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An experimental modeling of trinomial bioengineering- *crp*, rDNA, and transporter engineering within single cell factory for maximizing two-phase bioreduction

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ABSTRACT

A carbonyl reductase (*cr*) gene from *Candida glabrata* CBS138 has been heterologously expressed in cofactor regenerating *E. coli* host to convert Ethyl-4-chloro-3-oxobutanoate (COBE) into Ethyl-4-chloro-3-hydroxybutanoate (CHBE). The CR enzyme exhibited marked velocity at substrate concentration as high as 363 mM with highest turnover number ($112.77 \pm 3.95\ s^{-1}$). Solitary recombineering of such catalytic cell reproduced CHBE 161.04 g/L per g of dry cell weight (DCW). Introduction of combinatorially engineered *crp* (*crp**, F136I) into this heterologous *E. coli* host yielded CHBE 477.54 g/L/gDCW. Furthermore, using nerolidol as exogenous cell transporter, the CHBE productivity has been towered to 710.88 g/L/gDCW. The CHBE production has thus been upscaled to 8–12 times than those reported so far. qRT-PCR studies revealed that both membrane efflux channels such as *acrAB* as well as ROS scavenger genes such as *ahpCF* have been activated by engineering *crp*. Moreover, membrane protecting genes such as *manXYZ* together with solvent extrusion associated genes such as *glpC* have been upregulated inside mutant host. Although numerous proteins have been investigated to convert COBE to CHBE; this is the first approach to use engineering triad involving *crp* engineering, recombinant DNA engineering and transporter engineering together for improving cell performance during two-phase biocatalysis.

1. Introduction

Organic solvent mediated or biphasic biocatalysis has been an emerging area for bioproduction of potentially important compounds since most of the organic reactions are water limiting and need apolar solvents as carrier for both substrate and products [1–3]. However, solvent mediated biocatalysis has often found limitations since organic solvents are toxic to both enzyme and the host microbe especially *E. coli* [4–7]. For improving enzyme performance, several measures have been taken such as protein engineering, surface modification of the enzyme, adding crown ether or β-cyclodextrin as fold protectant, co-lyophilizing enzyme with excipients, enzyme micellization, enzyme immobilization or introduction of ionic liquids [1,3]. Nevertheless, this method encompasses several limitations such as it requires isolated enzyme which has limited self life outside cell. In addition, protein and surface engineering are hugely cost and resource intensive which may take several months to years to optimize even one enzyme's performance. As an alternative way, strain engineering has emerged as a reliable technique to improve host phenotype or productivity in organic solvent mediated biocatalysis [8–10]. In one strategic approach, it involves methodologies such as transposon mutagenesis or inserting solvent tolerant gene cassettes from extremophiles to host cell [8]. In other approach, metabolic engineering has been undertaken to improve cell productivity by changing its metabolic pathway [9,10]. However, these methods need prior knowledge about the genetic orchestration within both host and donor cell. Moreover, pivotizing cell metabolic pathway is a riskful, tedious and troublesome job. Thus evolution of optimum activity, selectivity and stability of target enzyme or its microbial host has always been a key search in biphasic biotransformation process [2].

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Global transcription machinery engineering has been introduced in last few years to improve cell tolerance against multiple stresses [11–15]. Seven global transcription factors are reported to be present inside *E. coli* which can regulate 1278 genes in the cell genome. Among these, cyclic AMP receptor protein (CRP) has been reported to regulate transcription of 444 genes [16,17]. So CRP could regulate genes more than any other global transcription regulator inside *E. coli* cell. Hence combinatorial engineering of CRP and subsequent enrichment selection through slow increment of stress [11,12] might converge into an optimum phenotype revealing best survival against organic solvent. As an example, Alper et al., 2006 improved ethanol tolerance in yeast by mutating one of its TATA binding protein gene *SPT15* which virtually engineered its global transcriptomic pathway [13]. Pursuing this concept, in our previous work, we mutated *crp* (*crp**) of *E. coli* genome and introduced *crp** encrypted plasmid inside *E. coli* cell to rewire entire transcriptome for enhancement of organic solvent tolerance under stress [4]. Thus, instead of protein engineering, strain engineering through gTME might be the superior answer to the stability and survival question in organic solvent mediated biocatalysis.

Although two-phase bioconversion has been practiced substantially in last few decades [18–23], optimization and potentiation of biocatalytic efficiency through strain or protein engineering is still a vacant area in this field. As some discrete attempts, rewiring metabolic pathway by transcription engineering either resulted in enhanced output for bioproduction [14,15] or improved cell phenotype against superincumbent stress which indirectly increased the titer value for bioproduction [13]. However, these cell engineering strategies for improved bioproduction has been tried with native cell pathway only. The strain engineering guided bioconversion involving heterologous pathways is still a virgin field to explore.

With a view to this, in this study, we have undertaken conversion of COBE ester to CHBE alcohol as a model to demonstrate the applicability of global transcription machinery engineering (gTME) for scaling up bioreduction with a highly efficient carbonyl reductase from *Candida glabrata* CBS138 [24]. Attempts have been made in this study to reconstruct our previously mutated *crp** [4] in low copy expression host (pACYC Duet-I) and reintroduce inside *E. coli* BL 21(DE3) for improving organic solvent tolerance during biphasic biocatalysis. Percent survival assay has been performed to reconfirm the cell survival under stress. Also, native genomic *crp* has been kept intact inside the genome in order to maintain the RNA polymerase bias for binding to the promoter and thereby initiating the desired protein expressions. In addition, we have incorporated nerolidol [25], a sesquiterpene extracellular transporter to improve substrate uptake inside the host cell. Biphasic biocatalysis in its optimized volumetric ratio for two solvents [14–19] has been used in this synthesis. Cofactor regeneration is used *via* NADPH/$NADP^+$/NADPH intracellular loop with glucose dehydrogenase from *Bascillus subtilis* as regenerating enzyme. To our knowledge, this is the first attempt to use trinomial engineering involving *crp* engineering, recombinant DNA engineering and transporter engineering together for maximizing output in biotransformation. Although this strategy is applied for COBE to CHBE bioproduction in this study, this is basically a *proof of the concept* approach which can be applied for other bioproductions as well.

2. Materials and methods

2.1. Reagents and chemicals

COBE and CHBE were procured from Sigma Aldrich (Aldrich, USA). Gel extraction kit and plasmid isolation kits were purchased from Qiagen (Qiagen, USA). All the restriction enzymes, digestion buffers and protein markers were from New England Biolabs (Ipswich, USA). The SDS-PAGE and DNA gel running assemblies were from Biorad (USA). pETDuet-I plasmid for enzyme over-expression and biotransformation was from Novagen (EMD Millipore, USA). Nerolidol (pubchem ID: 5284507) was procured from Sigma Aldrich (Aldrich, USA).

2.2. Experimental methods

2.2.1. PCR and construction of GDHCRpET Duet-I

The amplification of the *Candida glabrata* CBS138 carbonyl reductase (*cr*) gene (NC_006028.2, Gene id: CAGL0E05170 g) and glucose dehydrogenase (*gdh)* from *Bacillus subtilis subsp. subtilis str. 168* (NC_000964.3) were performed with suitable primers and subsequently cloned into pET Duet-I to construct GDHCRpET Duet-I. The detailed procedure of cloning has been provided in supplementary information.

2.2.2. CR enzyme over-expression and purification

The CR and GDH enzymes were over-expressed with 0.3 mM IPTG after growing the selected clones upto mid-exponential phase (OD_{600} = 0.6–0.8) at 37 °C, 200 rpm. The CR enzyme was purified from aqueous fraction of cell lysate using a Ni-His binding column (His-Gravitrap Column; GE Healthcare, USA) equilibrated with binding buffer. The binding buffer contained 10 mM PPB, 20 mM imidazole, 500 mM NaCl (pH 7.2) while the protein was eluted with 10 mM PPB, 200 mM Imidazole, 500 mM NaCl (pH 7.2). After desalting through PD-10 column (GE Healthcare, USA), the protein was finally purified and stored in a buffer containing 100 mM PPB, pH 7.5 at 4° C. The purity of the enzyme was finally checked by SDS PAGE (Supplementary Fig. S1)

2.2.3. Enzyme kinetics and its calibration with temperature and pH

A reaction system (1 ml) comprising the substrate COBE (0–4 mM); cofactor NADPH (200 μM) and enzyme CR (100–113 ng) within appropriate buffer was constructed to evaluate enzyme characteristics. The enzyme was calibrated against a pH range of 6.0–9.5 where pH 6.0-8.0 was maintained by 0.1 M potassium phosphate buffer, the higher range (pH 8.0-9.5) being maintained with Tris-HCl buffer. Variation of enzyme activity with temperature was also investigated through a bandwidth of 20 °C to 90 °C; where temperature attainment of the bioreactor system was ensured by equilibrating the reaction system with the corresponding temperature for 3 min. After suitable optimization, the final enzyme kinetics was performed spectrophotometrically at 340 nm at pH 7.5 and 30 °C, monitoring the reaction for 120s.

2.2.4. Construction of the *crp** strain of E. coli *BL21 (DE3)*

2.2.4.1. Cloning of genomic crp template from E. coli *DH5α.* The genomic *crp* template was chosen from *E. coli* DH5α and amplified *via* colony PCR. Briefly, the overnight grown cells were used as template for PCR and the genomic *crp* was amplified with following set of primers: *crp_sense* (5′-GAGAGGATCCATAACAGAGGATAACCGCGCATG-3′) and *crp_anti* (5′-AGATGGTACCAAACAAAATGGCGCGCTACCAGGTAACGCGCCA-3′). The amplified DNA was recycled from 1% agrose gel with gel extraction kit (Qiagen, Germany), and was verified by sequencing. The verified *crp* was digested by *Kpn*I and *Bam*HI and cloned into pACYC Duet-I for subsequent studies. The very low copy pACYC Duet-I has been chosen as *crp* carrier in order to mimic cell's genomic replication as well as plasmid's contribution as second compatible expression host with GDHCRpET Duet-I.

2.2.4.2. Random mutagenesis of crp. The random mutagenesis of *crp* was performed by error prone PCR (random mutagenesis kit,

Stratagene, USA) perceiving genomic *crp* as template. The method has been followed as described in our previous study [4]. Briefly, the manufacturer's protocol was pursued for mutagenesis through 30 cycles of PCR taking 20 ng genomic *crp* as template. The mutated *crp* was checked for correct length, thereafter purified from gel and subsequently cloned into pACYC Duet-I using *Kpn*I and *Bam*HI. Mutant library was created after transforming the cloned plasmids into *E. coli* DH5α by electroporation. Improved phenotypes were afterwards selected in *E. coli* DH5α background by enrichment selection as described earlier [4]. 0.2–0.25% (v/v) toluene was chosen as selection pressure in air-locked culture flaks while individual survived colonies were picked up after third round of selection. After extracting plasmids from the selected clones *via* minipreparation, the point/s of *crp* mutation were finally verified by DNA sequencing. Based on some initial growth analysis against toluene and cyclohexane together with our previous study results [4], the F136I *crp* mutant (*crp**) was chosen as desired phenotype for this study. Fresh *E. coli* BL21 (DE3) cells were co-transformed with *crp**pACYC and GDHCR pET Duet-I and allowed to grow at 37 °C, 200 rpm. The host genomic CRP was left undisturbed to induce normal protein expressions inside the cell. For routine analysis, genomic *crp* (*crp*WT) was also cloned into pACYC Duet-I and transformed into another fresh set of *E. coli* BL21 (DE3) cells harbouring GDHCR pET Duet-I. For all the subsequent studies (*crp** pACYC −GDHCR pET Duet-I) would be treated as mutant or engineered strain while (*crp*WT pACYC −GDHCR pET Duet-I) would be treated as wild type or unengineered strain.

2.2.5. Subjection of engineered strain in biphasic media, tolerance analysis

The tolerance of the engineered strain against butyl acetate media was evaluated by subjecting the strain (*crp** pACYC −GDHCR pET Duet-I) grown upto mid exponential phase (OD_{600} 0.6–0.8), into 50% butyl acetate (Butyl acetate: 100 mM PPB, 1:1 v/v) with respect to control (*crp*WT pACYC −GDHCR pET Duet-I) grown upto the same phase. The temperature and buffer pH were kept at optimum temperature and pH of CR enzyme in order to mimic the actual reaction conditions. The cells were allowed to grow at 37 °C, 200 rpm. Aliquots were drawn at specific time intervals from the culture and plated on to LB-agar plates after suitable dilution. Colonies were counted after 24 h post incubation at 37 °C. Tolerance is reported as percent survival of colonies with respect to time.

2.2.6. Quantitative real-time reverse transcription PCR (qRT-PCR)

The qRT-PCR of selected genes were performed after growing host *E.coli* cells (both mutant *crp** and WT *crp* encoded) up to OD_{600} 0.6–0.7 in biphasic media (0.1 M PPB, Butyl acetate 50% v/v) at 30 °C, 180 rpm. The RNA isolation from both the mutated and unmutated strains was performed using RNeasy Mini Kit (Qiagen, Germany) according to instructor's protocols. RNase free DNase-I (Qiagen, Germany) was used to eliminate genomic DNA from both the samples. The isolated RNA purity and integrity were confirmed by gel electrophoresis and extracted RNA was quantized by Eppendorf Biophotometer (Eppendorf, Germany).

qRT-PCR was carried out in two step system. Briefly, RNA of $OD_{260/280}$ 1.8–2.0 was used for cDNA synthesis. The cDNA synthesis was carried out with Gene Script cDNA synthesis kit (Gene Script, USA) with 1 μl reverese transcriptase and 500 ng isolated RNA. 2 μl of 1:200 dilution cDNA was used for quantitative analysis of real time DNA amplification with 12.5 μl of iQ-SYBR supermix (Bio Rad, CA, USA) and 1 μM of each primer pair. qRT-PCR was carried out with the following program: 3 min at 95 °C, 40 cycles at 95 °C for 15 s, followed by 60 °C for 30 s in a total reaction volume of 25 μl. The threshold cycle (C_t) values were determined for each gene while calibration of data was performed with Bacterial 16S rRNA gene (*rrsG*) as the housekeeping gene. $2^{-\Delta\Delta Ct}$ method was undertaken to calculate the fold change of mutant gene against WT.

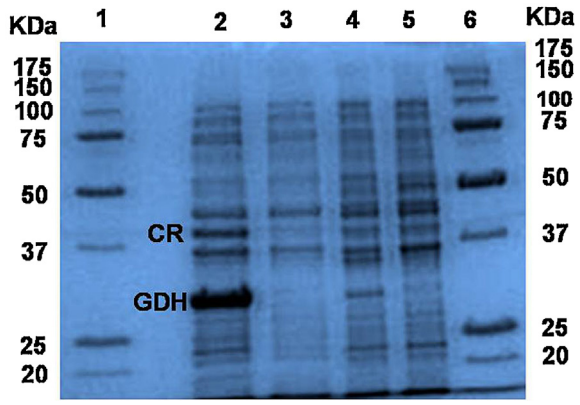

Fig. 1. *Protein expression in crp**pACYC-GDHCRpET Duet-I. Normal CR (39 KDa) and GDH (28 KDa) protein expressions have been occurred in the *crp*-engineered strain. However, intense bands of CR and GDH in *crp*-engineered strain suggests profound expression of the both the proteins in the mutated cell compared to control probably due to synergistic action of both CRP proteins in perturbing RNA polymerase binding with the *lac* promoter on the pET vector. Lane 1 and 6: Protein markers; Lane 3 and 5: Overnight cultures of *crp** and *crp*WT cells respectively harbouring GDHCRpETDuet-I plasmids; Lane 2 and 4: IPTG induced expression of *crp** and *crp*WT cells respectively harbouring GDHCRpETDuet-I plasmids.

2.2.7. Optimization of nerolidol concentration in biocatalytic media

Nerolidol concentration in the biocatalytic media was determined by putting various concentrations (0.5 mM ~ 1.5 mM) of nerolidol inside 1:1 butyl acetate: 0.1 M PPB media together with 0.30 g of native *E. coli* BL21 (DE3) cell pellets and 1 g/L COBE. The nerolidol induced COBE permeability has been evaluated by measuring COBE concentration from time to time in the extracellular media (Supplementary Fig. S2) by GC.

2.2.8. Biotransformation of COBE into CHBE via engineered strain together with nerolidol

The biotransformation reaction was set by adding 6% (60 g/L) COBE in butyl acetate with 0.33 g over-expressed immobilized cells in 1:1 butyl acetate: 100 mM PPB, pH 7.5 media, 18 mg $NADP^+$ and 0.1 g/L glucose. The reaction was carried out at 30 °C, 180 rpm. Biphasic reaction was carried out conceiving its advantage over monophasic reaction as described earlier [18–23]. Three bioreactions were performed involving engineered strain with nerolidol, engineered strain without nerolidol and *crp*WT strain without nerolidol. Fed batch culture was used to utilize strain tolerance in order to maximize bioconversion in a batch bioreactor. Suitable amount of COBE was added in the stirred bioreactor at specific time intervals in order to maintain COBE level at 60 g/L. Bioconversion was continued from 8 to 30 h and CHBE concentration was assessed by time specific sampling of reaction media followed by Gas Chromatography (GC) analysis. The detailed GC procedure has been described in supplementary information.

3. Results

3.1. Protein expression in crp* cell

The protein expression in the *crp*-engineered strain was evaluated on SDS PAGE gel (Fig. 1). From the figure, it can be observed that CR (39 KDa) and GDH (28 KDa) proteins have been expressed successfully in the *crp*-engineered strain. However, CR and GDH bands in *crp** cell lysate appears more intense compare to WT.

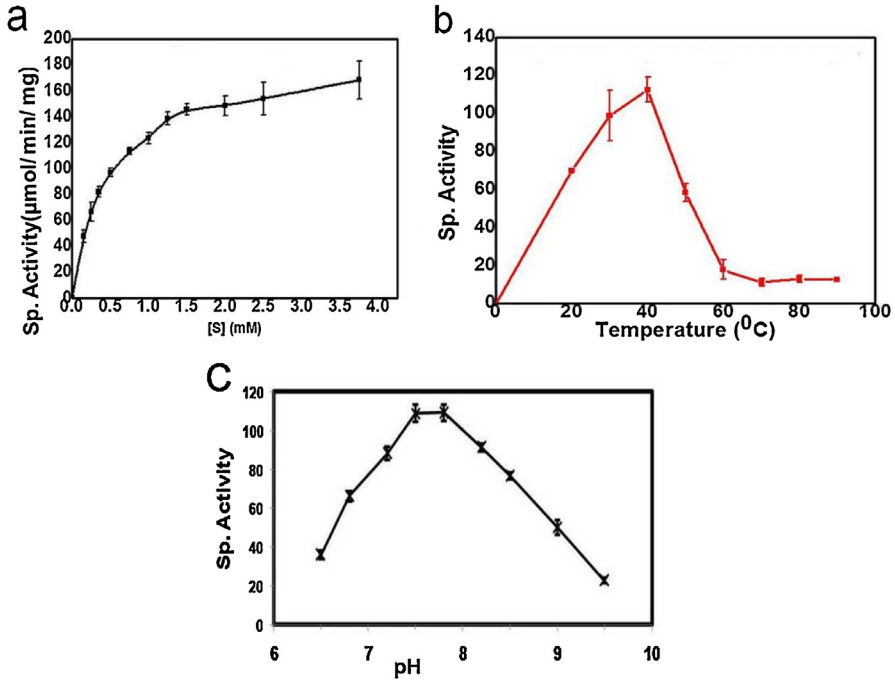

Fig. 2. Evaluation of specific activity, pH and temperature effects of carbonyl reductase. a. Determination of specific activity by Michaelis-Menton curve plot b. Effect of temperature, maximum activity is shown in between 30 °C–40 °C. c. Effect of pH, enzyme revealing optimum activities in between pH 7.5–8.2. The reaction was monitored in an assay mixture comprising 1 mM COBE and 0.2 mM NADPH within PPB buffer, pH 7.5. Each data point is the mean ± standard deviation of three independent observations.

Table 1
Amino acid substitutions in mutants.

Mutants	Amino acid substituions
M1	F136I
M2	T127N
M3	T127N, T208N

3.2. Enzyme purification and characterization

The enzymes were over-expressed by IPTG induction and purified by Ni^{+2} bound His-Gravitrap column (GE Healthcare, USA). The K_m was determined in the range of 0.42–0.46 mM having a mean of 0.45 ± 0.02 mM. In addition the specific Activity was found in the 173.49 ± 6.08 µmol of NADPH converted/min/mg of enzyme (Fig. 2a). The mean K_{cat} was calculated as 112.77 ± 3.95 s^{-1}. The enzyme showed marked activity at 30 °C–40 °C (Fig. 2b) and at pH 7.5 (Fig. 2c).

3.3. Transcription engineering to the cell E. coli BL21 (DE3)

The randomly mutagenized *crp* was introduced into fresh *E. coli* DH5α cells by electroporation to create a variant library of width 10^5–10^6. The variant library was screened in 250 ml cap-locked flasks at 37 °C, 200 rpm under 0.2%–0.25% (v/v) toluene pressure for 4–5rounds. Individual clones were selected from the third round and plasmid extractions were performed by miniprepration (Qiagen, USA). After routine insert DNA check by agarose gel electrophoresis, the amino acid substitutions were investigated by DNA sequencing. Three distinct mutants (M1–M3) were obtained (Table 1). Mutant plasmids were then re-introduced into fresh *E. coli* DH5α cells in order to nullify any phenotypic improvement due to background mutation. The phenotypic improvement were then verified against a strongly hydrophobic (toluene, 0.2%, v/v) and a moderately hydrophobic solvent (cyclohexane, 1.5%, v/v); detailed data not shown here. All of the mutants showed improved growth profile compared to *crp*WT-pACYC under aforementioned solvents.

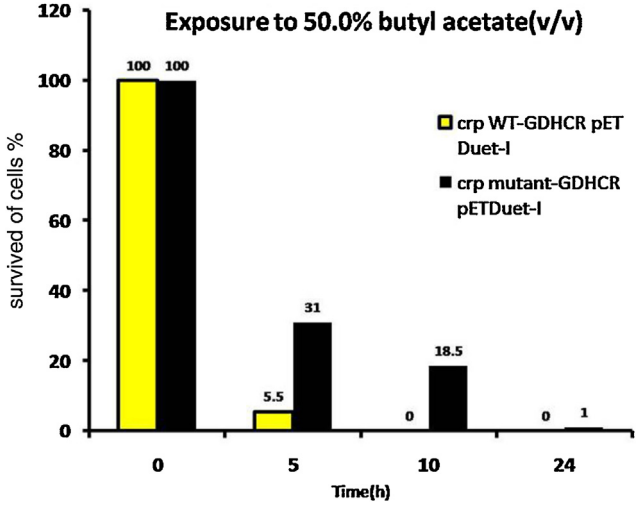

Fig. 3. Tolerance study *crp**pACYC-GDHCRpET-Duet-I in *n*-butyl acetate. The tolerance study has been evaluated with respect to WT CRP harbouring GDHCR-pET Duet-I.

Together with this, encouraged by our previous study results where F136I mutant showed cross tolerance against other stresses, M1 (F136I) has been chosen for further investigations.

3.4. Tolerance study in butyl acetate

The mutant-pACYC plasmids were transformed into fresh *E. coli* BL21 (DE3) cells for preliminary analysis of tolerance against organic solvent used in biocatalysis (butyl acetate). The tolerance study of *crp** BL21 (DE3) against 50% butyl acetate was performed by percentage survival analysis with respect to *crp*WT. Only 5.5% of WT cell has survived after 5 h on exposure to butyl acetate while the *crp* mutant cell survived 31% on solvent stress (Fig. 3). After 10 h and 24 h, the survival of *crp* mutant was found as 18% and 1%

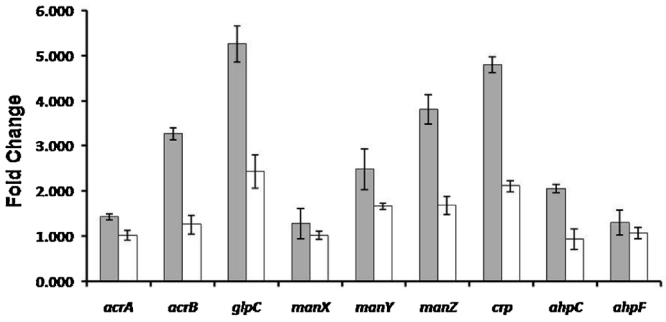

Fig. 4. qRT-PCR study of *crp** vs. WT host cell. All data points are the average of three biological replicates (n = 3). The grey bars denote mutant while the white bars denote WT.

respectively with respect to 0% of *crp*WT. Thus, *crp** cell proved to be more tolerant than *crp*WT in biphasic biocatalysis.

3.5. qRT-PCR

qRT-PCR analysis was performed in order to verify role of certain genes that exhibited significant response to elucidate organic solvent tolerance of mutant bacteria in our previous study (Fig. 4). As for analysis, we verified expression of genes such as *acrAB*, *manXYZ*, *glpC* and *crp*. As additional genes, we analyzed antioxidant gene expression profile such as *ahpC* and *ahpF*. Solvent tolerance contributing genes such as *acrB*, *manYZ* showed elevated expression in mutant during biphasic biocatalysis while *ahpC* has been found activated suggesting its antioxidant role in cell defense mechanism. *glpC* (anaerobic glycerol-3-phosphate dehydrogenase subunit C) has been revealed to be more than 2.0 times activated (also *acrB*) in mutant compared to WT. Most importantly, *crp*, the engineered global regulator, has also been activated more than 2.0 times in mutant during stress incumbent biocatalysis.

3.6. Finding optimum nerolidol concentration

The optimum nerolidol concentration was determined by testing various concentrations of nerolidol (0.5–1.5 mM) with 1 g/L COBE. Blank (without recombinant plasmid) *E. coli* BL21 (DE3) cells were used in order to nullify any CR or GDH enzymatic effect to accelerate COBE transport inside cell. Under this condition with 1.2 mM nerolidol, the extracellular COBE concentration declined from 1 g/L to 0.2 g/L in 4 h suggesting a decrease rate of 0.2 g/L/h. This has been the highest amongst all COBE decrease rates found with various concentrations of nerolidol (Supplementary Fig. S2). Thus 1.2 mM nerolidol is chosen as exogenous transporter of substrate in this study.

3.7. Biotransformation

In fed batch culture technique where suitable amounts of COBE was added at specific time intervals, the maximum CHBE concentration was reached at 250 g/L when *crp** encoded cell was used for biotransformation together with 1.2 mM nerolidol (Fig. 5a). However, when nerolidol was removed from the media, the CHBE concentration reached upto 158 g/L only with *crp** encrypted cell (Fig. 5b). Furthermore, when normal *crp* harbouring cell was used, the biotransformation reached 57 g/L and reached saturation within 6 h (Fig. 5c).

We normalized all of our biotransformation data per g of dry cell weight in order to compare the productivity with previous literatures. Most interestingly, without any engineering and permeation enhancer, our *(R)*-CHBE productivity reached 161.04 g/L/g DCW which already surpassed the previous productivities reported among literatures. However, on application of *crp* engineering and extracellular transporter as nerolidol, *(R)*-CHBE productivity reached 710.88 g/L/g DCW (Fig. 6) which is significantly higher than those of published contemporary works (Table 2).

4. Discussion

One of the main challenges in biphasic biocatalysis is cell viability during biocatalysis since organic solvents are toxic to the cells leading the death of the microbes [4–7]. Especially solvent entry is of major concern for those microbes where cofactor regeneration is one of the key features of biotransformation. Hence attempt has been taken in this study to improve host cell phenotype by *crp* engineering thereby reprogramming entire cell performance (Fig. 7) within solvent-water two-phase during biotransformation.

For *crp* engineering, we used combinatorial method of mutagenesis as it can create largest possible sets of mutation on a given subset of gene or protein at a time that can be manifested through properly transformed cells (library size at least 10^5–10^6) [11–15]. The advantage of error prone PCR being that all the possible amino

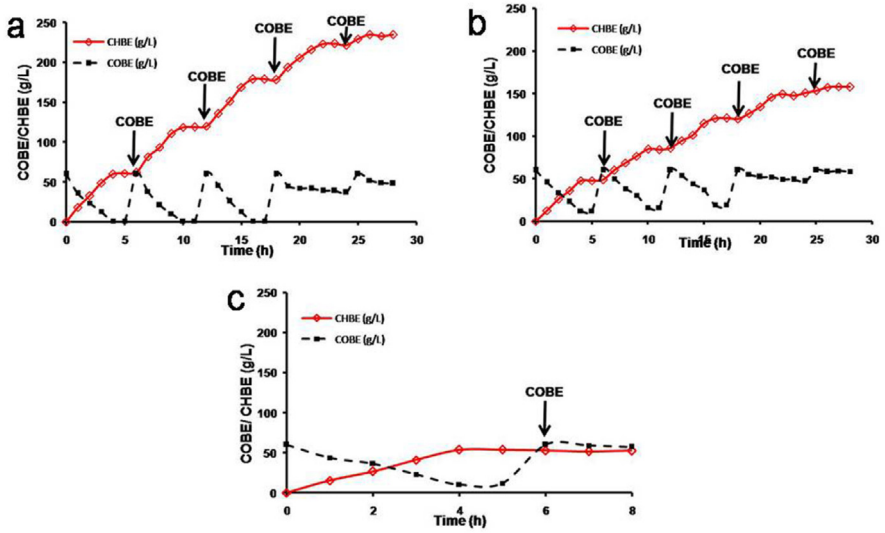

Fig. 5. Biotransformation with *crp**/*crp* strain with or without nerolidol. a. *crp** with nerolidol b. *crp** without nerolidol c. *crp*WT strain without nerolidol.

Table 2
Comparative batch production profile of CHBE amongst contemporary works.

Name of the publication	Output	Our work
	(CHBE production)	(CHBE production)
[37]	49.8 g/L/gDCW	161.04 g/L/gDCW
[38]	19.72 g/L/h/gDCW	45.23–27.27 g/L/h/gDCW
[39]	0.166 g/L/gDCW	161.04 g/L/gDCW
[40]	265.6 g/L/gDCW	161.04 g/L/gDCW
[41]	Nothing is mentioned, Only V_{max} is mentioned	161.04 g/L/g DCW
[42]	2.76 g/L/gDCW	161.04 g/L/gDCW

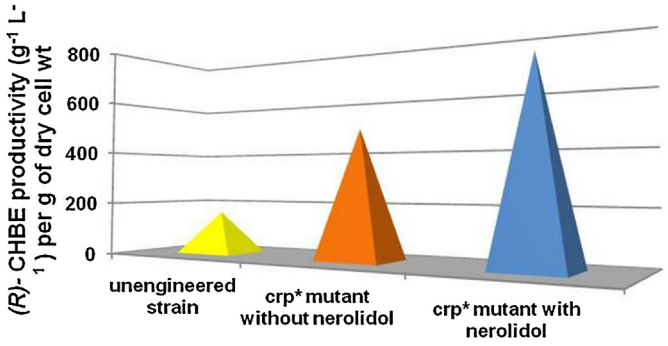

Fig. 6. Cell wt. normalized batch productivity of three systems. *crp*WT strain without nerolidol, *crp** without nerolidol, *crp** with nerolidol. The batch productivity has been increased about 8 times (~477 g/L) when *crp** strain was used without nerolidol compared to control (*crp*WT strain without nerolidol, ~58 g/L). However, when *crp** strain was used with nerolidol, batch productivity hiked to ~12 times (711 g/L).

acid combinations can be perturbed through a single cycle PCR optimizing the reaction time and condition for a particular gene or protein. For example, CRP has 610 amino acids and one amino acid can be mutated to 19 possible other essential amino acids. Thus through error prone PCR, we can create a library size of 19^{610} possible amino acids. This output is larger than any other method of random mutagenesis. We have chosen toluene as selecting solvent in this study. It is because toluene being extremely hydrophobic, helped to select highly improved phenotype which showed enhanced tolerance against a multitude of solvents.

We kept host genomic *crp* direct regulator of RNA polymerase (RNAP, EcoCyc, Regulon DB), integrated in mutant cell for maintaining the RNA polymerase bias over the *lac* promoter of our expression vector. Interestingly, the intense bands of both the proteins in our mutant might be due to the dual CRP regulation of RNAP which underwent stronger binding with our expression vector to result in more intense protein expressions.

The mutant *crp* (F136I) encoded plasmid has been introduced inside fresh *E. coli* BL21 (DE3) and cell tolerance has been evaluated against 50% butyl acetate (Butyl acetate: Buffer 1:1 v/v). From percentage survival assay against the same suggested that the cell tolerance has improved markedly due to introduction of *crp** inside

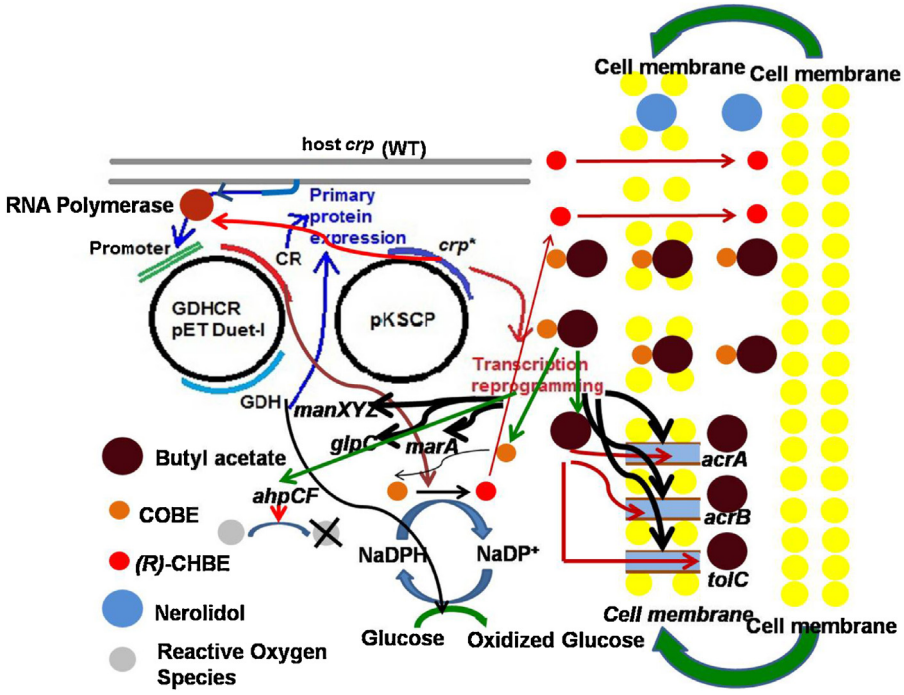

Fig. 7. Metabolic and biotransformation pathway inside *E.coli* BL21 (DE3) harbouring *crp**pACYC-GDHCRpET Duet-I. The host genomic *crp* is kept integrated inside the genome in order to regulate RNA polymerase to bind with the promoter thereby inducing normal protein expression of both CR and GDH inside host cell. The mutated *crp* (*crp**, F136I) does transcriptional reprogramming of genetic subset inside host cell which in turn upregulates transporter channels such as *acrA*, *acrB* and *tolC* encoding extrusion channels. Under stress, *crp** also encodes stress responsive genes such as *manXYZ*, *glpC*, *marA* andothers. Nerolidol, the sesquiterpine compound, is incorporated inside intra-membrane space of the microbe and thus augments membrane permeability for extracellular substrate. COBE with butyl acetate enters into the cytosol where butyl acetate gets detached from COBE and exported outside through extrusion channels. COBE later forms *(R)*-CHBE by action of CR and GDH with cofactor regeneration ($NADP^+$ –-> NADPH). The CHBE further, permeates through the fluidic cell membrane and drives in to the extracellular organic media wherefrom product collection is performed. Most interestingly, prolonged aeration inside bioreactor may produce oxidative stress by producing free radicals and reactive oxygen species (ROS). *crp** most probably, upregulates de-oxidization pathway such as encoding alkyl hydroperoxidase (*ahpCF* encoding enzyme) which detoxify the ROS from the cell [7].

the cell. The enhancement of solvent tolerance is probably due to over-expression of solvent efflux channels such as *acrA*, *acrB* as revealed by our qRT-PCR results which also concurred with our previous findings [4]. Activation of such kind of efflux channels help cell pump-out organic solvent from inside. In addition, genes like *glpC* which encodes a Glycerol-3-phosphate dehydrogenase is upregulated in our *crp** host that might provide the cells an additional machinery to extrude organic solvent from intracellular cytosol [26]. Furthermore, upregulation of mannose PTS permease, *manXYZ*, probably rendered the cells less adherent to the hydrocarbon phase [4]. Activation of all these solvent tolerant defense machineries probably contributed in enhanced cell performance during biphasic biocatalysis.

CRP is an autoregulator and is itself upregulated to keep cellular metabolism integrated at pressure and nutrient deficit condition [17]. In pursuit, we tried to investigate the *crp* expression profile in our mutant during biocatalysis. The qRT-PCR data suggests that the *crp* expression in mutant has been upregulated more than 2.0 times compared to WT under stress which in turn, might have triggered the cell nutrition pathway during starving condition. But why one amino acid mutation in CRP can cause transcriptomic profile of so many genes? Kim et al., 1992 acknowledged that on binding with cAMP, CRP undergoes conformational changes which binds with the promoter DNA and activates transcription of genes [27]. The authors also reported that residues135-138 forms a hinge region which holds the two domains together. Moreover, Fas et al., 2015 revealed that F136 plays an important role in stabilizing the inter-domain hinge [28]. We propose that F136I substitution eventually changed the hinge orientation resulting in altered conformation of CRP. The altered conformation of CRP subsequently brought changes in its binding with the promoter thus causing differential expression of genes inside the cell.

Another key challenge in long term fed batch biocatalysis is to relieve oxidative stress due to prolong aeration, temperature and solvent stress during biotransformation. Cumulative effect of organic solvent stress, stressed operations of metabolic pathways thereof and oxygen pressure inside bioreactor creates reactive oxygen species (ROS) inside the cell which is responsible for cell death and necrosis. Alkyl hydroperoxide reductase (AhpCF) a key ROS scavenger of *E. coli* [29–31] and Regulon DB shows that CRP is regulator of AhpCF. Also, Ferrante et al., 1995 reported that recombinant *E. coli* encoding AhpCF could combat organic solvent stress in *E. coli* [7]. Our qRT-PCR results in this study revealed that mutated *crp** upregulated AhpCF guided antioxidant gene expression more than 2.0 times compared to WT. Probably this is one of the antioxidant pathways that led to improve oxidative stress tolerance of the mutant which indirectly helped it improve solvent tolerance. This phenomenon has also been acknowledged by contemporary workers [29,30]. Thus our CRP guided strain engineering has successfully maintained its protein expression pathways, yet invariably activated cell defense machineries to prolong cell survival during two-phase biocatalysis. Since previous reports acknowledged the role of mar-sox-rob regulon in the upregulation of organic solvent tolerant genes and found *mar-sox-rob* sequence at the upstream of certain genes such as *tolC* [32–35], we propose that *crp* engineering has activated the same regulon which ultimately led to upregulation of associated genes.

Any biocatalytic performance depends on its enzyme's efficiency to convert substrate into product at a given condition. Choosing suitable enzyme for bioconversion is therefore major bottleneck of the process. *Candida glabrata* CR has been already been reported for its efficiency in biocatalysis, however the full potential of this enzyme in biocatalysis has never been optimized [24]. In this study, we have purified and characterized this enzyme from cloned constructs. Its optimum temperature and pH for activity has also been identified. Especially its high specific activity, low K_m and high K_{cat} have made it a suitable candidate enzyme for our bioconversion. Moreover, since any CR gene uses cell cofactor as hydrogen pool, we have complemented the CR with GDH from *Bascillus subtilis* for cofactor regeneration. (supplementary Fig. S3).

Since microbial cell membrane is a semi-permeable membrane and acts as limiting barrier for substrate entry inside the cell, rate of substrate permeation is a rate determining parameter for whole cell biotransformation. However, amongst a subset of membrane proteins, rational approach of transporter engineering either requires prior knowledge to this or requires identification of proper transporter through numerous expression or knockout approaches [36]. This strategy being a tedious, time and cost intensive job, introduction of exogenous transporter is always a key strategy in biocatalysis. Nerolidol is a sesquiterpene compound reported to increase substrate permeability by getting incorporated inside bacterial cell membrane, thus altering its fluidity and pore diameter [25]. One key advantage of using nerolidol is that it increases cell permeability by chemical interaction with cell membrane, not by gene regulation. Thus it is less likely that nerolidol guided cell permeability could be affected by *crp* engineering. In this study, 1.2 mM of nerolidol has been observed to maximize rate of entry of COBE inside the blank host cell. Further increment of nerolidol concentration reduced rate of substrate uptake (Supplementary Fig. S3) either due to nerolidol toxicity or due to intracellular deposition of nerolidol which retarded the diffusion of the compound itself inside the cell. Although nerolidol has been reported to increase uptake of antimicrobial compound [25], this is the first attempt till date to use the compound to improve substrate uptake during biotransformation.

This study is therefore is a balanced and amalgamated approach for using three pivotal approaches of biotransformation- strain engineering, recombinant DNA technology and transporter engineering (Fig. 7). The previous approaches [18–23] only relied on isolation of novel active enzymes and using them for biotransformation while some recent contemporary studies tried to change cofactor or adding solubility enhancer such as β-cyclodextrin [37,38] for improving biotransformation. However, none of the works was able to unlock full cellular potential for maximizing output in biotransformation. In this study, we have tried to perturb a set of speed-breaking steps in biocatalysis and the resulting output has superseded all the contemporary works of CHBE bioproduction (Table 2). The recent contemporary works have been taken as benchmarks for comparisons as the authors of those reports claimed to have already outdone the outputs of previous related studies in this field. Actually, individual and separate attempts of using strain engineering or transporter engineering over microbes have been undertaken for bioproduction (although very few attempts are reported). However, employing the engineering triad in an optimized package within a single microbial cell is really lacking especially in the field of biotransformation. We claim that, this study is an attempt to fill in the gap of biotransformation with aforedescribed trinomial bioengineering that can be employed for any further kind of bioproduction as well.

5. Conclusion

The present investigation reveals detailed study of a biocatalytic system having one of the highest carbonyl reductase activities. When *crp* mutation had been performed, improved cell tolerance mediated biotransformation yielded 477 g CHBE/L/gDCW. Further incorporation of nerolidol, a sesquiterpene membrane transporter, improved the bioproduction upto 710.88 g/L/gDCW. Dual CRP regulations inside variant proved successful, yet intense enzyme expressions for biotransformation. Mechanistic investigations inside the host revealed upregulation of solvent efflux

channels as well as antioxidant machineries that might contribute towards outstanding tolerance of the host against multiple stresses inside bioreactor. Thus this study lays out a promising, novel and first time approach for clubbing three musketeers of bioengineering- strain engineering, rDNA technology and transporter engineering for maximizing output in biotransformation. As a result, it renders the batch productivity outstandingly higher and makes the process more commercially feasible than any of the previous ones.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijbiomac.2016.12.001.

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