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Bio-transformation of Glycerol to 3-Hydroxypropionic Acid Using Resting Cells of *Lactobacillus reuteri*

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Abstract *Lactobacillus reuteri* grown in MRS broth containing 20 mM glycerol exhibits 3.7-fold up-regulation of 3-hydroxypropionic acid (3-HP) pathway genes during the stationary phase. Concomitantly, the resting cells prepared from stationary phase show enhancement in bio-conversion of glycerol, and the maximum specific productivity (q_p) is found to be 0.17 g 3-HP per g CDW per hour. The regulatory elements such as catabolite repression site in the up-stream of 3-HP pathway genes are presumed for the augmentation of glycerol bio-conversion selectively in stationary phase. However, in the repression mutant, the maximum q_p of 3-HP persisted in the stationary phase-derived resting cells indicating the role of further regulatory features. In the production stage, the external 3-HP concentration of 35 mM inhibits 3-HP synthesis. In addition, it has also moderated 1,3-propanediol formation, as it is a redox bio-catalysis involving NAD^+/NADH ratio of 6.5. Repeated batch bio-transformation has been used to overcome product inhibition, and the total yield (Ypx) of 3-HP from the stationary phase-derived biomass is 3.3 times higher than that from the non-repeated mode. With the use of appropriate gene expression condition and repeated transfer of biomass, 3-HP produced in this study can be used for low-volume, high-value applications.

Introduction

Whole-cell bio-catalysis, which uses non-growing resting cells for the production of industrial chemicals, has recently gained much interest. The desired enzymatic reaction can be controlled independently from the limitations imposed during growth. Minimal side reactions and the consequent fewer by-products are additional advantages. Furthermore, co-factor economy and their availability for the desired reaction can be manipulated easily [1]. Resting cells can be immobilized like enzymes and be used for several rounds of conversion unlike the growing cells. In this background, whole-cell bio-catalysis is applied for the production of different kinds of products [2–5].

The 3-hydroxypropionic acid (3-HP), a structural isomer of lactic acid, is one of the biomass-derivable platform chemicals. Both fermentative (e.g., Fed-batch cultivation) and whole-cell bio-catalysis (e.g., resting cells) have been employed to produce 3-HP from sustainable carbon sources such as corn sugar and biodiesel-derived surplus glycerol [6–10]. The resting cells of lactic acid bacteria (LAB), *Rhodococcus erythropolis,* recombinant *Escherichia coli*, and *Klebsiella pneumoniae* have also been studied for 3-HP production. In addition to its use in the production of bio-acrylic acid, 3-HP is known for its low-volume, high-value applications in food preservation, drug delivery, medical sutures, etc. [11, 12]. Recently, full-fledged GRAS compliance is expected by regulatory bodies for infant and food applications of biomass-derived chemicals (http://goo.gl/WcixgI). The latest advances in metabolic engineering of LAB are one such endeavor. The resting cells of *Lactobacillus reuteri* (*L. reuteri*) have been explored for the synthesis of food industry products, such as conjugated

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linoleic acid, reuterin, etc., because of its established probiotic safety [13, 14].

In *L. reuteri*, 3-HP is produced by the oxidation of glycerol and the simultaneous reduction of glycerol to 1,3-propanediol (1,3-PD) is resourceful for co-factor regeneration (Fig. 1a). The drain of carbon from glycerol to by-products such as lactic acid, acetic acid, and succinic acid is negligible in comparison with other natural and recombinant producers of 3-HP. The absence of conversion of glycerol to dihydroxyacetone (DHA) phosphate provides this sort of advantage [15]. Energetic weak acid export out of the cell is facilitated by the formation of ATP during 3-HP synthesis, and it is an essential attribute in pathway thermodynamics of 3-HP formation [16]. The genes responsible for 3-HP synthesis are located in propanediol utilization (pdu) operon (Fig. 1b) and are subjected to catabolite repression [17]. Over-expression studies of its transcriptional regulator known as *pocR* does not report improvement in 3-HP production [18]. Rather, recent reports have shown that the de-regulation of catabolite repression enhances 3-HP productivity [19]. Other than the gene expression-centric approaches, the prevention of substrate inhibition is found to be useful for 3-HP production [20]. In *L. reuteri*-based whole-cell bio-catalysis, the foremost optimization criterion is to increase the specific productivity and yield of 3-HP from biomass because of the weaker process economics involved in producing higher quantities of biomass from the expensive De Man, Rogosa, and Sharpe (MRS) broth. In this study, 3-HP pathway gene(s) expression, catabolite repression, and product inhibition are addressed to enhance the yield of 3-HP from biomass.

Materials and Methods

Strains and Cultivation Conditions

Lactobacillus reuteri strains used in this study and their phenotypes are listed in the supplementary Table 1. The MRS broth containing 20 g glucose/l was used for anaerobic propagation of cultures at 37 °C. The absorbance at 600 nm in UV–Vis spectrophotometer was used to measure the optical density (OD), and cell dry weight was estimated using the previously established co-correlation, 1 $OD_{600\ nm} = 0.33$ g CDW/L [21].

Preparation of Resting Cells

Lactobacillus reuteri was cultivated in a 1.4-L working volume fermenter using 5 % inoculum. In order to facilitate the expression of glycerol bio-conversion genes, 20 mM glycerol was used as an inducer. Nitrogen was flushed into the reactor to maintain the anaerobic environment. Biomass was cultivated at 37 °C and 100 rpm. The pH was maintained at 5.5 using 1 M NAOH or 1.5 M H_3PO_4. Periodically, samples were withdrawn for measuring the growth. During mid-log and ($t = 4$ h) and stationary ($t = 12$ h) phases of growth, fermenter was drained aseptically for the preparation of resting cells. Briefly, the culture was centrifuged at 6000 g and 20 °C and the cell pellet was washed twice using 100 mM sodium phosphate buffer (pH 7.4). In a 100-ml rubber stopper vial, the washed cell pellet was re-suspended to a final absorbance ($A_{600\ nm}$) of 20 in 50 ml phosphate buffer (pH 7.4) containing 150 mM glycerol. Nitrogen was flushed inside the vial using sterile air filter (0.2 µm) and kept statically at

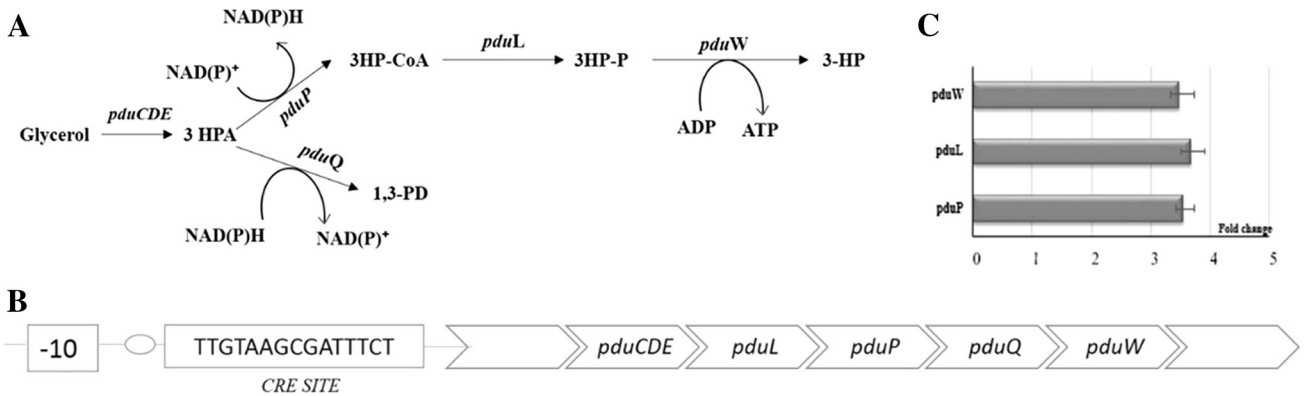

Fig. 1 **a** Propanediol utilization (*pdu*) operon of *Lactobacillus reuteri*: Catabolite repression element (CRE) is represented as CRE site following the −10 region. In the CRE mutant, by means of recombineering approach, the repression site is modified as T**GAATTC**CGATTTCT [41]. **b** Bio-conversion pathway of glycerol to 3-HP: *pduCDE* (vitamin B12-dependent glycerol dehydratase), *pduP* (Co-A and $NAD(P)^+$-dependent propionaldehyde dehydrogenase), *pduL* (phosphotransacetylase), *pduW* (propionate kinase), *pduQ* (NAD(P)H-dependent 1,3 propanediol dehydrogenase). **c** Up-regulation of 3-HP pathway genes in stationary phase

37 °C. At regular intervals, 2 ml of samples were withdrawn for supernatant analysis in HPLC.

HPLC Analysis of Glycerol Bio-Conversion

The concentration of glycerol and end products of glycerol metabolism was analyzed using an analytical HPLC-RID (Shimadzu) equipped with an organic acid analysis column, Aminex HPX 87H (300 × 78 mm, Bio-Rad USA). The mobile phase of 35 % acetonitrile in 5 mM sulfuric acid at 0.4 ml/min and 37 °C was used for isocratic elution. Refractive index was measured at 50 °C. Samples from batch cultures and resting cell experiments were centrifuged, and the supernatant was filtered using 0.22-µm polyether sulfone filter membranes prior to HPLC analysis. The standard solutions of glycerol and 1,3-propanediol were procured from Sigma Aldrich, and 3-HP was obtained from TCI chemicals. Since 3-HPA was not available commercially, it was produced by previously used method [21].

Quantitative PCR of 3-HP Pathway Genes

Expression of *pduP, pduW*, and *pduL* was studied using the primers listed in supplementary Table 2 synthesized by IDT. Total cellular RNA was isolated using Qiagen kit from the cell pellets stored using RNA protect® reagent for bacteria. Cell lysis was supplemented using 50 units of mutanolysin (MP Biomedicals) and 20 mg/ml of lysozyme and was carried out at 37 °C for 30 min. Synthesis of cDNA was carried out using M-MuL reverse transcriptase (NEB), and real-time PCR analysis was performed with SYBR® green master mix (Life Technologies) using our previously explained method [22]. In order to normalize the gene expression data, 16S rRNA of *L. reuteri* was used as an internal reference gene [23]. ΔΔCT was calculated based on difference between the ΔCT of a gene transcript in the mid-log phase ($t = 4$ h) and stationary phase (t = 12 h). The relative change in expression was compared using the equation, fold change $= 2^{-\Delta\Delta CT}$.

NAD^+/NADH Ratio Estimation

Pyridine nucleotides were extracted from 2 ml of resting cells at regular time intervals during the course of bio-transformation. Briefly, acidic condition (0.2 M HCL) was used to extract oxidized co-factors and basic condition (0.2 M NAOH) was used for obtaining reduced co-factors [24]. The extracts were neutralized, and the supernatant was assayed using enzymatic cycling assay. The reaction was carried out using a scale-down procedure [25] in kinetic mode using a plate reader by recording absorbance at 570 nm for 10 min. Alcohol dehydrogenase (EC 1.1.1.1; 500 U/mL), thiazolyl blue (MTT, 4.2 mmol/L), and phenazinium methyl sulfate (PES, 16.6 mmol/L) required for the cycling assay were obtained from MP Biomedicals, USA. Following the kinetic read, the change in absorbance per unit time ($\Delta A/t$) for each standard nucleotide concentration was obtained. A linear fit equation for the estimation of unknown concentration in extracted samples was further obtained by plotting the calculated $\Delta A/t$ versus respective standard nucleotide concentration.

Effect of 3-HP Addition, Repeated Batch Bio-transformation, and pduP Assay

In the product inhibition experiment, 3-HP was added from 10 to 50 mM in the resting cell medium at 0 and 1.5 h of bio-transformation. Effect of such exogenous addition was studied using HPLC analysis of supernatants at regular intervals. Repeated batch bio-transformation was carried out in a 250-ml static flask flushed with sterile nitrogen and kept at 37 °C. In order to recycle the cells and remove supernatant intermittently, the static flask was connected to a micro-filtration module having 0.22 µm pore size and an operating pressure of 1.2 bar. Culture broth from 12 h (stationary phase) was passed into the micro-filtration unit, and the cells were washed twice using phosphate buffer (pH 7.4) to obtain the active biomass. Finally, the biomass was re-suspended to a cell density of 20 ($A_{600\ nm}$) in 100 ml of phosphate buffer (pH 7.4) having 100 mM glycerol. For every 2 h, the supernatant was removed by passing the bio-transformation media into the micro-filtration unit, and fresh phosphate buffer having 100 mM glycerol was added for the subsequent cycles. For each cycle (2 h) of bio-transformation, the dead time spent in micro-filtration was approximately 30 min. At regular time points, the supernatant analysis was carried out in HPLC. The assay of *pduP* was carried out using propionaldehyde as a substrate using previously established protocol [20].

Results

The whole-cell bio-catalysis using *L. reuteri* comprises two phases namely the production of active biomass and the subsequent bio-conversion of glycerol. Resting cells' bio-transformation profiles for the cells harvested from mid-log and stationary phases of growth are compared in Fig. 2a. The maximum specific productivity (q_p) of 3-HP is 2.54-fold higher in stationary phase-derived cells, and the corresponding increase in maximum glycerol uptake rate is also observed. The increase in the specific productivity of 3-HP can be due to the increase in enzyme activities of 3-HP pathway during stationary phase. Expression of *pduP*, *pduW*, and *pduL* has shown a 3.7-fold up-regulation

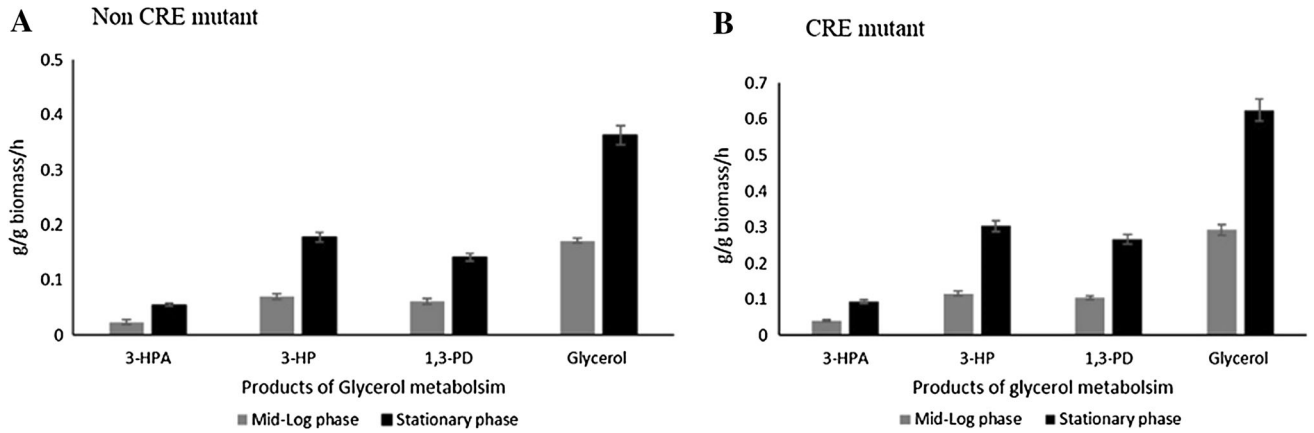

Fig. 2 **a** Maximum specific formation and consumption rates of products, namely 3-hydroxypropionaldehyde (3-HPA), 3-hydroxypropionic acid (3-HP), 1,3-propanediol (1,3-PD), and substrate (glycerol), respectively, in mid-log and stationary phase-derived resting cells of *L. reuteri* ATCC 55730, non-CRE (catabolite repression element) mutant. **b** Similar profile in the CRE mutant, *L. reuteri* RPRB 3007

in the stationary phase cells (Fig. 1c). The simultaneous increase in 1,3-PD and 3-HPA concentration is because of the fact that the *pduQ* and *pduCDE* along with *pduP, pduL*, and *pduW* are arranged in a single transcription unit encoded by a polycistronic mRNA.

In the whole-cell bio-catalysis, inhibition by substrates and products is a regulatory process associated during bio-conversion step. Glycerol to 3-HP formation encompasses three metabolic steps namely glycerol to 3-HPA, 3-HPA to 3-HP, and regeneration of redox equivalents through 1,3-PD formation. The end product(s)-related inhibition may target any of these three steps. When 3-HP is added externally at 0 h and also in the middle of the production (1.5 h), a profound metabolic change is observed. In samples containing more than 35 mM externally added 3-HP, the absence of synthesis of 3-HP, diminished 1,3-PD, and subtle change in 3-HPA concentration are observed at 3 h. The depletion of 1,3-PD up to 73 % lesser than the control suggests that the external addition of 3-HP not only influences the 3-HPA to 3-HP conversion but also regulates the 3-HPA to 1,3-PD conversion. The absence of 3-HP formation may essentially disrupt the NAD^+/NADH ratio of 6.5 ± 0.2, maintained during bio-conversion (Fig. 3), and therefore 1,3-PD synthesis is moderated. Since the formation of reuterin is not a redox bio-catalysis, its synthesis is not influenced during external addition of 3-HP.

3-HP is further produced using repeated batch bio-transformation to overcome the product inhibition. In Fig. 4a, the concentration profiles of 3-HP and residual glycerol are shown for the first four cycles. After two cycles, the volumetric consumption rate of glycerol tends to decline (Fig. 4b). It can be due to the lack of vitamin B12 *de novo* synthesis required for reuterin formation and

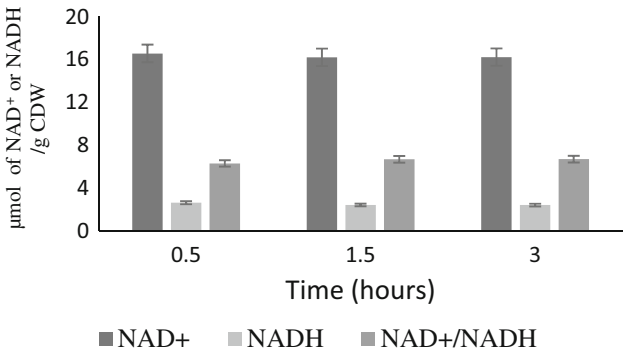

Fig. 3 NAD^+/NADH ratio during bio-transformation of glycerol

loss in cellular viability. The total yield (*Y*px) from the biomass after reuse of cells is 3.3 times more than the single batch of bio-conversion. The total yield based on the substrate is 0.275 g 3-HP/g glycerol, and it is certainly lesser because of the inevitable co-product 1,3-PD and unconverted precursor reuterin. While scaling up this process, the tradeoff between surplus glycerol and production costs can be a critical factor.

Discussion

Downtime in the current micro-filtration process can be reduced through alternates like magnetic separation technology [26] and cellular immobilization strategies [27]. Since the reuterin is also known to inhibit 3-HP formation, periodical removal of the bio-transformation media containing products is quite a useful strategy to enhance the conversion of glycerol. Aerobic regeneration of NAD^+ can be an alternate to increase the yield, but the diminished

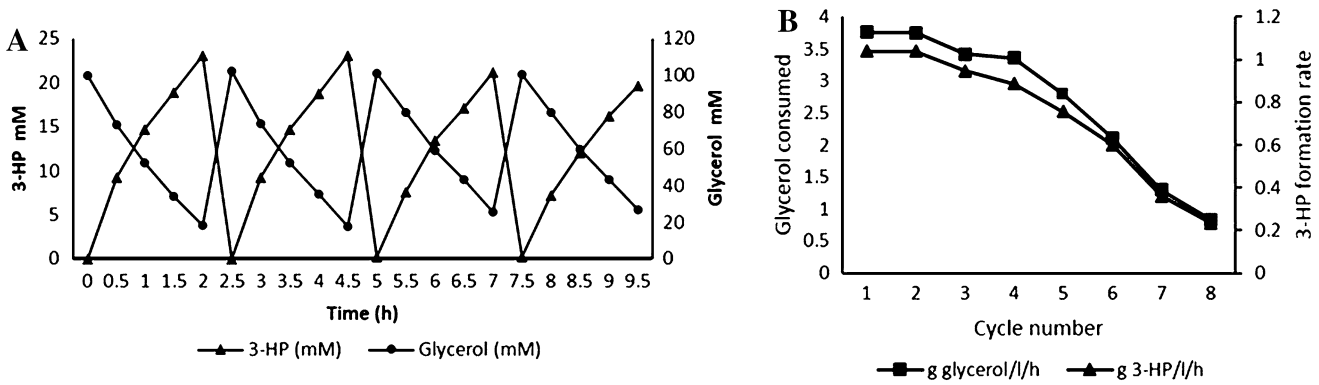

Fig. 4 **a** Concentration profile of glycerol and 3-HP for first four cycles of bio-transformation. Each cycle of bio-transformation is carried out for 2 h. **b** Volumetric consumption rate of glycerol and volumetric formation rate of 3-HP in each cycle of bio-transformation

precursor (3-HPA) production and glycerol consumption [28] under aerated conditions may be a disadvantage. Anaerobically, nitrate is used as an alternate electron acceptor in 1,3-PD-deficient strain of *Klebsiella* species [29], and the same may be used in *L. reuteri* to increase the yield of 3-HP.

In similarity to the growth phase-dependent up-regulation in *L. reuteri*, stationary phase of *Clostridium phytofermentans* and *Planctomycete* shows enhanced propionic acid and saccharide metabolism in their respective micro-compartments [30, 31]. Regulatory factors such as catabolite-responsive element (CRE) in the *pdu* operon may be limiting the 3-HP formation in logarithmic phase-derived resting cells. In order to test this hypothesis, 3-HP formation in CRE mutant is analyzed in different growth phases. Alike its wild-type strain (ATCC PTA 6475), the stationary phase-derived cells remain as the highest 3-HP producer (Fig. 2b). In carboxysomes' biogenesis, multiple levels of regulatory events are responsible for the concerted expression of its enzymes and not only limited to carbon catabolite repression. The up-regulation of micro-compartmental genes in the stationary phase of *L. reuteri* may also have a complex regulation involving several factors such as depletion/absence of certain types of sugar [32], change in the environmental pH [33], and the biological necessity to retain certain molecules within the cell for further processing [17].

In the course of bio-transformation, it is observed that, after 6 h, the enzyme activity of *pduP* decreases from the initial 0.57 protein to 0.23 U/mg protein. Similarly, the absolute concentration of pyridine nucleotides is also in the downtrend during the course of bio-conversion. Redox biocatalysis not only demands continuous exchange of co-factors but also necessitates concerted synthesis of both co-enzymes and co-factors. Implying the same, the external supply of nicotinic acid is sought for dihydroxyacetone in resting cells of *E. coli* [1]. Depletion of amino acid pool size and the absence of enzyme synthesis are a kinetic challenge for the metabolic pathways in resting cells to endure the reaction(s) in maximum velocity. Alternatively, bio-transformation using growing cells can be used. *L. reuteri* is not capable of growing in glycerol as a sole carbon and energy source, even in the presence of complex ingredients of MRS broth. Repression is suggested in earlier studies for the absence of 3-HP formation in batch and carbon-limited chemostat cultures [34]. The removal of catabolite repression is previously shown to have altered the fluxes across glycerol metabolic pathway [19]. However, the absence of 3-HP formation in batch cultures of CRE mutant [35] suggest detailed thermodynamic feasibility analysis. If glucose and glycerol are to be co-oxidized biologically, the competition toward the co-factor $NAD(P)^+$ is obviously evident. In order to circumvent this, *L. reuteri* prefers glycerol as an electron acceptor [36] and reduces it to 1,3-PD. The absence of dihydroxyacetone-mediated oxidative route [15] and the presence of more than one 1,3-PD oxidoreductases in the genome of *L. reuteri* [37] facilitate the reduction to 1,3-PD. Rather, the oxidative 3-HP formation is thermodynamically confronted. eQuilibrator, a biochemical thermodynamics calculator, was used to estimate the equilibrium constant (K'_{eq}) of the reactions in which NADH can be regenerated during the fermentation of glucose and glycerol [38].

$$\text{Glucose} + 2\text{NAD}^+ + 2\text{ADP} + 2\text{Phosphate} \rightleftharpoons 2\text{Pyruvate} + 2\text{NADH} + 2\text{ATP} + 2\text{H}_2\text{O} \quad (1)$$

For the above oxidation reaction at pH 7 and an ionic strength of 100 mM,

$$K'_{eq} = 1.5 \times 10^{14} \quad (2)$$

$$\text{Glycerol} + \text{NAD}^+ + \text{ADP} + \text{Phosphate} \rightleftharpoons 3 - \text{hydroxypropionic acid} + \text{NADH} + \text{ATP} + \text{H}_2\text{O}. \quad (3)$$

For the above oxidation reaction at pH = 7 and an ionic strength of 100 mM,

$$K'_{eq} = 7.9 \times 10^{9} \quad (4)$$

$$K'_{eq} = e^{-\Delta G^{\circ}/RT} \quad (5)$$

$$\Delta G^{\circ}_{eqn1} \ll 0. \quad (6)$$

Thermodynamic feasibility encompassing cellular physiology and co-occurrent cellular processes is more realistic than the standalone reaction feasibility [39]. Highly feasible pathways are bound to have $\Delta G^{\circ} \ll 0$ [40], and therefore, to increase the kinetic feasibility of regenerating NAD(P)H through 3-HP formation, it is essential to increase the amount of enzymes involved in the conversion. The mutation in CRE may not be sufficient enough to provide the large quantity of enzymes, and hence paralogous or heterologous over-expression of the pathway enzymes may be required to obtain 3-HP in batch cultures itself. The over-expression of heterologous aldehyde dehydrogenases such as *puuC* from *E. coli* can be used because of its low k_m for 3-HPA, a kinetic wizard for overcoming the thermodynamic obstacle. In addition, *puuC* has not been reported for neither substrate nor product inhibition.

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