Homologous overexpression of Hydrogenase and Glycerol dehydrogenase in *Clostridium pasteurianum* to enhance hydrogen production from crude glycerol

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Abstract

This study reports engineering of a hypertransformable variant of *C. pasteurianum* for bioconversion of glycerol into hydrogen (H_2). A functional glycerol-triggered hydrogen pathway was engineered based on two approaches: (1) increasing product yield by overexpression of immediate enzyme catalyzing H_2 production, (2) increasing substrate uptake by overexpression of enzymes involved in glycerol utilization. The first strategy aimed at overexpression of *hydA* gene encoding hydrogenase, and the second one, through combination of overexpression of *dhaD1* and *dhaK* genes encoding glycerol dehydrogenase and dihydroxyacetone kinase. These genetic manipulations resulted in two recombinant strains ($hydA^{++}$/$dhaD1K^{++}$) capable of producing 97% H_2 (v/v), with yields of 1.1 mol H_2/mol glycerol in *hydA* overexpressed strain, and 0.93 mol H_2/mol glycerol in *dhaD1K* overexpressed strain, which was 1.5 fold higher than wild type. Among two strains, $dhaD1K^{++}$ consumed more glycerol than $hydA^{++}$ which proves that overexpression of glycerol enzymes has enhanced glycerol intake rate.

Keywords: Biohydrogen, Crude glycerol, Clostridium pasteurianum, Overexpression

1. INTRODUCTION

Concerns of energy security and climate change risk due to rampant use of fossil fuels have been the main driving force for quest of alternate renewable biofuels. Various initiatives have been implemented by the governments of developing countries to encourage the use of biofuels (or blended petroleum fuels) as a transport fuel. In India, a target of 20% blending of biodiesel and bioethanol with diesel and petrol is envisaged by 2022. Bioethanol and biodiesel have gained popularity as alternate liquid biofuels because of ease of blending with conventional fuels, and compatibility of blended fuels with conventional IC engines. Further, biodiesel production holds a strong market potential due to availability of wide variety of feedstock (about 350 oil-bearing crops) for its production. Biodiesel (or fatty acid methyl esters) is produced through one-step reaction, i.e. transesterification. This reaction produces huge amount of glycerol as a byproduct. Use of this glycerol to produce other fuels or value-added products would increase revenue of biodiesel industry. Bioconversion of glycerol to products like 1, 3-propanediol, ethanol, butanol, hydrogen, succinic acid has been reported by previous authors (Biebl et al., 2001; Dabrock et al., 1992; Khanna et al., 2012). Another valuable product feasible from bioconversion of waste glycerol is biohydrogen, which is a highly versatile source of renewable energy. Glycerol is efficiently metabolized to hydrogen by strict anaerobes such as *Clostridium acetobutylicum*, *Clostridium pasteurianum*, *Clostridium paraputrificum*, *Clostridium thermocellum* (Sarma et al, 2016).

Numerous strategies for enhancing the yield of valuable products through bioconversion of glycerol have been reported by previous authors. However, not much work has been done in genetic engineering of Clostridial flora. Two major hurdles in genetic accessibility of Clostridial strains are: (1) Gram positive bacteria characterized by presence of thick network of peptidoglycan in the cell wall which acts as a barrier to DNA transformation, and (2) presence of unknown restriction-modification systems which destroys any incoming foreign DNA (Mermelstein et al., 1992, Richards et al., 1988, Trevors et al., 1992, Gozalez-Pajuelo et al.,

2005). Although few industrial strains such as *C. acetobutylicum* and *C. beijerinckii* have been genetically explored for biofuel production but studies on *C. pasteurianum* is still limited. As evident from early investigations, efforts were made to genetically manipulate *C. pasteurianum* since 1979 when Clarke et al., developed a method to regenerate protoplasts. Further, identification of CpaAI - a Type-II restriction endonuclease, as a barrier to gene transfer in *C. pasteurianum* (ATCC 6013) has motivated researchers to study the importance of DNA methylation as a technique to protect the foreign DNA (Jennert et al., 2000; Mermelstein et al., 1992; Klapatch et al., 1996, Richards et al., 1988). Recently, new strategies have been attempted to modify the metabolic pathway of *C. pasteurianum* to boost the productivity of its metabolites (Papoutsakis et al., 2008; Pyne et al., 2013-2015). Initially Schwarz et al. (2017) tested various strains of *C. pasteurianum* for DNA transformation by the electroporation protocol published by Pyne et al. (2013) and highlighted the fact that *C. pasteurianum* DSM 525 could be repeatedly transformed with low efficiencies, and that few transformants were most likely a result of the presence of rare mutant variants within the culture that are highly competent for DNA transfer. Thus, Schwarz et al. (2017) proposed methods for screening hypertransformable *C. pasteurianum* DSM 525 strains and *C. pasteurianum* H4 is one of them, which has been used for this study.

Hydrogenase is the key enzyme involved in catalyzing the reversible reaction of protons to molecular hydrogen (H_2) (Kim et al., 2011; Vignais and Colbeau, 2004). Genome sequence of *C. pasteurianum* reveals the presence of four hydrogenases coded by CLPA_c07060-70, CLPA_c00280, CLPA_c33960 and CLPA_c37830 (Poehlein et al., 2015). Among these, CLPA_c07060 and CLPA_c07070 encode the small and large subunit of [Ni Fe]-hydrogenase, respectively. BLAST searches and the reports by Pyne et al. (2014) affirm the respective gene of CLPA_c00280 as *hydA*. Further knock-down of *hydA* by Schwarz et al. (2017) clearly shows that deletion of the hydrogenase encoded by CLPA c00280 is tightly involved with the fermentative pathway in *C. pasteurianum*. Therefore, attempts were made in this study to

overexpress the *hydA*.

Under anaerobic conditions, bioconversion of glycerol is mediated through the *dha* system, that involves initial enzymes glycerol dehydrogenase (*dhaD or gldA*), oxidizing glycerol to dihydroxyacetone and dihydroxyacetone kinase (*dhaK*), phosphorylating dihydroxyacetone to dihydroxyacetone-phosphate (DHAP) which then subsequently enters glycolysis and fermentation (Biebl et al., 1999; Malaviya et al., 2012). The byproducts of the oxidative branch of metabolic pathway are ethanol, butanol, acetic acid, butyric acid, hydrogen and succinic acid. This pathway also provides energy and NADH for the reductive pathway of glycerol conversion leading to formation of 1,3-PDO. Metabolic flux analysis in *Escherichia coli* showed that during anaerobic bioconversion of glycerol, the glycolytic flux was exclusively controlled by dihydroxyacetone kinase and glycerol dehydrogenase (Cintolesi et al., 2012). Numerous efforts to engineer glycerol mediated pathways for production of various value-added products, such as biomaterials, biofuels and biochemicals have been reported in the literature.

In this study, we have used crude glycerol resulting from transesterification of soybean oil (Sarma et al., 2016) as fermentation substrate. Our previous study on metabolic flux analysis (Sarma et al., 2017) explained that doubling the glycerol uptake flux channeled metabolic flux towards pyruvate node thereby increasing the flux towards generation of hydrogen. This could be initiated by overexpression of the immediate genes of glycerol utilization pathway viz., *dhaD* and *dhaK*. To the best of our knowledge, there is no previous study on over-expression of *hydA*, *dhaD1* and *dhaK* in *C. pasteurianum* for enhancing hydrogen yield. This study presents the fermentation patterns of *C. pasteurianum* with overexpressed *hydA* and *dhaD1-dhaK*.

2. MATERIALS AND METHODS

2.1 Aerobic strains and culture conditions

Cultures of *Escherichia coli* were revived in LB-medium agar plates and incubated at 37 °C. The liquid cultures were grown in Erlenmeyer flasks with shaking at 200 rpm. Various

antibiotic concentrations after filter sterilization were used for selection of recombinant strains, viz. chloramphenicol (12.5 µg/mL in liquid cultures and 25 µg/mL in agar plates), kanamycin (50 µg/mL) and erythromycin (500 µg/mL)

2.2 Anaerobic strains and culture conditions

Clostridium pasteurianum-H4 (DSM 525) were revived in Reinforced Clostridial Medium (RCM) agar plates and incubated at 37 °C in an anaerobic workbench (Don33 Whitley, Yorkshire, UK) maintained under anaerobic conditions by supply of N_2:H_2:CO_2 in a ratio of 80:10:10% v/v. The RCM agar plates and liquid medium were left over-night in the anaerobic workbench, to pre-reduce medium before inoculation. Various antibiotic concentrations were used (after filter sterilization) for selection of recombinant strains, viz. thiamphenicol (25 µg/mL) and erythromycin (50 µg/mL).

2.3 Medium used

Reinforced Clostridial Medium (RCM) agar was used as solid growth medium for *C. pasteurianum*. The composition of RCM is same as described in Sarma et al. (2016) with pH maintained at 6.8.

2xYT was used as liquid growth medium composed of the following constituents (g/L): yeast extract (10); tryptone (16); sodium chloride (5) added to distilled water and pH adjusted to 6.2 followed by autoclaving. After sterilization, 100 mL of 50%w/v glycerol solution was added.

Biebl Medium was used for fermentation experiments and was composed of the following components (added to 1 L of distilled water): KH_2PO_4, 0.5 g; K_2HPO_4, 0.5 g; $(NH_4)_2SO_4$, 5 g; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2 \cdot 2H_2O$, 0.02 g; $FeSO_4 \cdot 7H_2O$, 0.00914 g and yeast extract, 1 g (Biebl et al., 2001). The pH was adjusted to 6.2 with HCl and autoclaved**.** After sterilization, 2 mL of SL7 micronutrient solution, 120 mL of 50% v/v glycerol, 250 µL of 0.1 mg/mL biotin (filter sterilized), 20 mL 250 g/L $CaCO_3$ and 1 mL 2 mg/mL resazurin (all stock solutions were sterilized separately). The composition of trace element SL7 solution is as follows (added to 1L H_2O): $MnCl_2 \cdot H_2O$, 100 mg; $ZnCl_2$, 70 mg, H_3BO_3, 60 mg; $NaMoO_4 \cdot 2H_2O$, 40 mg; $CoCl_2 \cdot 6H_2O$,

28.7 mg; $CuCl_2 \cdot 2H_2O$, 20 mg; HCl (25 %), 1 mL; $NiCl_2 \cdot 6H_2O$, 20 mg (Biebl and Pfennig, 1981) The solution was filter sterilized by passing through 0.2 µm filter.

BSH Medium (a modified form of Basal solution for hydrogen production) was optimized in our previous study with following composition (g/L): yeast extract 1.48, peptone 2.7, K_2HPO_4 0.68, sodium chloride (NaCl) 1.46, KH_2PO_4 4.035, vitamin solution 10 mL and trace solution 10 mL (Sarma et al., 2016, 2017, Chen et al., 2005, Junghare et al., 2012). The composition of trace element solution was as follows (cocn. g/L): $ZnSO_4 \cdot 7H_2O$ 0.05, $CuCl_2 \cdot 6H_2O$ 0.05, $MnO_4 \cdot 7H_2O$ 0.01, $N(CH_2COOH)_3$ 4.5, H_3BO_3 0.01, $CaCl_2 \cdot 2H_2O$ 0.01, $AlK(SO_4)_2$ 0.01, Na_2MoO_4 0.01, $MgCl \cdot 6H_2O$ 0.2, $CoCl_2 \cdot 6H_2O$ 0.2, $FeCl_3$ 0.1.The vitamin solution comprised of the following constituents (concn., g/L): citric acid 0.02, para–amino benzoic acid 0.01, riboflavin 0.025, folic acid 0.01. The pH of the medium was maintained at 7.0 using 1 M sodium hydroxide. The anaerobic environment was maintained in the serum bottles by flushing the medium continuously with 99.99% (v/v) pure nitrogen, then sealed and crimped with butyl–rubber stoppers before autoclaving. The medium was then inoculated with 10% v/v pure culture with the help of sterile disposable syringe and incubated at 37 °C with shaking at 150 rpm. After inoculation, aliquots of 2 mL culture broth were periodically withdrawn and stored at –20°C for HPLC analysis of glycerol and other metabolites. Similarly, gaseous samples were collected periodically from the headspace to quantify hydrogen and carbon–dioxide content.

2.4 Batch fermentation

Growth experiments were conducted in 100 mL serum flasks with 50 mL working volume (the growth medium). Prior to experiments, these flasks were autoclaved for sterilization. Serum bottles were placed in anaerobic cabinet for 24 h prior to inoculation. Falcon tubes with 20 mL growth medium were used for growth of pre-cultures. The optical density of the solution was monitored, and microbial cultures in mid-exponential phase were used for inoculation of serum bottles. Post inoculation, the serum bottles were sealed with sterile stoppers and were incubated with shaking at 150 rpm and 37 °C. Samples of the solution and the gas

accumulated in the head space were withdrawn for analysis using a sterile syringe.

2.5 Standard molecular biology methods

Standard protocols described by the respective supplier were followed for plasmid DNA purification (NEB Biolabs Monarch Plasmid Miniprep Kit #T1010L); genomic DNA purification (GenElute™ Genomic DNA Extraction Kit (Sigma Aldrich, UK)); gel extraction (NEB Biolabs Monarch Gel Extraction Kit #T1020L); PCR clean up (Jena Bioscience PCR Purification Kit # PP-201L). For PCR amplification of DNA fragements, Dream Taq Green PCR Master Mix (Thermo Scientific™, UK) and Phusion® High-Fidelity DNA Polymerase (NEB, UK) was used. Colony PCR of *E. coli* cells was performed by picking half of a colony from plate followed by resuspension in 10 µL Dream Taq Green PCR Master Mix with specific primers. However, the colony PCR protocol for clostridial clones varied from that of gram negative cells. A single colony was picked from RCM plate followed by resuspension in 50 µL of colony lysis buffer (20 mM Tris-HCl, pH 8; 2 mM EDTA; Triton-X 100 - 1% w/v) and microwaved for 2 min, followed by vortexing. 1 µL of this mixture was added as a template DNA to 9 µL of Phusion® High-Fidelity DNA Polymerase PCR mix with specific primers. The standard PCR program was used for amplification, with initial denaturing step extended to 10 min at 98°C for efficient cell lysis. Standard protocols of cloning and transformation into *E. coli* were followed. Standard procedures given in the supplier handbook were used for restriction digestion by Fast Digest™ restriction enzymes (Thermo Scientific™, UK) but incubation time was increased to 1 h. Quick Ligation™ Kit (NEB, UK) was used for ligation reactions and incubated for at least 1 h at room temperature. Based on the published genome sequence of *C. pasteurianum* DSM 525 (Poehlein et al., 2015) oligonucleotide primers were designed (Table 2) to amplify the *hydA* and *dhaD1-dhaK* gene.

2.6 Analytical methods

Samples of fermentation mixture were thawed followed by centrifugation (12,000 rpm, 20 min). Supernatant was filtered through a membrane filter with 0.22 µm pore size. The HPLC

apparatus (Perkin Elmer, Series 200) comprised of a RI detector, a pump and a vacuum degasser. Residual glycerol in the sample was determined using HPLC that employed a Rezex RCM Monosaccharide calcium–column (300 mm × 8 µm × 7.8 mm, Phenomenex, India). The column oven temperature was set up at 35°C. Mobile phase in the HPLC was ultra–pure water (flow rate = 0.5 mL/min). Quantifications of other metabolites (acetate, butyrate, lactate, ethanol, butanol, 1,3-propanediol) in the fermentation samples were done using a Hi–Plex H column (300 mm × 8 µm × 7.7 mm, Agilent, India) with column oven temperature maintained at 60°C. 0.01 M H_2SO_4 in ultra–pure water (18.2 MΩ·cm resistivity at 25°C) was used as the mobile phase at a flow rate of 0.5 mL/min. Standard calibration plots were used to quantify glycerol consumed during fermentation process.

Gas Chromatograph (Ceres800 plus, Thermo scientific) was used to quantify and determine the composition of the product gas collected from the headspace of the batch reactors. The GC apparatus is equipped with a TCD detector, maintained at 200^{o}C, Porapak Q (60/80 mesh) GC column placed in an oven thermostated at 45^{o}C and the injector maintained at 200^{o}C. Carrier gas in the GC was argon (flow rate = 30 mL/min). Gas samples collected from the headspace of the serum bottles were injected to determine the concentration of H_2 and CO_2. The cumulative volume of hydrogen gas was calculated as detailed in our previous paper (Sarma et al., 2017).

2.7 Preparation of crude lysate

60 ml of overnight grown cultures of both *C. pasteurianum*-H4 wild and recombinant strains were harvested by centrifugation (10 min, 5000 rpm) at 4°C. The pellet was washed with 0.05 M sodium phosphate buffer, pH 7.5 followed by resuspension in 5 mL of the same buffer. The cells were lysed by application of ultrasound (Model: VCX-500) and sonicated for 10 min (9 s on and 5 s off per cycle) at 4°C followed by centrifugation at 12000 rpm for 10 min, 4°C. The resulting supernatant was used as the crude cell lysate and used for SDS-PAGE and activity assay.

2.8 Hydrogenase assay

The activity of hydrogenase was assayed by analysis of H_2 produced by reduction of sodium dithionite from methyl viologen (MV). The assay mixture consist of 2.25 mL working volume composed of 3 mM MV , 50 mM Tris-Cl buffer (pH 6.8), and crude lysate which was purged with 99.99% pure nitrogen gas for 3 min followed by addition of 0.25 mL of 230 mM sodium dithionite at 37 °C which initiated the enzymatic reaction (Tamiya et al., 1955). The amount of H_2 produced was measured after every 5 min using a gas chromatograph.

2.9 Glycerol dehydrogenase assay (GDH)

The activity of GDH enzyme was assayed in the crude lysate by measuring the reduction rate of 2,6-dichlorophenol-indophenol (2,6-DCIP) (Li et al., 2010). Time profile of The reduction of 2,6-DCIP was measured using UV–Vis spectrophotometer at absorbance (600 nm) at 30°C. The assay mixture with a reaction volume of 0.8 mL comprised of 50 mM potassium phosphate buffer (pH 6), 0.325 mM of phenazine methosulfate (PMS), 0.25 mM DCIP and 100 µL of crude extract, was incubated for 5 min in the cuvette. The reaction was initiated by addition of 200 µL of pre-warmed glycerol at 30 °C. One unit of GDH activity was defined as the amount of enzyme that catalyzes the reduction of 1 µM of DCIP per min at 30 °C. Protein concentration was quantified by Bradford method (1976) using BSA (bovine serum albumin) as a standard.

3. RESULTS AND DISCUSSION

3.1 Construction of recombinant hydrogenase from *C. pasteurianum*

In *C. pasteurianum* it was possible to overexpress the putative main hydrogenase *hydA* (CLPA_c00280) which shows 71% identity with the well-described *C. acetobutylicum hydA* (Santangelo et al., 1995). The gene was amplified (Fig. 1A) from genomic DNA of *C. pasteurianum* DSM 525(H4) (Schwarz et al., 2017) with primers hydA_F and hydA_R (Table 2). The *hydA* gene was then cloned into a pMTL80000 modular shuttle plasmid developed by

Heap et al. (2009). The plasmid pMTL82251 was used and slightly modified by deletion and insertion of few bases followed by insertion of the glycerol promoter (Pgl_GL) from *C. pasteurianum* glycerol operon (CLPA_c22720 to CLPA_c22820) and was renamed as Q5_pMTL_82251_PGL by Minton and co-workers. Both the plasmid and the amplified *hydA* was digested by NdeI and NheI (Fig. 2B) and ligated overnight. The ligation mixture was transformed into *E. coli* TOP10 cells using calcium chloride heat shock method (Ausubel et al. 1987). The erythromycin resistant colonies were selected on the LB agar plate supplemented with 500 μg/mL erythromycin and positive clones were screened by colony PCR followed by DNA sequencing. After successful screening of positive clones carrying *hydA*, the plasmid DNA was protected against restriction by *in vivo* methylation with *E. coli* carrying plasmid pMTL-CR1 expressing the *M. BepI* methylase. The methylated plasmids were then transformed into *C. pasteurianum* by electroporation and the erythromycin resistant colonies were selected on the freshly prepared RCM agar plate supplemented with 40 μg/mL erythromycin. Positive clones of *C. pasteurianum* carrying *hydA*-Q5_pMTL_82251_GL plasmid were screened by colony PCR with primers 82XXX-LR and traj_F (primers in the plasmid backbone) amplifying a band of 2385 bp (Fig. 2D) against the clones with empty Q5_pMTL_82251_GL plasmid (without *hydA* insert) with a band of 861 bp (Fig. 1C).

3.2 Construction of recombinant glycerol dehydrogenase-dihydroxyacetone kinase from *C. pasteurianum*

The *dhaD1-dhaK* (2936 bp) gene is amplified (Fig. 2A) from genomic DNA of *C. pasteurianum* DSM 525 (H4) by using primers DhaD1K_Pgs_F and DhaD1K_R. The vector Q5_pMTL82251_GL was digested with NdeI and FspI (5241 bp) (Fig. 2B) and the cloning was carried out by using the NEB Gibson Assembly Cloning Kit (NEB #E5510). The ligation mixture was transformed into *E. coli* TOP10 cells using calcium chloride heat shock method (Ausubel et al. 1987). The erythromycin resistant colonies were selected on the LB agar plate supplemented with 500 μg/mL erythromycin and the positive clones in *E. coli* TOP10 were

screened by colony PCR with primers 9C_DhaK_sco_F and 82XXX-LR (Table 2) amplifying a band of 1272 bp (Fig. 2C) followed by DNA sequencing. After successful screening of positive clones carrying *dhaD1-dhaK*, the plasmid DNA was methylated by transformation into *E. coli* carrying plasmid pMTL-CR1 expressing the *M. BepI* methylase. The methylated plasmids were then transformed into *C. pasteurianum* by electroporation and the erythromycin resistant colonies were selected on the freshly prepared RCM agar plate supplemented with 40 μg/mL erythromycin. Positive clones of *C. pasteurianum* carrying *dhaD-dhaK*-Q5_pMTL_82251_GL plasmid were screened by colony PCR with primers 82XXX-LR and traj_F amplifying a band of 3526 bp (Fig. 2D) against the clones with empty Q5_pMTL_82251_GL plasmid (without *dhaD-dhaK* insert) with a band of 861bp (Fig. 1C).

3.3 Batch fermentation

To analyze the fermentation patterns of hydrogenase (*hydA*), dihydroxyacetone kinase and glycerol dehydrogenase (*dhaD1K*) overexpression, *C. pasteurianum hydA*$^{++}$, *C. pasteurianum dhaD1K*$^{++}$ and *C. pasteurianum* DSM525 H4 wild type were grown in 100 mL serum flasks with 50 mL Biebl medium or BSH medium with pure and crude glycerol as the substrate. The crude glycerol fermentation profiles are described in Fig. 3 and 4. Both Biebl and BSH fermentation media were used for the fermentation experiments with pure and crude glycerol as substrate and compared. The BSH medium (as optimized in our previous study) was used for glycerol fermentation by recombinant and wild type strains of *C. pasteurianum.* The fermentation data presented in Table 3 and 4 infers nearly similar profiles with both fermentation media, which suggest that both of them are suitable for fermentative hydrogen production from glycerol. The biomass profiles are also nearly similar suggesting that components of both media are significant for growth and metabolism of *C. pasteurianum* cells.

3.3.1 Hydrogenase overexpressed phenotype

Pure and crude glycerol with a final concentration of 60 g/L was used for fermentation. Glycerol is not entirely exhausted in the cultures of either strain. However consumption in the

dhaD1K^{++} strain is much faster than the wild type and the *hydA*$^{++}$ strain. This is due to high activity of the enzymes acting on glycerol immediately after intake, viz. glycerol dehydrogenase and dihydroxyacetone kinase. The *hydA* recombinant produced higher amount of acetate and negligible amount of lactate and butyrate as compared to wild type. The lactate production reduced to 56% and butyrate to 75% of the wild type production (Table 3). The final acetate concentration after 27 h of fermentation in the *hydA* recombinant increased by 45% of that of wild type. Further stoichiometrically, higher hydrogen production is associated with higher acetate production, and from this study it could be inferred that the highest hydrogen is produced in the *hydA*$^{++}$ strain, which again produces higher amount of acetate. Thus, it could be concluded that improvement in hydrogen production in the *hydA*$^{++}$ strain was caused by reduction in the amount of lactic acid and enhancement in acetic acid. It seems apparent that the enhanced hydrogenase activity caused over-oxidation of NADH to NAD+, and consequently the depletion of NADH to reduce pyruvic acid to lactic acid. Similar observations were also reported by Morimoto et al. (2005) for hydrogen production by *C. paraputrificum*. Butyrate production was observed in WT strain but it decreased in both overexpressed strains after 24 h (Fig. 4E). Similar butyrate profiles were observed by Schwarz et al. (2017). The solvent profiles of *hydA*$^{++}$ strain suggested that there was a reduction in all the solvents viz. 1,3-propanediol, ethanol and butanol as compared to the WT strain. The total acids produced in the *hydA*$^{++}$ strain increased by 57% and total solvents decreased by 15% as compared to the wild type (Table 3 and 4). Thus *hydA*$^{++}$ strain is associated with increase in acid and hydrogen production along with decreased solvents compared to the wild type. This is in accordance with that hydrogen is produced in acidogenic phase and reduces during solventogenic phase of fermentation. A marked increase of 80% in the cumulative hydrogen volume in the *hydA*$^{++}$ strain is observed as compared to the wild type. This corresponds to a yield of 1.1 moles of H_2 per mole of glycerol after 24 h of fermentation using crude glycerol.

The overexpression of *hydA* thus resulted in increase in hydrogen production in *C.*

pasteurianum along with changes in the formation of other fermentation products. These results clearly demonstrate that hydrogenase is the rate limiting enzyme of hydrogen production in *C. pasteurianum*, which is in accordance with findings of Morimoto et al. (2005), but in contrast with the findings of Klein et al. (2010) who have reported that overexpression of hydrogenase has no effect on hydrogen and other metabolites in *C. acetobutylicum*. Therefore, the role of *hydA* as the key enzyme in hydrogen formation varies from organism to organism. However, in *C. pasteurianum* the key role of *hydA* in fermentative pathway was identified by Schwarz et al. (2017).

Metabolic engineering strategies have been applied to hydrogenases mostly for increasing solvent production or decreasing by-product formation such as acids. Some previous studies in this area are as follows: Pyne et al. (2015) have reported knock-down of *hydA* via anti-sense RNA in *C. pasteurianum*. Cooksley et al. (2012) have attempted disruption of *hydA* in *C. acetobutylicum*, while Biswas et al. (2015) have studied deletion of the hydrogenase maturase gene (*hydG)* in *C. thermocellum*. Das and co-workers (Mishra et al., 2004; Khanna et al., 2010) have also attempted overexpression of hydrogenase in *Enterobacter cloacae* (IIT-BT08) which resulted in 1.2 fold higher yield of hydrogen as compared to the wild type. The authors have precisely demonstrated that [FeFe] hydrogenase in *E. cloacae* played an important role in enhancing the hydrogen yield. The hydrogenase activity of the recombinant strain was 1.3 fold higher than the wild strain.

3.3.2 *dhaD1-dhaK* overexpressed phenotype

Glycerol dehydrogenase and dihydroxyacetone kinase are the significant enzymes that play key role in glycerol uptake pathway. The genes for glycerol dehydrogenase (*dhaD*), dihydroxyacetone kinase (*dhaK*), glycerol dehydratase, and 1,3-propanediol oxidoreductase (*dhaT*) are encoded in one and the same regulon named *dha* (Forage and Lin, 1982; Tong et al., 1991). These enzymes has been metabolically explored in microorganisms particularly for fermentative production of 1,3-propanediol (1,3-PDO), 1,2-propanediol (1,2-PDO), and 2,3-

butanediol (2,3-BDO) from glycerol (Jung et al., 2011; Clomburg et al., 2011; Yang et al., 2013; Wong et al., 2014; Maervoet et al., 2016). Overexpression of *dhaD* or *gldA* has resulted in improved yield of 1,2-PDO in *E. coli* and *S. cerevisiae* (Jung et al., 2011; Clomburg et al., 2011). Yazdani and Gonzalez showed that simultaneous overexpression of *dhaKLM* and *gldA* increased glycerol utilization and ethanol synthesis (Yazdani and Gonzalez, 2008). Glycerol consumption is partially strengthened by overexpression of the glycerol uptake genes: glycerol dehydrogenase (*gldA*) and dihydroxyacetone kinase (*dhaKLM*) in *E. coli* which enhanced ethanol yield under microaerobic condition (Wong et al., 2014). Moreover, it has been reported that there are two genes encoding glycerol dehydrogenase in *Klebsiella pneumoniae* viz., *dhaD* and *gldA* (Wang et al., 2014). Similarly, in *C. pasteurianum* glycerol dehydrogenase is encoded by two genes *dhaD1* and *dhaD2* which was depicted from the complete genome of *C. pasteurianum.* In this study we have cloned the *dhaD1* gene.

Fermentation experiments were carried out with *C. pasteurianum dhaD1K* overexpressed strain (*dhaD1K*$^{++}$) with crude and pure glycerol (60 g/L) as the substrate. Higher glycerol consumption could be observed in the fermentation experiments with both crude and pure glycerol with *dhaD1K* recombinant strain (Table 3 and 4). However, only the expression of glycerol dehydrogenase protein was observed in the SDS-PAGE gel. Although proper PCR amplification of the entire *dhaD1K* gene was observed (Fig. 2D), but no activity or protein expression was observed for dihydroxyacetone kinase which might be because of some unexpected inhibition of transcription or translation such as abnormal secondary structure formation. Thus the overexpression of *dhaD1K* gene resulted in higher production and activity of glycerol dehydrogenase, which was manifested in terms of enhanced glycerol metabolism and simultaneous increase in the product yields. Similar reduction in lactate and butyrate was also observed and acetate production increased as compared to the wild type. This strain showed an interesting solvent profile with almost 68% increase in 1,3-PDO and 13.5% increase in butanol production, as compared to wild type (WT). Higher 1,3-PDO production reveals that the

overexpression of the immediate enzymes of glycerol metabolism have redirected the pathway towards 1,3-PDO formation rather than towards acetyl-CoA. Due to higher solvent production in this strain, the hydrogen production is slightly reduced as compared to the *hydA* recombinant strain. The fermentation profiles revealed that cumulative hydrogen volume increased by 75% in the *dhaD1K*$^{++}$ strain, which correspond to a yield of 0.93 mol H_2/ mol glycerol.

From these results it could be concluded that under anaerobic conditions combined overexpression of glycerol dehydrogenase and dihydroxyacetone kinase in *C. pasteurianum* showed an increased 1,3-PDO production along with hydrogen, as compared to the wild type. The importance of *dhaD* and *dhaK* in 1,3-PDO production has been widely explored in *K. pneumonia* but no such report exists that addresses their significance in hydrogen production in any other species. Horng et al. (2010) disrupted *dhaD* and *dhaK1* in *K. pneumoniae*, which significantly lowered 1,3-PDO production in the mutant than the wild strain. These results are consistent with the findings of Wei et al. (2014) that *DhaK1* or *dhaK2* mutation inhibited *dha* regulon expression and *DhaK3* mutation reduced glycerol utilization, as compared to the wild strain. Luo et al. (2011) knocked out *dhaD* in *K. pneumoniae*, and the mutant also shows poor 1,3-PDO production ability. They suggested that this phenomenon is due to unbalanced NADH. Thus, these findings ascertain that *dhaD* and *dhaK* have significant role in glycerol utilization and *dha* regulon expression which has been supported by our present findings that overexpression of these genes regulate the production of propanediol and hydrogen. Results presented in Tables 2 and 3 show slight increase in biomass of the recombinant strains as compared to the wild type. This observation indicated that combined overexpression of *dhaD1K* affected glycerol uptake rate through the oxidative pathway of *dha* regulon. Increased glycerol uptake regulated energy utilization by the cell thereby increasing the cell growth. On the other hand, a marginal increment in biomass content further ascertains that major fraction of carbon is utilized in the formation of cell metabolites rather than cell growth, which resulted in increase in yield of acetate, butanol, 1,3-PDO and hydrogen. Stoichiometrically, one mole of glycerol

produces 1 mole of acetic acid and one mole of carbon dioxide with 3 moles of hydrogen. From Tables 2 and 3, it can be concluded that 0.65 M initial glycerol yielded only 0.076 M CO_2 and 0.068 M of acetate (in $hydA^{++}$) which is only 10% of the initial glycerol. This implies that most of the carbon is diverted to some other products involved in other pathways like TCA cycle or pentose phosphate pathway. This paves the way for further research on 13C flux analysis to determine all the metabolites and pool size of each intermediate which would further help in channeling the carbon flux solely towards hydrogen and other desired products.

In this study, the protein expression of the recombinant strains was evaluated by SDS-PAGE analysis and was compared with the wild type strain with empty Q5_pMTL_82251_GL plasmid. As described in section 3.1, the putative hydrogenase gene *hydA* (CLPA_c00280), encodes for a protein of around 64 kDa (Gorwa et al., 1996). It could be inferred from the SDS-PAGE gel that a protein of 64 kDa was highly expressed in the recombinant strain $hydA^{++}$ which corresponds to hydrogenase gene. Similarly a protein of approx. 42 kDa is expressed in the recombinant strain $dhaD1K^{++}$ which corresponds to glycerol dehydrogenase (Kohl et al., 1996; Yamada-Onodera et al., 2006). The expression of these genes could be further studied by using RT-PCR but due to limitations of instrumentation in our lab we were not able to use the technique. Hence, we have performed SDS-PAGE analysis to study the expression of the genes at protein level. Similar approaches have also been adopted by other authors (Khanna et al., 2011; Zhao et al., 2010).

Further the enzyme activities of the over-expressed proteins were determined by hydrogenase and glycerol dehydrogenase assay. The maximum specific hydrogenase and glycerol dehydrogenase activity obtained was 0.58±0.41 µmol $H_2.min^{-1}$(mg protein)$^{-1}$ and 4.6±0.32 U.(mg protein)$^{-1}$ respectively for $hydA^{++}$ and $dhaD1K^{++}$ recombinant strains which was found to be around 3 fold higher than that of the wild strain. The bar chart depicting the activities is provided in supplementary material. Higher activities of these key enzymes suggested that hydrogen production was accelerated by increased oxidation of ferredoxin by

hydrogenase and increased uptake of glycerol by glycerol dehydrogenase. Although the recombinant strains in this study could not produce the theoretical maximum hydrogen i.e. 3 mole per mole of glycerol, but the yield obtained (1.1 and 0.93 mol H_2/mol glycerol) was higher than several previous studies (Table 1 of Sarma et al., 2016). There is a wide prospect to further increase the yield of biohydrogen from *C. pasteurianum* by the application of other metabolic strategies and use of different modes of fermentation in future studies.

4. CONCLUSIONS

In present study, we have successfully attempted the overexpression of the key genes responsible for hydrogen production from glycerol. To the best of our knowledge, this is the first study for homologous overexpression of these genes (*hydA, dhaD1-dhaK*) in *C. pasteurianum* and its effect on fermentative hydrogen production. It is revealed that *hydA* plays crucial role in enhancing hydrogen yield by simultaneous increase in acetic acid yield and reduction in lactic acid yield. Similarly, overexpression of *dhaD1K* also enhanced glycerol metabolism and hydrogen production, as compared to wild type strain, thereby explaining its importance in the glycerol hydrogen pathway.

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Supplementary material

E-supplementary data of this work can be found in online version of the paper.

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TABLES AND FIGURES

Table 1. Strains and plasmids used in this study

Name	Properties	Reference
C. pasteurianum DSM 525	Wild type strain	DSMZ
C. pasteurianum DSM525-H4	Hypertransformable strain based on DSM 525	Schwarz et al., 2017
E. coli TOP10	Strain used for initial screening of clones	
E. coli TOP10xCR1	Strain harbouring plasmid CR1 with M. BepImethylase	Schwarz et al., 2017
pMTL82251	*E. coli-Clostridium* shuttle vector (pBP1, ermB, ColE1 traJ, MCS, TCpafdx	Heap et al., 2009
Q5_pMTL82251_Pgl	231 bp fragment comprising promoter from glycerol operon of *C. pasteurianum* (Pgl) cloned into the NotI/NdeI recognition sites of pMTL82251	Minton and group.
Q5_pMTL82251_ Pgl _*hydA*	1795 bp fragment comprising of *hydA* gene of *C. pas*teurianum cloned into NdeI/NheI recognition sites of Q5_pMTL82251_Pgl	This study
Q5_pMTL82251_ Pgl _*dhaD-K* :	2936 bp fragment comprising of *dhaD1-dhaK* gene from glycerol operon (oxidative path) of *C. pasteurianum* cloned into NdeI/FspI recognition sites of Q5_pMTL82251_Pgl	This study

Table 2. Oligonucleotides used in this study (Underlined sequences indicate added restrictions sites)

Oligonucleotide	**Sequence 5' to 3'**
hydA_F	GGGAATTCCATATGAAAACAATAATTATAAATGGTGTACAG
hydA_R	CTAGCTAGCGATCAACTTATATTAGCAAGAAATGAACTTTTTGCCAGCATTATGAAAATATTGTTTTTTCATAAGCTAGCTAG
6Q_hydA_F	GAACGGCGCGCGCGTTA
ShydA	GGAAGATGTGTTAATGCCTGTG
S82251_R	CTAAGGATTCAGAACGGCGCGCGCGCCGTTCTGAATCCTTAG
traj-F	GGAGGATTACAACGGCGCT
82XXX-LR	ATCCAGGGTGCTATCTTCG
DhaD1K_Pgs_F	TTGATAAAGGAGGATTACATATGAGAAAAGCATTTATTTGTCCAACTAAATAT
DhaD1K_R	CCATTCAGGCTGCCTACTTTATAACCTCTGAAATCTCTGTGAATATTACAC
9C_DhaKsco-F	ACTTTATAACCTCTGAAATCTC

Table 3. Product and substrate profiles of *hydA*$^{++}$, *dhaD1K*$^{++}$ overexpressed crude glycerol fermentations with wild type (WT) as control in serum bottles.

Carbon source	60 g/L (650 mM) crude glycerol					
Medium	Biebl			BSH		
Strain	*hydA*$^{++}$	*dhaD1K*$^{++}$	WT	*hydA*$^{++}$	*dhaD1K*$^{++}$	WT
Product concentrations [mM]						
Carbon source used[mM]	420.54±5.4	450.11±2.3	390.31±3.1	418.34±2.5	445.66±3.4	350.23±2.6
[a]Lactate	2.51±0.2	3.90±0.1	5.7±0.3	5.45±0.5	5.73±0.2	7.19±0.2
[b]Acetate	68.69±1.1	55.39±0.8	37.68±0.4	64.82±1.1	51.12±0.5	36.12±0.5
[c]Butyrate	0.51±0.0	0.33±0.7	2.10±0.5	11.27±0.8	10.53±0.4	12.10±1.0
[d]1,3-Propanediol	56.70±1.2	105.10±0.8	62.40±0.7	53.21±1.1	86.33±0.2	60.40±0.3
[e]Ethanol	17.6±0.3	17.11±0.6	32.12±1.2	15.61±1.1	10.56±0.4	10.12±0.7
[f]Butanol	88.8±1.5	112.23±1.1	98.83±0.8	44.21±1.4	63.21±1.1	66.43±0.7
[g]CO2	76.21±2.3	76.34±3.2	65.56±2.1	75.45±3.1	75.34±1.8	63.7±4.4
[h]Biomass	38.21±0.3	39.81±0.4	35.32±0.1	37.54±0.3	39.44±0.2	34.12±0.1
Total Carbon[*]	349.23	410.21	339.69	307.56	342.26	290.18

[*]Total Carbon = (a+b+c+d+e+f+g+h)

Table 4. Product and substrate profiles of *hydA*$^{++}$, *dhaD1K*$^{++}$ overexpressed pure glycerol fermentations with wild type (WT) as control in serum bottles.

Carbon source	60g/L (650 mM) pure glycerol					
Medium	Biebl			BSH		
Strain	*hydA*$^{++}$	*dhaD1K*$^{++}$	WT	*hydA*$^{++}$	*dhaD1K*$^{++}$	WT
Product concentrations [mM]						
Carbon source used[mM]	408.91±2.1	440.34±3.2	375.54±1.4	380.91±1.2	410.34±2.5	340.54±3.2
[a]Lactate	2.44±0.3	4.22±0.1	4.58±0.6	4.78±1.1	5.43±1.0	7.32±0.5
[b]Acetate	61.12±0.3	45.21±0.4	31.13±0.4	55.23±0.1	43.21±1.1	28.67±1.0
[c]Butyrate	0.56±0.3	1.56±0.8	1.12±0.3	7.87±0.2	7.63±1.3	8.34±1.1
[d]1,3-Propanediol	45.34±0.8	93.44±0.5	58.06±0.6	26.25±1.3	73.44±0.5	61.28±1.1
[e]Ethanol	22.11±1.0	23.12±0.8	35.40±0.4	10.89±0.5	12.67±1.1	12.23±0.4
[f]Butanol	71.87±0.1	115.13±2.1	106.12±0.2	60.87±0.4	61.12±0.0	58.12±1.2
[g]CO2	74.54±3.2	71.32±2.5	63.34±1.4	71.12±3.5	76.34±2.8	62.21±3.2
[h]Biomass	36.12±0.3	38.67±0.1	33.42±0.7	35.23±0.2	36.53±0.3	32.15±0.2
Total Carbon[*]	314.1	392.67	333.17	272.24	316.37	270.32

[*]Total Carbon = (a+b+c+d+e+f+g+h)

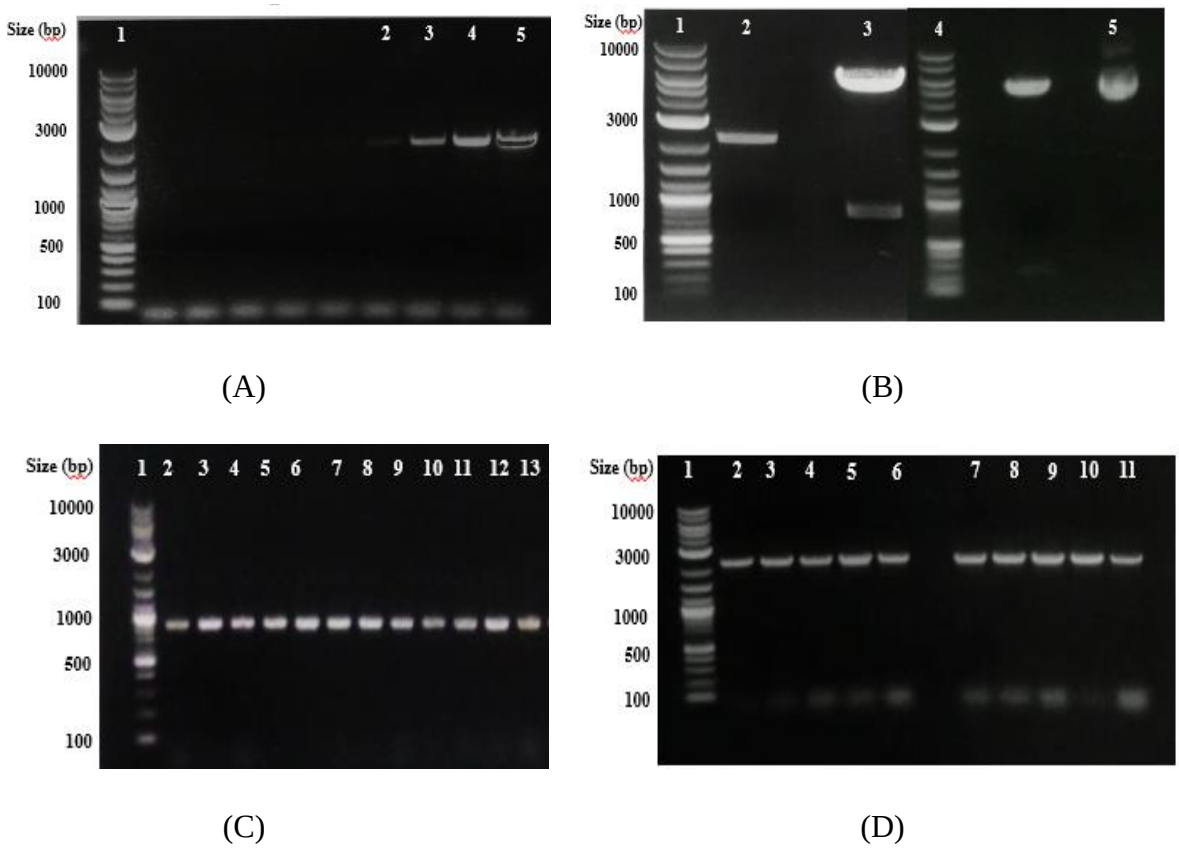


Figure 1. Cloning and overexpression of *hydA* in Q5_pMTL82251_GL vector. (**A**) PCR amplification of *hydA* from *C. pasteurianum* genomic DNA. Lane 1: 2-log DNA ladder, Lane 2-5: Gradient PCR amplified band of *hydA* showing size of around 2100 bp. (**B**) Restriction digestion of insert and plasmid with NdeI and NheI. Lane 1,4: 2-log DNA ladder, Lane 2,3: Digested hydA insert and Q5_pMTL82251_GL vector respectively, Lane 5: Undigested Q5_pMTL82251_GL vector. (**C**) Clone confirmation by colony PCR of *C. pasteurianum* harbouring empty-Q5_pMTL82251_GL plasmid giving a band of 861bp. Lane 1: 2-log DNA ladder; Lanes 2 to 13: PCR product. (**D**) Clone confirmation by colony PCR of *E. coli* and *C. pasteurianum* harbouring*hydA*-Q5_pMTL82251_GL plasmid giving a band of 2385bp. Lane 1: 2-log DNA ladder; Lanes 2-6: PCR product of *hydA* amplified from *E.coli* TOP10, Lanes 7-11: PCR product of *hydA* amplified from *C. pasteurianum* H4.

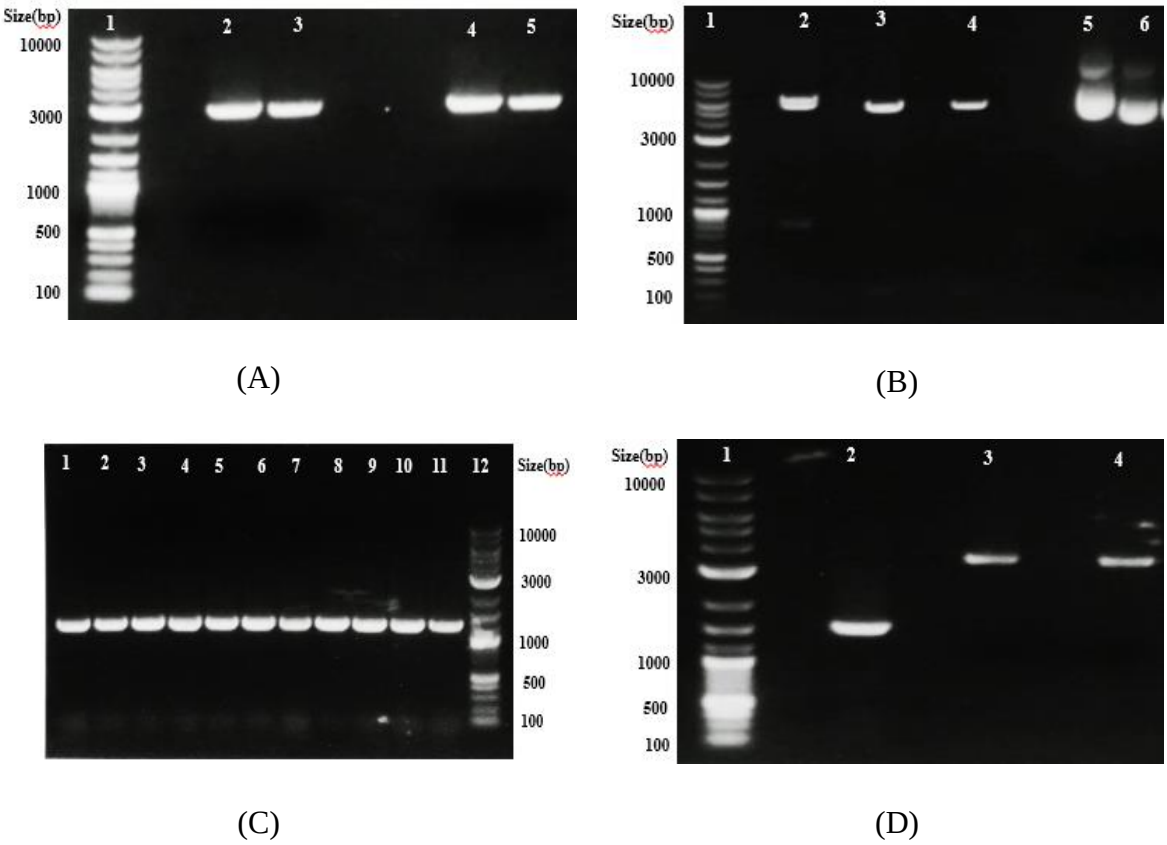


Figure 2. Cloning and overexpression of *dhaD1-dhaK* in Q5_pMTL82251_GL vector. (**A**) PCR amplification of *dhaD1-dhaK* from *C. pasteurianum* genomic DNA. Lane 1: 2-log DNA ladder, Lane 2-5: PCR amplified band of *dhaD1-dhaK* showing size of around 2936 bp. (**B**) Restriction digestion of plasmid with NdeI and FspI. Lane 1: 2-log DNA ladder, Lane 3,4: Digested Q5_pMTL82251_GL vector, Lane 5,6: Undigested Q5_pMTL82251_GL vector. (**C**) Clone confirmation by colony PCR of *E. coli* harbouring *dhaD1-dhaK*_Q5_pMTL82251_GL plasmid with primers 9C_DhaK_sco_ F and 82XXX-LR giving a band of 1272 bp. Lanes 1-11: PCR product, Lane 12: 2-log DNA ladder. (**D**) Clone confirmation by colony PCR of *C. pasteurianum* harbouring *dhaD1-dhaK*_Q5_pMTL82251_GL plasmid giving a band of 3526 bp. Lane 1: 2-log DNA ladder; Lane 2: PCR product with 9C_DhaK_sco_F and 82XXX-LR primers, Lanes 3,4: PCR product 82XXX-LR and traj_F primers.

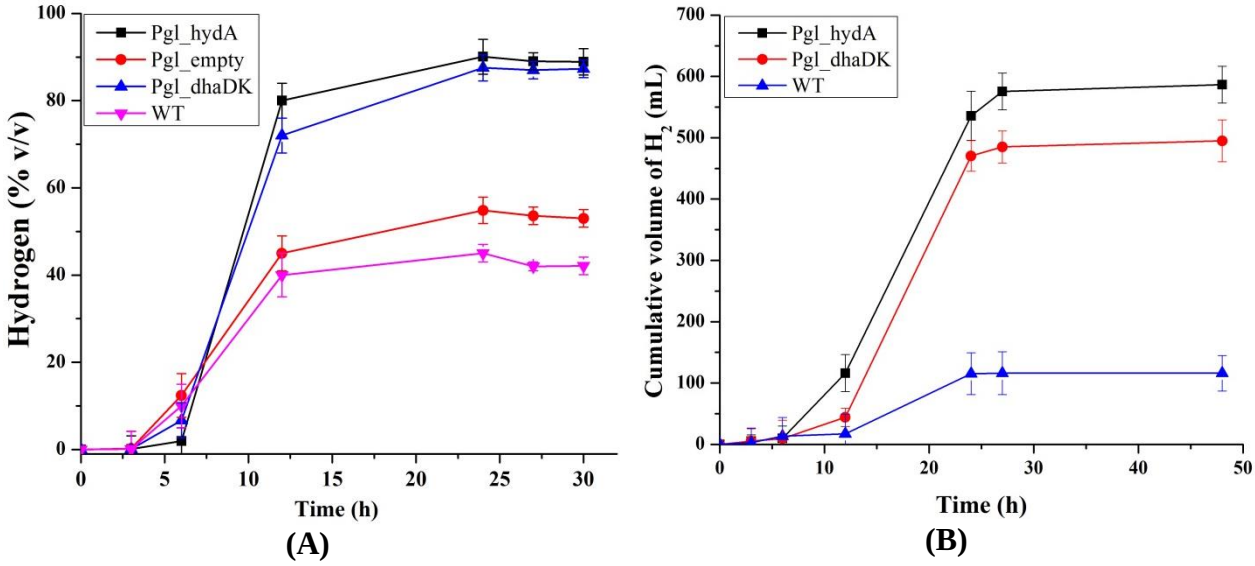
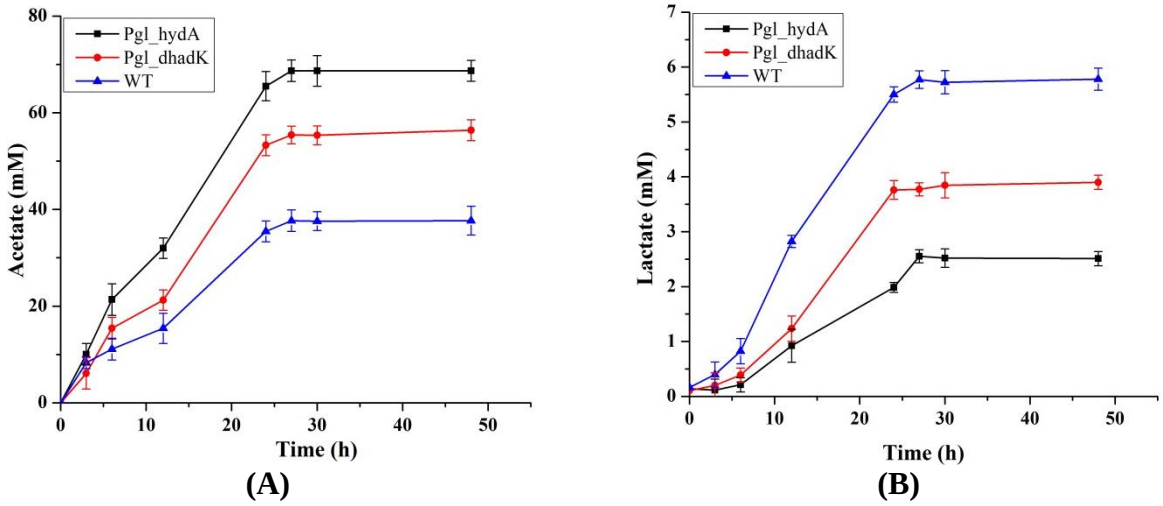


Figure 3. Time profiles of hydrogen production from crude glycerol fermentation by *C. pasteurianum*-H4(WT); *C. pasteurianum*-H4-*hydA* (Pgl_hydA); *C. pasteurianum*-H4-*dhaDK* (Pgl_dhaDK) and *C. pasteurianum*-H4 (Pgl_empty). (A) % H_2 v/v (B) Cumulative volume of H_2



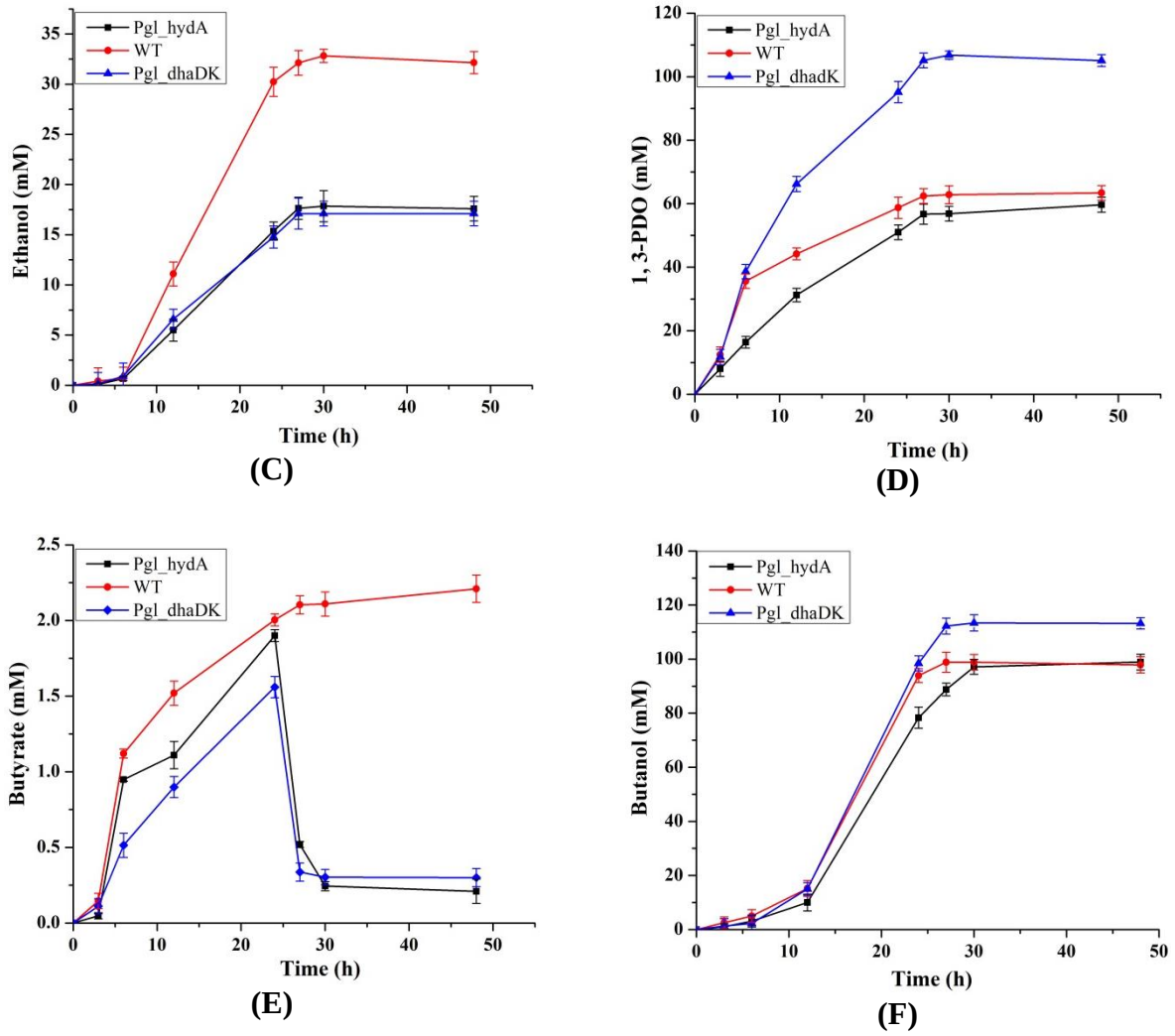


Figure 4. Solvent and acid profiles for crude glycerol fermentations of *C. pasteurianum DSM525-H4*(WT); *C. pasteurianum*-H4-*hydA* (Pgl_hydA); *C. pasteurianum*-H4-*dhaDK* (Pgl_dhaDK); (A) Acetate;(B) Lactate; (C) Ethanol; (D) 1,3-Propanediol; (E) Butyrate; (F) Butanol. (Note: The time profiles of the wild type *C. pasteurianum*-H4 and *C. pasteurianum*-H4 harbouring the empty vector Q5_pMTL82251_GL were similar as seen in Fig. 3A and Fig. 5 and 6 hence only the time profiles of wild type have been included in the fermentation graphs to reduce complexity and for direct comparison with the wild type strain).

SUPPLEMENTARY MATERIAL

Homologous overexpression of Hydrogenase and Glycerol dehydrogenase in *Clostridium pasteurianum* to enhance hydrogen production from crude glycerol

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S1. Supplementary Methods

S1.1 Methylation of plasmid and its transfer into *C. pasteurianum*

For high frequency transformation into *C. pasteurianum* strain, *in vivo* methylation of plasmid DNA was carried out by transformation into *E. coli* Top 10_CR1 (dam+, dcm+), which harbors a plasmid CR1 having a gene encoding the M. BepI methyltransferase of *Brevibacteriumepidermidis* (The activity of the restriction enzyme *Cpa*II (a*Mbo*I/*Dpn*II-type restriction endonuclease) was insignificant as the *E. coli* host (Top10) was dam+. Thus, M. BepI methyltrasferase facilitated *in vivo* methylation of the external cytosine residues (5'-mCGCG-3') of the *Cpa*A II DNA recognition site . After plasmid isolation from *E. coli* host, the methylated plasmids (0.5–5 μg DNA) were electroporated into *C. pasteurianum* (Schwarz et al., 2017).

S1.2 Preparation of electro-competent *C. pasteurianum*

The electro-competent cells of *C. pasteurianum* were prepared with a slightly modified protocol as reported by Pyne et al. (2013). The protocol consists of the following steps: (i) serially diluted (10^{-3}) cultures were grown overnight in 2x YTG medium; (ii) main-culture (100 mL) was inoculated to a final OD_{600} = 0.05; (iii) sucrose and glycine were added to a final concentration of 0.4 M and 1.25% w/v respectively, when the OD_{600} = 0.3-0.4; (iv) culture was further grown to OD_{600} = 0.6-0.8; (v) culture was pelleted down and the pellet was washed with ice-cold sucrose-magnesium-phosphate (SMP) buffer (270 mM sucrose, 1 mM $MgCl_2$, 5 mM NaH_2PO_4, pH 6.5).Thereafter, the pellet was dissolved in 3000 µL SMP buffer, and aliquots were added in vials for five transformations.

S1.3 Electro-transformation into *C. pasteurianum*

Electro-transformation was carried out by addition of 100% ethanol (30µL) and methylated plasmid DNA (5µg)into 580 µL of competent cells in a pre-chilled 4 mm cuvette. The mixture was incubated for 5 min in ice inside the anaerobic chamber, and electroporated immediately with an exponential decay pulse of 1800 V without parallel resistance (Pyne et al., 2013; Schwarz et al., 2017). Transformations were immediately recovered in 10 mL 2x YTG and grown overnight inside the anaerobic chamber followed by plating dilutions on RCM plates with antibiotics.

S1.4 SDS-PAGE analysis

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed with samples diluted 2X SDS-PAGE loading buffer. The samples were boiled for 5 min at 95 ℃, cooled and the loaded onto a 12% (w/v) resolving gel. Electrophoresis was carried out at 100 V for 4 h. Protein concentration was determined by the method of Bradford (1976), using bovine serum albumin as a standard and equal amount of protein was loaded.

S2. Supplementary Figures

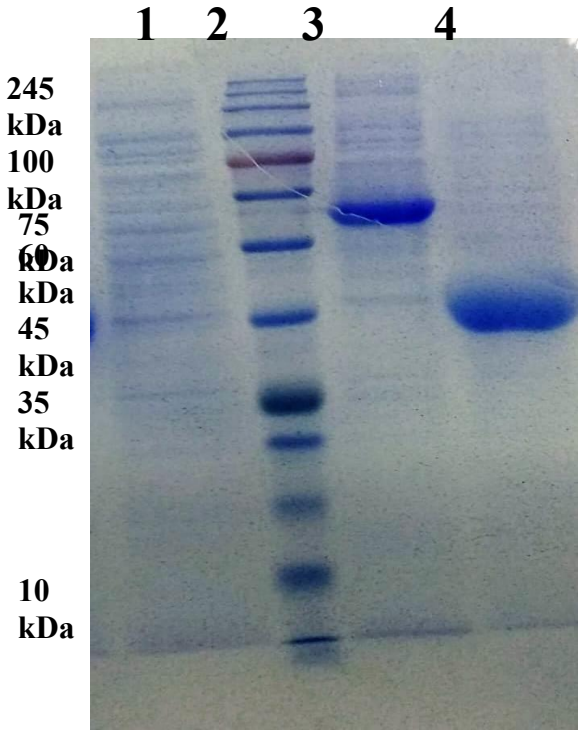
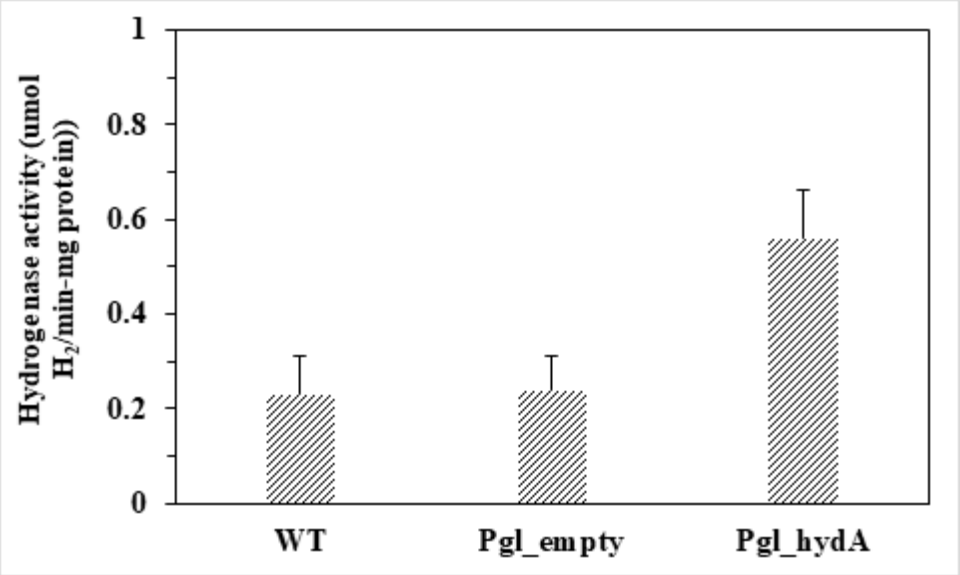
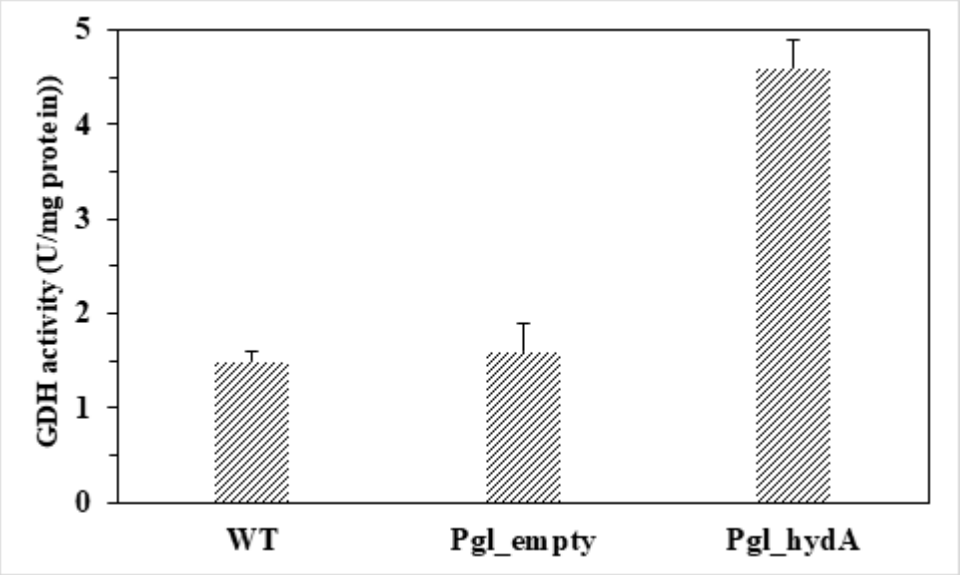


Figure S1. SDS-PAGE of recombinant hydrogenase and glycerol dehydrogenase protein. *Lane-1*: *C. pasteurianum*-H4 strain carrying empty Q5_pMTL_82251_PGL plasmid; *Lane-2*: mol. Wt. marker (Thermo); *Lane-3*: *C. pasteurianum*-H4 strain carrying Q5_pMTL_82251_PGL/*hydA* plasmid; *Lane-4*: *C. pasteurianum*-H4 strain carrying Q5_pMTL_82251_PGL/*dhaD1K* plasmid



(A)



(B)

Figure S2. (A) Comparison of hydrogenase activity assay. (WT: Wild type *C. pasteurianum*-H4; Pgl_empty: *C. pasteurianum*-H4 strain carrying empty Q5_pMTL_82251_PGL plasmid; Pgl_hydA: *C. pasteurianum*-H4 strain carrying Q5_pMTL_82251_PGL/*hydA* plasmid). **(B)** Comparison of glycerol dehydrogenase assay. (WT: Wild type *C. pasteurianum*-H4; Pgl_empty: *C. pasteurianum*-H4 strain carrying empty Q5_pMTL_82251_PGL plasmid; Pgl_dhaDK: *C. pasteurianum*-H4 strain carrying Q5_pMTL_82251_PGL/*dhaD1K* plasmid)

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[1] Pyne, M.E., Moo-Young, M., Chung, D.A., Chou, C.P., 2013. Development of an electro-transformation protocol for genetic manipulation of *Clostridium pasteurianum*. Biotechnol. Biofuels. 6:50.10.1186/1754–6834–6–50.

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[3] Tamiya, N., Koxdo, Y., Kameyama, T., Akabori, S., 1955. Determination of hydrogenase by the hydrogen evolution from reduced methylviologen. The J Biochem. 42, 613–614.