i>gdrAB</i> (<i>K. pneumonia</i>) and <i>gabD</i> (AEV60207.1) from <i>Pseudomonas fluorescens</i>	This study
pSPC13	pTrcHis:: <i>dhaB</i> , <i>gdrAB</i> (<i>K. pneumonia</i>) and <i>gox0499</i> (AAW60278.1) from <i>Gluconobacter oxydans</i>	This study
pSPC14	pTrcHis:: <i>dhaB</i> , <i>gdrAB</i> (<i>K. pneumonia</i>) and <i>gox1122</i> (AAW60890.1) from <i>Gluconobacter oxydans</i>	This study
pSPC15	pTrcHis:: <i>dhaB</i> , <i>gdrAB</i> (<i>K. pneumonia</i>) and <i>ald2</i> (CAA89806.1) from <i>Saccharomyces cerevisiae</i>	This study
pSPC16	pTrcHis:: <i>dhaB</i> , <i>gdrAB</i> (<i>K. pneumonia</i>) and <i>ald3</i> (DAA10065.1) from <i>Saccharomyces cerevisiae</i>	This study
pSPC17	pTrcHis:: <i>dhaB</i> , <i>gdrAB</i> (<i>K. pneumonia</i>) and <i>ald4</i> (DAA11133.1) from <i>Saccharomyces cerevisiae</i>	This study
Strain		
W3110	Expression host	ATCC 27325
SH003	Recombinant host W3110 harboring <i>dhaB</i> , <i>gdrAB</i> and <i>aldH</i>	This study
SH004	<i>ΔackA-pta</i> , <i>ΔyqhD</i> of recombinant host W3110 harboring <i>dhaB</i> , <i>gdrAB</i> and <i>aldH</i>	This study
SH401	<i>ΔackA-pta</i> , <i>ΔyqhD</i> of recombinant host W3110 harboring <i>dhaB</i> , <i>gdrAB</i> and <i>gabD4</i>	This study
SH501	<i>ΔackA-pta</i> , <i>ΔyqhD</i> of recombinant host W3110 harboring <i>dhaB</i> , <i>gdrAB</i> and mutant <i>gabD4</i>	This study

Table 2. 3-HP production of candidate aldehyde dehydrogenases

Aldehyde dehydrogenase	3-HP (g/L)	Aldehyde dehydrogenase	3-HP (g/L)	Aldehyde dehydrogenase	3-HP (g/L)
SPC1_ <i>hapE</i> (<i>P.fluorescens</i>)	n.d.	SPC7_ <i>sad</i> (<i>E.coli</i>)	n.d.	SPC13_ <i>gox0499</i> (<i>G.oxydans</i>)	n.d.
SPC2_ <i>gabD4</i> (<i>C.necator</i>)	9.75±0.92	SPC8_ <i>gabD3</i> (<i>K.pneumoniae</i>)	n.d.	SPC14_ <i>gox1122</i> (<i>G.oxydans</i>)	n.d.
SPC3_ <i>gabD3</i> (<i>C.necator</i>)	0.21±0.13	SPC9_ <i>rsph17025</i> (<i>R.sphaeroides</i>)	n.d.	SPC15_ <i>ald2</i> (<i>S.cerevisiae</i>)	n.d.
SPC4_ <i>gabD2</i> (<i>C.necator</i>)	n.d.	SPC10_ <i>uga2</i> (<i>S.cerevisiae</i>)	n.d.	SPC16_ <i>ald3</i> (<i>S.cerevisiae</i>)	n.d.
SPC5_ <i>gabD1</i> (<i>C.necator</i>)	n.d.	SPC11_ <i>gabD</i> (<i>P.aeruginosa</i>)	n.d.	SPC17_ <i>ald4</i> (<i>S.cerevisiae</i>)	0.17±0.10
SPC6_ <i>gabD</i> (<i>E.coli</i>)	n.d.	SPC12_ <i>gabD</i> (<i>P.fluorescens</i>)	0.49±0.37	Control_ <i>aldH</i> (<i>E.coli</i>)	6.75±0.49

*“n.d.” stands for “not detected” at the Minimum Reporting Level (the concentration of 3-HP < 0.001 g/L)”

Table 3. The highest specific activity of mutant GabD4 at position 209, 210, 269, 270, 271, and 335

Aldehyde dehydrogenase	$V_{\max}$ (U/mg protein)	$k_{\text{cat}}/K_m \times 10^3$ (M ⁻¹ S ⁻¹)
AldH	38.16±2.13	57.28±3.69
GabD4	55.12±1.37	71.48±1.26
Mutant GabD4_E209Q	60.43±1.42	93.22±4.13
Mutant GabD4_V210L	49.23±2.23	62.27±2.57
Mutant GabD4_E269Q	74.16±2.92	87.36±2.04
Mutant GabD4_M270R	53.62±1.48	55.76±3.08
Mutant GabD4_L271Q	50.36±2.78	44.24±4.58
Mutant GabD4_E335Q	57.77±1.54	49.33±4.69

Table 4. The specific activity of mutant GabD4 at position 209 and 269 by saturation mutagenesis

Aldehyde dehydrogenase	$V_{\max}$ (U/mg protein)	$k_{\text{cat}}/K_m \times 10^3$ (M ⁻¹ S ⁻¹)
AldH	38.16±2.13	57.28±3.69
GabD4	55.12±1.37	71.48±1.26
Mutant GabD4_E209G	52.59±1.69	76.35±5.48
Mutant GabD4_E209A	54.05±3.25	53.48±1.09
Mutant GabD4_E209S	50.03±1.38	63.75±4.09
Mutant GabD4_E209T	53.37±1.14	82.38±2.89
Mutant GabD4_E209C	52.09±1.41	72.13±3.81
Mutant GabD4_E209V	53.81±1.34	67.21±0.89
Mutant GabD4_E209L	49.04±2.83	98.01±2.74
Mutant GabD4_E209I	53.13±1.11	74.34±3.94
Mutant GabD4_E209M	52.58±1.22	63.04±2.17
Mutant GabD4_E209P	48.36±1.13	55.36±5.12
Mutant GabD4_E209F	49.57±2.85	51.48±2.14
Mutant GabD4_E209Y	59.03±1.44	44.16±3.45
Mutant GabD4_E209W	53.86±1.29	50.23±4.25
Mutant GabD4_E209D	57.67±2.02	74.25±2.01
Mutant GabD4_E209N	53.56±1.04	43.72±4.27
Mutant GabD4_E209Q	60.43±1.42	93.22±4.13
Mutant GabD4_E209H	55.71±1.18	60.35±6.42
Mutant GabD4_E209K	54.60±2.16	42.18±1.62
Mutant GabD4_E209R	58.64±1.02	55.69±4.43
Mutant GabD4_E269Q	74.26±2.92	87.36±2.04
Mutant GabD4_E269K	56.28±1.87	46.25±3.84
Mutant GabD4_E269R	60.23±2.41	55.47±2.32
Mutant GabD4_E209,269Q	78.07±1.78	162.34±1.38
Mutant GabD4_E209,335Q	69.73±2.01	93.46±2.43
Mutant GabD4_E269,335Q	72.16±2.48	141.09±3.24
Mutant GabD4_E209,269,335Q	63.72±2.30	69.48±5.39

Table 5. Recent studies for biological production of 3-HP

Carbon source	Recombinant strain	Titer (g/L)	Productivity (g/L/h)	Reference
glycerol	<i>Lactobacillus collinoides</i> 17	2.8	0.07	Graai-Ibabe et al., 2008
glycerol	<i>E. coli</i> BL21_dhaB_aldH	0.6	0.02	Raj et al., 2008
glycerol	<i>E. coli</i> BL21_dhaB_aldH	31	0.43	Raj et al., 2009
glycerol	<i>E. coli</i> SH254_dhaB_KGSADH	38.7	0.54	Rathnasingh et al., 2009
glucose	<i>E. coli</i> (BX3_0240)	49	0.71	Lynch et al., 2011
glycerol	<i>K. pneumoniae</i> AK_pduP	1.4	0.06	Luo et al., 2011
glycerol	<i>K. pneumoniae</i> Cu	1.9	0.08	Luo et al., 2012
glycerol	<i>K. pneumoniae</i> WM3 pUC18kan_aldHec	24.4	1.02	Huang et al., 2012
glycerol	<i>K. pneumoniae</i> WM3 pUC18kan_aldHec	48.9	1.75	Huang et al., 2013
glycerol	<i>K. pneumoniae</i> J2B ΔdhaT_KGSADH	16.3	0.3	Ko et al., 2012
glycerol	<i>K. pneumoniae</i> ΔdhaTΔyqhD_dhaB_puuC	28.1	0.58	Ashok et al., 2012
glycerol	<i>K. pneumoniae</i> J2BΔldhA_KGSADH	22.7	0.38	Kumar et al., 2012
glucose & glycerol	<i>E. coli</i> BL21_dhaB_dhaR_aldH	14.3	0.26	Kwak et al., 2012
glucose & glycerol	<i>E. coli</i> W3110 ΔackA-ptA ΔyqhD_dhaB_mutant gabD4	71.9	1.8	This study

Figure Captions

Fig. 1 Various building blocks from 3-HP

Fig. 2 Optimum (A) pH and (B) temperature on GabD4 activity

Fig. 3 3D-structural modeling of GabD4, showing the location of mutations; C283 is active site residue and E209, 269 and 335 were mutated positions.

Fig. 4 Reaction mechanism for the dehydrogenase activity. E-SH indicates the enzyme with the active site cysteine as the nucleophile. E-NAD complex to form the thiohemiacetal intermediate, which is then oxidized to an acyl intermediate.

Fig. 5 (A) 3-HP production and (B) cell growth of C117A, C144A, C169A, C283A and GabD4; C117A (◆), C144A (▲), C169A (■), C283A (■) and GabD4 (●).

Fig. 6 (A) Cell growth and (B) 3-HP production of AldH, GabD4 and mutated GabD4 in flask; GabD4 (◆), AldH (■), E209Q (■), E269Q (▲), E335Q (x), E209Q/E269Q (), E209Q/E269Q (●),*E269Q/E335Q (|), E209Q/E269Q/E335Q (■) (C) Cell growth and 3-HP production of SH004, SH401, and SH501_E209Q/E269Q in 5 L bioreactor; open circle (○) OD of SH401, open square(□) OD of SH501_E209Q/E269Q, open triangle (△) OD of SH004, close circle(●) 3-HP production of SH401, close square(■) 3-HP production of SH501_E209Q/E269Q, and close triangle(▲) 3-HP production of SH004.

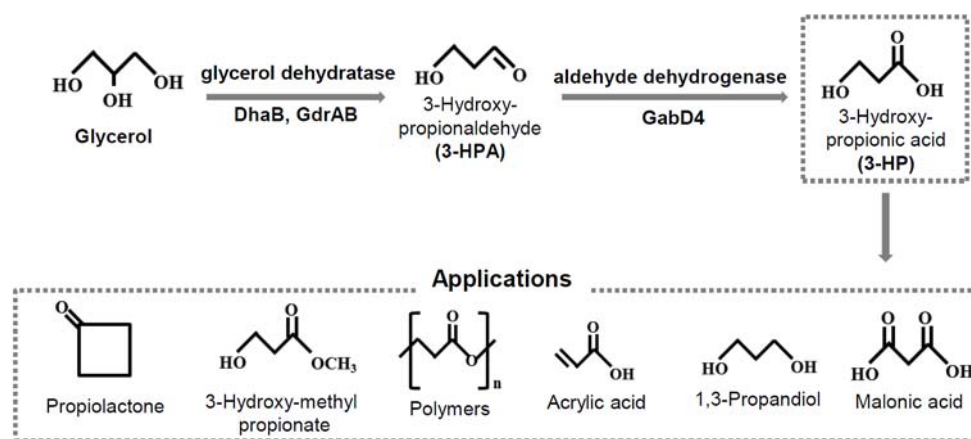


Figure 1

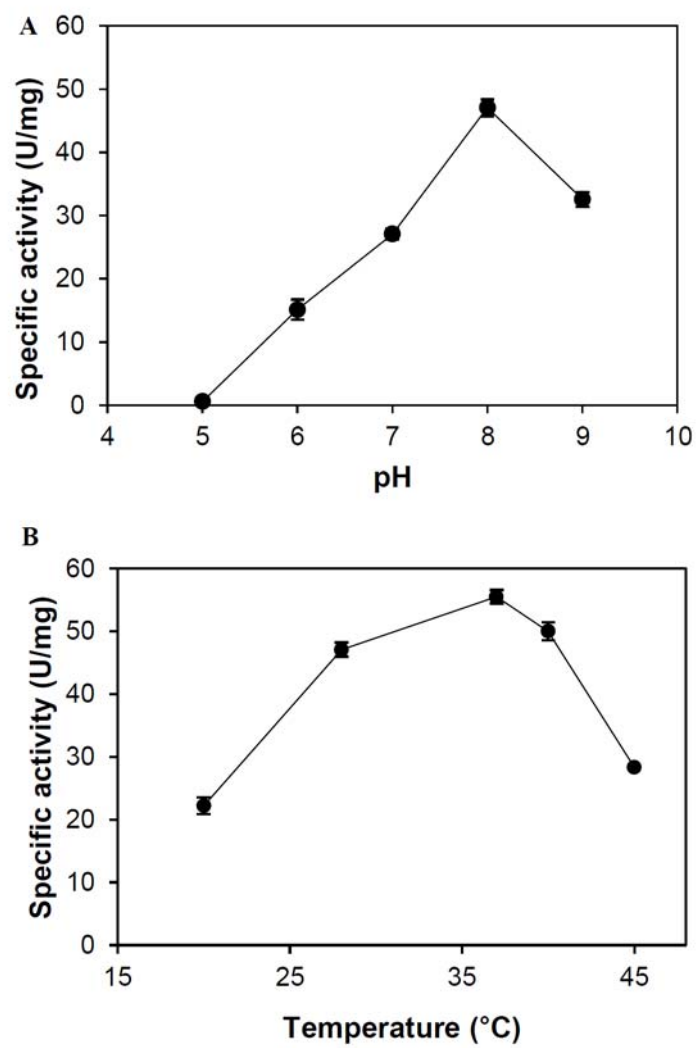


Figure 2

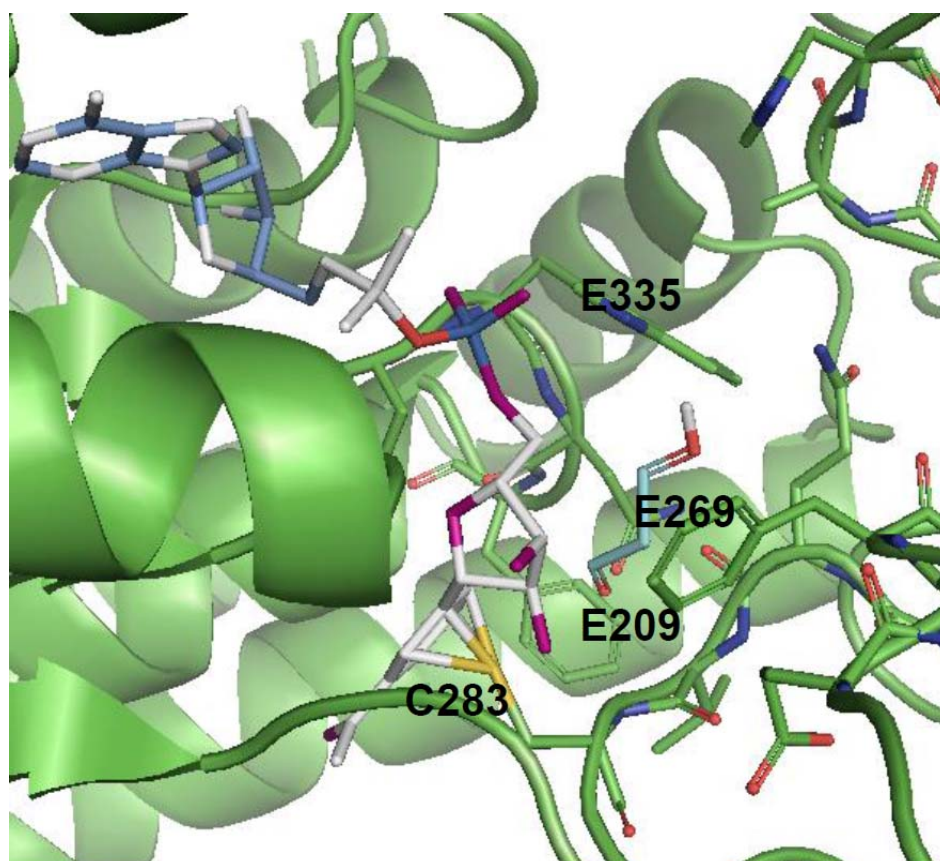


Figure 3

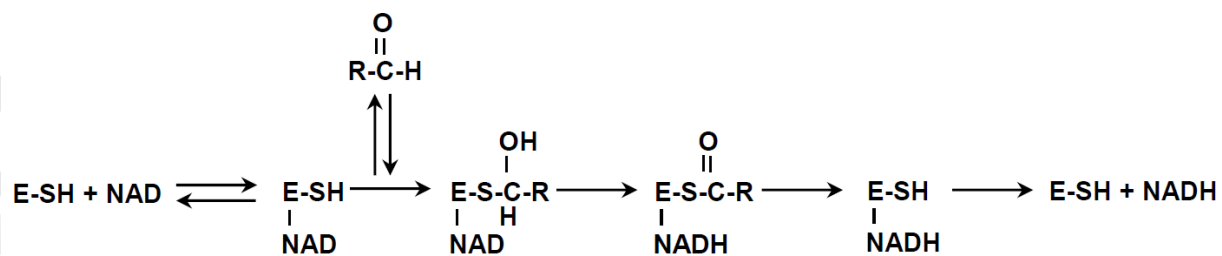


Figure 4

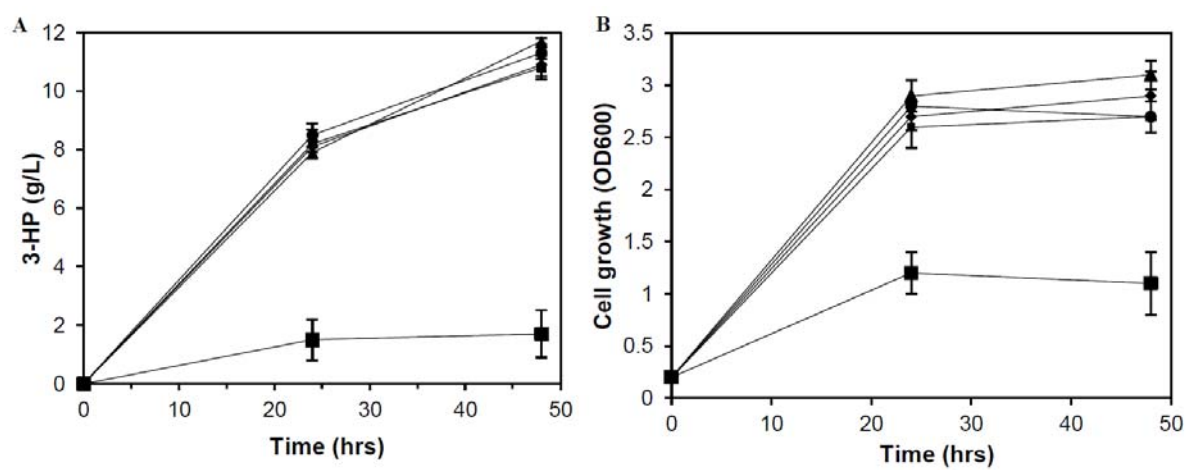


Figure 5

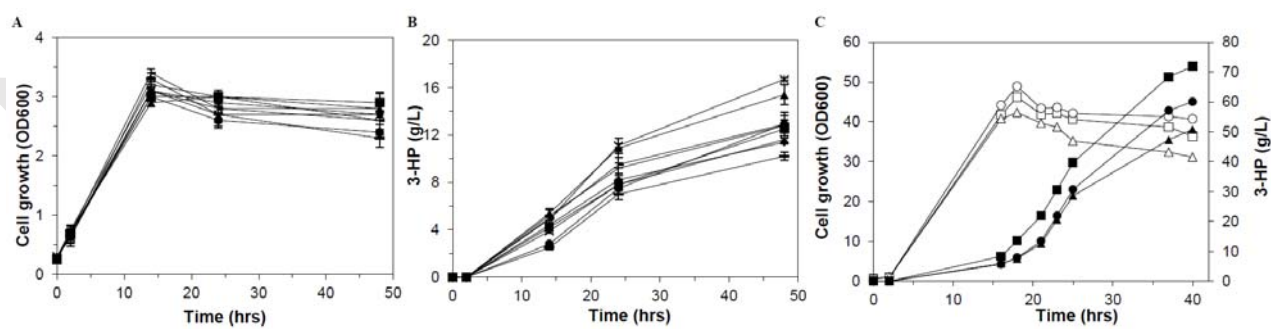


Figure 6