ENHANCED BIOHYDROGEN PRODUCTION BY OVEREXPRESSION OF HYCE AND HYCG IN ENTEROBACTER AEROGENES AB91102

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ABSTRACT

Metabolic engineering is considered as one of the key methodology for improving hydrogen yield from dark fermentation. In this study, two strains with over-expression of hydrogenase subunits, Enterobacter aerogenes AB91102/hycE and E. aerogenes AB91102/hycG by manipulation of hycE and hycG genes in the expression vector pET28-Pkan, were developed to further improve hydrogen (H_2) production from this strain. Optimization of anaerobic batch fermentations is achieved and the yield of 0.988 and 0.959 mol-H_2/mol-glucose by hycE and hycG over-expressed strains was respectively obtained, which was only 0.850 mol-H_2/mol-glucose in the control strain harboring empty plasmid, in the range of pH 6.0-6.5 at 33-37 °C. Metabolic flux analysis was also investigated by measuring H_2, glucose consumption and other metabolites including formate, succinate, 2,3 butanediol, lactate, acetate and ethanol. The results demonstrate that over-expression of large and small subunits of hyc3 operon may significantly enhance hydrogen production from dark fermentation by increasing the catabolism of formate and activity of hydrogenase. We describes the use of metabolic engineering strategies to overcome the limitations, increase yields and substrate utilization.

KEYWORDS

hycE, hycG, Biohydrogen, E. aerogenes, over-expression.

1. INTRODUCTION

The world is tackled with thoughtful environmental problems, many of them related directly or indirectly to excessive utilization of fossil fuel. Renewable energy is derived from sources that are constantly replaced by natural processes such as wind, water, geothermal, and solar. The use of hydrogen as a fuel has been of great interest since its combustion yields only heat and water, although shift to H_2 as a fuel would also require a new association. Hydrogen provides more energy per unit mass than all other combustible energy sources [1].

Numerous efforts have been made to improve the hydrogen molar yield from dark-fermentation. For instance, Deleting the enzymes that consume hydrogen [2], alteration in metabolic pathways to redirect energy flow [3], changing the metabolic pathways [4], heterogeneous over-expression of subunits of hydrogenase [5] and over-expression of hydrogenase or activity subunit have been shown to improve hydrogen production [6]. The key enzyme involved in the metabolism of H_2 is hydrogenase. This enzyme catalyzes the simplest chemical reaction: $\boldsymbol{2H^+ + 2e^- \leftrightarrow H_2}$. The reaction is reversible, and its direction depends on the redox potential of the components able to interact with the enzyme.

Of all the fermentative hydrogen producers, *Enterobacter* spp. has attracted much, *Enterobacter* spp. studied for hydrogen production include *E. asburiae*[7], *E. cloacae*[8], *E. aerogenes*[9, 10], etc. High growth and production rate makes E. aerogenes a strong candidate for large-scale hydrogen production [11].

Hydrogenase evolves molecular hydrogen under the collaboration of several subunits, among whom the principal activity subunits are the large and small subunits encoded by *hycE* and *hycG*, respectively [2]. The large subunit of hydrogenase 3, *hycE* has been subjected to random protein engineering for enhanced hydrogen production by error-prone polymerase chain reaction (epPCR) and DNA shuffling, and showed eight folds higher yield as compared with the wild type [12].

Therefore, in this study, two strains with over-expression of small and large subunits of hydrogenase, *E. aerogenes* AB91102/hycE (AB-E) and *E. aerogenes* AB91102/hycG (AB-G), were constructed to enhance hydrogen production from this strain. The distribution of carbon sources was further analyzed under batch fermentation, specific enzymatic activities were also monitored to study the effects and mechanism of these gene manipulations on H_2 production.

2. MATERIALS AND METHODS

2.1 Strains, vectors and culture media

In the following experiments, the microorganism used for enhancement of bio-hydrogen production was *Enterobacter aerogenes* AB91102. It was purchased from the China Center for Type Culture Collection in Wuhan University, Wuhan, P.R. China. Composition of the fermentation medium for the hydrogen production (W/V %) was as:Glucose, 1.5g; tryptone, 0.5 g; yeast extract, 0.2g; NaCl, 0.2g; K_2HPO_4, 0.15g; $MgCl_2{\cdot}6H_2O$, 0.06g; $FeSO_4{\cdot}7H_2O$, 0.02g, and in addition 1% trace elements were also added containing (g/L) $ZnSO_4{\cdot}7H_2O$, 0.06 g; $AlK(SO_4)_2$, 0.01 g; $N(CH_2COOH)_3$, 0.03g;H_3BO_3, 0.02 g; $CaCl_2$, 0.02 g; Na_2MoO_4, 0.02 g; $MnSO_4{\cdot}7H_2O$, 0.03 g; $CoCl_2$, 0.1g.

Luria-Bertani medium (LB), was used for screening of the preferred organisms containing (%): yeast extract, 0.5 g; tryptone, 1 g; and NaCl, 1 g. Desired transformants and recombinants were selected by adding Kanamycin (50μg/mL). The strains and plasmids used in this study are listed in **Table 1**, while the primers for PCR amplification are given in **Table 2**.

2.2 Overexpression of hycE and hycG

The plasmid pET-28kan was modified to express homologous protein in *E. aerogenes* AB91102, using the previously studied methods with some modifications [13, 14]. The promoter T7 was replaced by Pkan which was essential for the replication of kanamycin resistant gene in the plasmid pET28a. The gene *hycE* was manipulated into plasmid pET-28kan as per following procedure. The gene *hycE* was cloned by PCR using the genomic DNA of *E. aerogenes* AB91102 (wild type) with primers hycE-Fw and hycE-Rv. Reaction mixture of PCR comprised of, 1 μL of 10 μmol primers mixed with 2 μL of 10 μmol purified genomic DNA, 25 μL 2x Es Taq Master Mix and sterile ddH_2O to finally produce a 50 μL PCR reaction mixture. The PCR conditions were 94 °C for 10 min, followed by 30 cycles of 94 °C for 45 sec, 60 °C for 30 sec and 72 °C for 1.5 min with a final step of 72 °C for 10 min (**Table 3**). The *NotI/XhoI* fragment of the *hycE* gene from the chromosome was ligated into the *Not*I and Xho*I* sites of vector pET-28kan to produce pET-28kan-*hycE*, andthe recombinant plasmid was confirmed by double digestion with *NotI* and *XhoI*. Composition of ligation reaction is listed in **Table 4**.

The same procedures were followed for the cloning of the gene fragment of hycG, accept for the PCR conditions, where temperature for annealing was set to 58 ℃ and the time for amplification at 72 ℃ was set to 45 seconds.

Table 1 Strains and plasmids used in this study

Strains and plasmids	**Genotype and relevant characteristics**	**References**
Strains		
E. aerogenes CCTCC AB91102	Wildtype	[10]
AB-C	Control, carrying plasmid pET-28Pkan Kan^r	This study
AB-E	*E. aerogenes* with *hycE* Kan^r	This study
AB-G	*E. aerogenes* with *hycG* Kan^r	This study
Plasmids		
pET-28(+)	Expression plasmid	EMD Biosciences
pET-28kan	Expression plasmid Kan^r	[13]
pET-28kan-*hycE*	Expression plasmid of $hycEKan^r$	This study
pET-28kan-*hycG*	Expression plasmid of $hycGKan^r$	This study

Table 2 Primer sequences used in this study

Primers	**Sequence***	**Reference**
hycE-Fw	TTGCGGCCGCATGTCTGAAGAAAAAATCGGTCA	This study
hycE-Rv	CCCTCGAGTTTCAGCGGCGAGTT	This study
hycG-Fw	TTGCGGCCGCATGAGCAACTTACTAGGCCC	This study
hycG-Rv	CCCTCGAGTCGGATACGGCGCC	This study
Pkan-fw	GCGAGATCTGTATCTCAGTTCGGTGTAGG	[13]
Pkan-rv	GCGGAATTCAACACCCCTTGTATTACTG	[13]

* DNA sequences underlined are the restriction sites, NotI is used in forward and XhoI in reverse primers for both the gene fragments *hycE* and *hycG*.

Table 3 Summary of PCR reagents and PCR conditions

PCR reagents		**PCR Conditions**		
2x Es Taq Master Mix	25 μL	94 °C	5 min	1 cycle
hycE-Fw (10 μM)	1 μL	94 °C	30 sec	30 cycles
hycE-Rv (10 μM)	1 μL	60 °C	30 sec	
E. aerogenes genomic DNA	1 μL	72 °C	90 sec	
ddH_2O	22 μL	72 °C	10 min	1 cycle
Total volume	50 μL	4 °C	Hold	1 cycle

The *hycE* and *hycG* gene fragments were placed under the control of *Pkan*. The plasmids pET-28kan-*hycE* and pET-28kan-*hycG* were primarily transformed into *E. coli* DH5-α one by one for amplification and the mutant strains, *E. coli*/pET28kan-*hycE* and *E. coli*/pET28kan-*hycG* were selected by addition of 50 μg/mL kanamycin in LB media. Then, amplified plasmids pET-28kan-*hycE* and pET-28kan-*hycG* were electro-transformed into *E. aerogenes* separately.Electric pulse was performed on BIORAD Gene Pulser Xcell, under the following parameters: Voltage 2500v, Capacitance 25μF, Resistance 200Ω, and 2mm cuvette was used to construct *E. aerogenes* AB91102/pET-28Pkan-*hycE* (AB-E) and *E. aerogenes*/pET-28Pkan-*hycG* (AB-G). By using the

same approach, empty plasmid without insertion of any gene was also transformed into *E. aerogenes* for the construction of *E. aerogenes* AB91102/pET-28Pkan (AB-C) used as a control.**Fig. 1** shows the steps of plasmid construction.

Table 4 Ligation conditions of the gene of interest with its corresponding plasmid. This reaction was achieved by incubation of the following mixture at 16 °C for 12 hours.

Ligation reaction	
Sol I	5.0 µL
Gene (hycE / hycG)	4.5 µL
Plasmid (pET28-Pkan)	0.5 µL
Total volume	10 µL

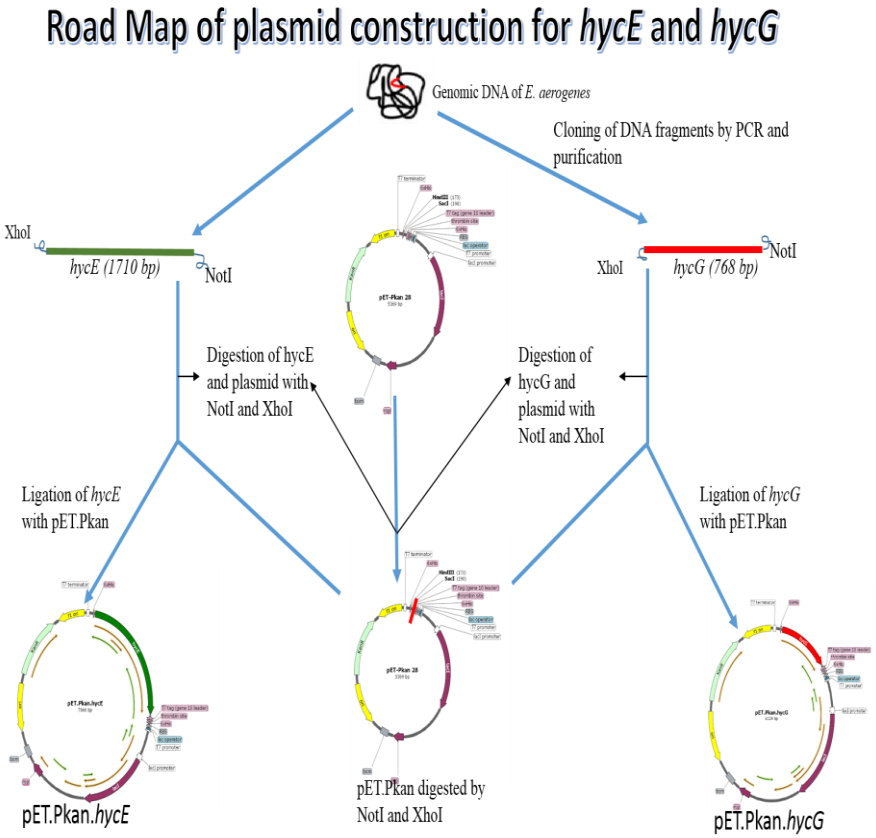

Fig. 1 flow diagram of plasmid construction for homologous over-expression of *hycE* and *hycG* to get pET-Pkan/*hycE* and pET-Pkan/*hycG* by insertion of gene fragments cloned from genomic DNA of *E. aerogenes* into pET28-Pkan.

2.3 Anaerobic batch fermentation experiments

150 mL flat bottomed flasks were used for anaerobic batch fermentation experiments. Each flask was filled with 70 ml fermentation medium and 10% (v/v) LB overnight seeding culture was added as an inoculum to start the fermentation. The headspace air and trace amounts of oxygen in the fermentation medium were displaced by nitrogen gas to ensure anaerobic conditions, and the batch fermentations were carried out in a magnetic stirring thermostatic water bath under shaking for 14 h at 37 °C.

2.4 SDS-PAGE analysis

The recombinant strains AB-E, AB-G and control AB-C were cultured overnight in 20 mL seed LB medium containing kanamycin in an anaerobic environment. Harvested bacteria were all inoculated into 1 L of LB medium without antibiotics at 16 °C, when OD600 reached between 0.5 to 0.6 nm, 0.4 mmol/L Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added into engineered and control strains to induce protein expression. After 20 hours shaking culture, the cells were centrifuged at 12,000×g under 4 °C for 10 minutes and lysed in a high-pressure cell disruption system (One Shot Model, Constant systems, UK). The lysates were centrifuged at 12,000×g for 10 minutes at 4 °C and the cell free extracts were separated by 10% SDS-PAGE.

2.5 Measurement of Gas Composition

The total volume of the gas produced by the anaerobic shaking flask culture was measured via measuring cylinders and the total gas was collected by water displacement method. The composition of the gas was measured by using a gas chromatography (FULI GC-9750, China) with a packed column (2.0 mml, D. 200 mml, carboxen-1004, 80-100 mesh) and a TCD detector. The working temperatures of the detector and the column were 60 °C and 110 °C, respectively. The concentration of hydrogen is calculated by the formula derived below:

$$Y = 7 \times 10^{6}x + 352155$$

Volume of hydrogen gas = $V(H_2)$ = V(gas) x density

Concentration of H_2 = n = $\frac{V(H2)}{Vm}$

Where; **Y** is the average peak area, in this study taken as average of triplicate readings
x is the density of hydrogen in term of percentage (%)
V(gas) is the total volume of gas collected at the end of fermentation in mL
$V(H_2)$ is the volume of hydrogen gas in mL
Vm is a constant value equals to 22.4
nis the concentration of hydrogen gas in mol

2.6 Metabolites Analyses

The analyses of glucose, formate, lactate, succinate, 2,3-butanediol, acetate and ethanol were performed using a high performance liquid chromatography (HPLC) (SSI Model 2300-525, USA) equipped with a sulfonated polystyrene divinyl benzene column (BioRad Aminex HPX-87H, USA) and a refractive index detector. Sulfuric acid of 10 mmol/L was used as a mobile phase. The column temperature was 55 °C and the flow rate was 0.5 mL/min.

3. RESULTS AND DISCUSSIONS

3.1 Respective over expression of hycE and hycG in Enterobacter aerogenes AB91102

In this approach, the *hycE* and *hycG* genes from *E. aerogenes* AB91102 were cloned in the expression vector pET-28kan individually under the control of Pkan promotor. The modified plasmids were confirmed by PCR and *NotI/XhoI* double digestion, and then transformed into *E. aerogenes* AB91102 by electroporation. SDS-PAGE analysis showed there were marked bands with similar molecular masses respectively corresponding to the large and small subunits of the hyc operon, encoded by *hycE* and *hycG*, respectively in the mutant strains. Furthermore, SDS PAGE analyses for the cell extracts of the IPTG induced overexpressed single gene revealed the existence of an abundant protein (molecular weight: approximately 65 kDa) in AB-E and another

abundant protein (molecular weight: approximately 28 kDa) in AB-G [15], while these proteins were not found in large excess in IPTG induced AB-C which was used as a control **(Fig. 2).** The results of SDS PAGE run after induction of mutants and wild type cells with IPTG showed that *hycE* and *hycG* in AB-E and AB-G were highly over-expressed, while both these proteins were not found in AB-C in high amounts.

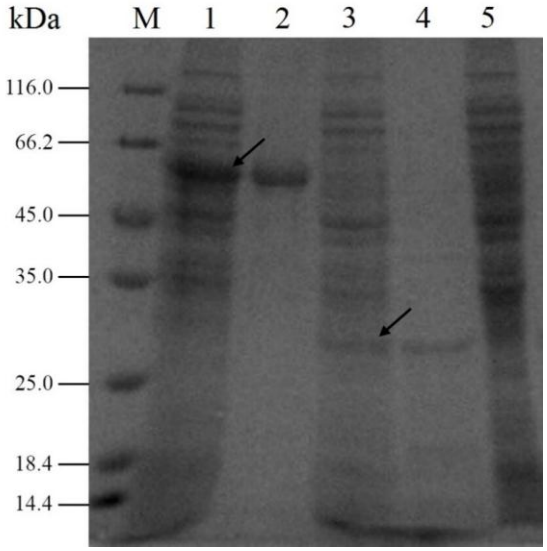

Fig. 2 SDS PAGE analysis after over-expression of hycE and hycG genes in E. aerogenes AB91102. Lane M, Molecular weight marker for proteins; Lane 1, cell free extract of AB-E; Lane 2, purified *hycE* protein; Lane 3, cell free extract of *hycG*; Lane 4, hycG protein purified from *hycG* over-expressed strain AB-G. All strains including control strain were induced by IPTG for over-expression of the desired proteins.

3.2 Improvement of specific hydrogen production rate by genetic modifications

In order to improve volumetric biohydrogen production, the *hycE*overexpressed strain (AB-E) and the *hycG*overexpressed strain (AB-G) were successfully constructed with the purpose of enhanced expression of hyc enzymes to facilitate hydrogen producing pathways. Strain AB-E showed the maximum H_2 yield of 0.988 mol/mol-glucose as compared to the control strain AB-C which was only 0.850 mol/mol-glucose (see **Table 5**). In contrast, strain AB-G yielded 0.959, which was 16% and 13% respectively more than that of the control strain AB-C **(Fig. 3).**

H_2 is usually produced from formate hydrogen lyase, while acetyle-CoA plays vital role in the production of ethanol and acetate. Formate seems a signature molecule in fermenting *E. aerogenes* cells and features that regulate formate metabolism controlled by hydrogenase for H_2 production. Thus, the difference in H_2 yields of the mutant strains (**Fig. 3**) can be explained by their metabolic flux distribution (**Table 5**). Most of the hydrogenases consist of several genes, in *E. aerogenes* native hydrogenase 3 is encoded by an operon of 8 kb in size termed as *hycABCDEFGHI* (*hyc* operon). It is difficult to clone such a large operon by PCR. So as to enhance the hydrogenase activity, only the large subunit of Hyd 3, namely, *HycE*, and a small subunit *hycG* was over-expressed, leading to enhance the hydrogen production. In addition to the *hycE* gene, all the genes in the *hyc* operon, excluding *hycA* which is a repressor of FHL complex, provide full Hyd 3 activity[16]. *HycG* which encodes small subunit of Hyd 3, plays vital role in the electron transport within the FHL complex.

The over expression of large and small subunits of *hycABCDEFGHI* operon, namely *hycE* and *hycG*, respectively in *E. aerogenes* genome increased hydrogen production. Simultaneously, formate excretion was decreased (**Table 5**). The consumption of glucose was increased as a result of overexpression of hydrogenase subunits. Finally, growth rate and cell metabolism were aided together with improved hydrogen production.

Table 5 Metabolite concentrations of *E. aerogenes* AB91102 (wild strain), AB-C (control), AB-E and AB-G (mutants) after anaerobic chemostat cultivation (n=3)

	AB91102	**AB-C**	**AB-E**	**AB-G**
Glucose consumed (mmol/L)	51.7±2.32	51.3±2.24	55.48±2.88	54.85±2.61
Hydrogen conc. (mmol/L)	45.28±2.26	43.64±2.25	54.83±3.12	52.61±2.89
H_2 via FHL pathway (mmol/L)	36.37±1.62	35.82±1.58	43.19±2.1	41.67±2.12
H_2 via NADH pathway (mmol/L)	8.61±0.85	7.82±0.91	11.64±1.2	10.94±0.81
H_2 yield (mol H_2/mol-glucose)	0.875±0.05	0.850± 0.05	0.988±0.06	0.959±0.06
Succinate (mmol/L)	15.615±0.82	15.55±0.53	16.85±0.48	17.34±0.31
Formate (mmol/L)	5.49±0.22	5.58±0.23	0.85±0.15	0.94±0.16
2,3-butanediol (mmol/L)	7.88±0.38	7.74±0.37	7.98±0.38	8.48±0.42
Lactate (mmol/L)	27.921±1.51	27.66±1.51	30.55±1.32	31.83±1.36
Ethanol (mmol/L)	21.81±0.82	21.12±0.81	25.89±1.1	23.95±0.96
Acetate (mmol/L)	20.35±1.01	19.58±1.01	23.08±1.44	21.96±1.45
Et/Ac ratio	1.07	1.07	1.12	1.09

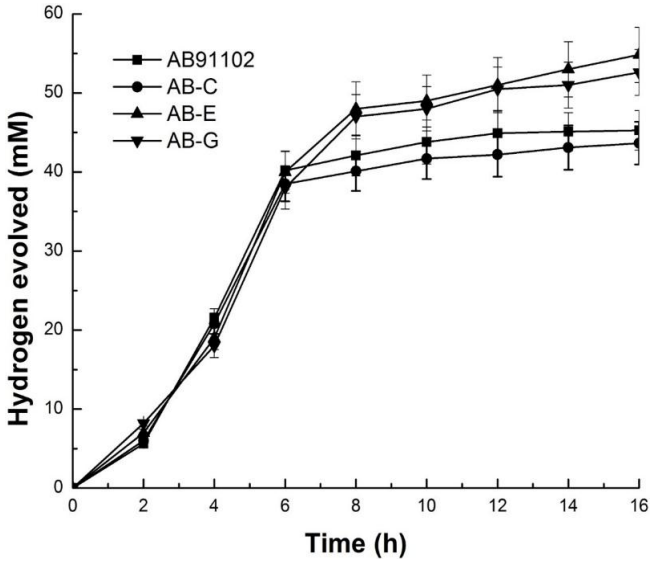

Fig. 3 Comparison of time courses of hydrogen evolved among the wild strain AB91102, control strain AB-C and mutants AB-E and AB-G in batch experiments (*n*=3).

3.3 Effects of pH and temperature on H_2productionactivity

Effects of pH were studied in the range 5.0 - 8.0 at 37 °C initially 15g/L glucose was used in the fermentation medium as a substrate. Optimum activity was revealed by all mutant and wild type strains in the pH range 6.0 - 6.5. The effects of temperature were observed in the range 25 – 40 °C at constant pH: 6.5. The maximum hydrogen molar yield (mol of H_2 /mol-glucose) obtained at

37 °C and pH 6.5 was respectively 0.988 and 0.959 for the mutant strains AB-E and AB-G respectively in comparison with AB-C which was only 0.850 mol H_2/mol-glucose.

3.4 Anaerobic Batch Fermentation

To study the change in hydrogen production rate and yield, metabolic flux and carbon balance, batch fermentation experiments were implemented anaerobically with the strains AB-C as a control, and genetically engineered strains AB-E and AB-G in 150 ml flat bottomed flasks after removal of oxygen and head space air by N_2 rehabilitation independently. Utilization of glucose, growth rate and the concentrations of important metabolites produced after anaerobic batch fermentation, including formate, succinate, 2,3 butanediol, lactate, acetate, ethanol, Et/Ac ratio and hydrogen were also investigated (**Fig. 4**). H_2 is usually produced from formate hydrogen lyase system in Enterobacteriaceae, while acetyl-CoA plays vital role in the production of ethanol and acetate. Formate shows a signature molecule in fermenting E. aerogenes AB91102 cells and features that regulate formate metabolism controlled by hydrogenase and subsequently increase H_2 production [17]. Increase in the production of ethanol and acetate is essential to increase the H_2 yield if hydrogenase is hyper-activated to break the formate into H_2 and CO_2[18, 19]. Thus, the difference in H_2 yields of the mutant strains (**Fig. 3**) can be explained by their metabolic flux distribution (**Table 5**).

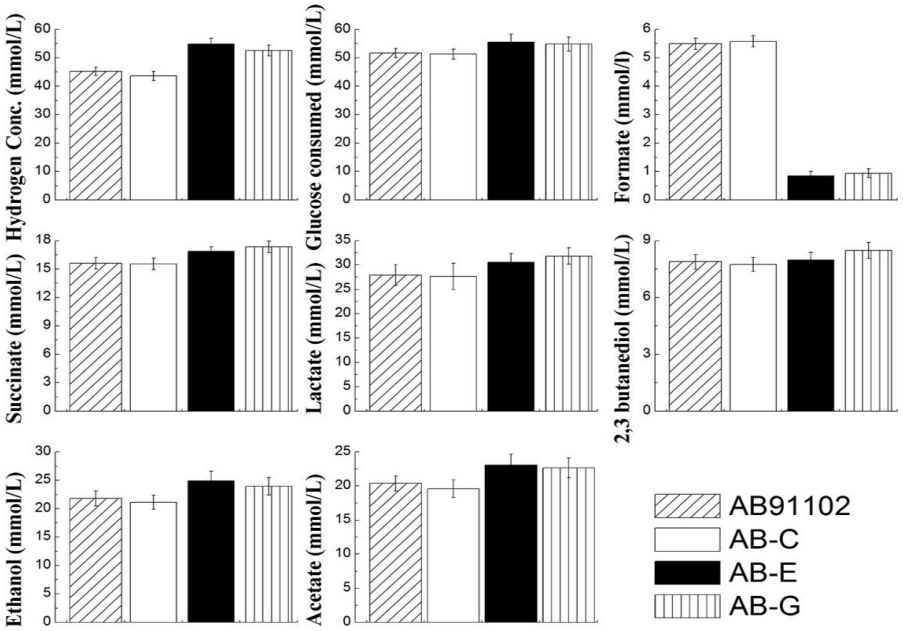

Fig. 4 Results of anaerobic batch fermentation; the metabolic products were analyzed after 16 hours cultivation and the results shown were the average of triplicate experiments.

Hydrogen production by *E. aerogenes* has traditionally been thought to occur mainly through the formate hydrogen lyase (FHL) metabolic pathway, however, in recent studies it has been evidenced that an alternative pathway for hydrogen production also exists in these bacteria, which is NADH pathway [15]. Measurements of hydrogen from both these pathways, formate hydrogen lyase (FHL) and NADH were also performed. In the control strain AB-C, 35.82 mmol/L of hydrogen was produced (see**Fig. 5**),among which, 82% was produced through FHL pathway and the rest by NADH pathway. AB-E evolved 54.83 mmol/L of hydrogen, out of which 79% was produced through FHL pathway. AB-G produced 52.61 mmol/L of H_2, where the proportion of FHL and NADH was approximately 4:1. **(Fig. 5)**.

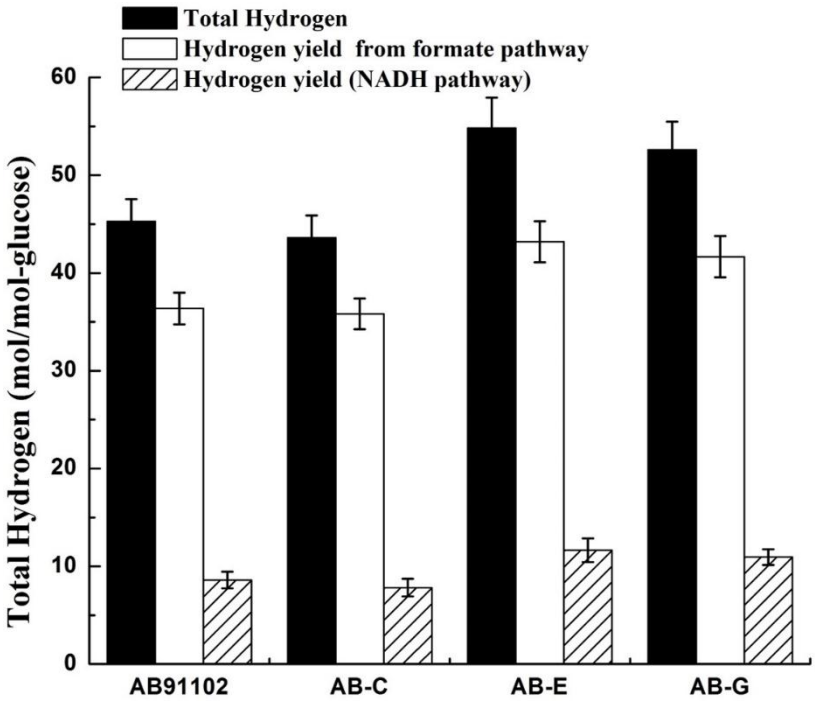

Fig. 5 Hydrogen yield from glucose via formate pathway and NADH pathway by AB91102 wild strain, AB-C, AB-E and AB-Gin batch experiments (n=3).

3.5 Redirection of metabolic flux

Overexpression of *hycE* and *hycG* led to a significant change in the hydrogen yield per mole of glucose consumed, which were increased by 16% and 13% in AB-E and AB-G, respectively, Two-way ANOVA in Origin Pro8 was performed at 0.05 level, the means of hydrogen production yields from all mutants when compared with the mean HMY from the control strain were found to be significantly different.Moreover, increase in succinic acid (8% and 11%), lactate (10% and 15%) and ethanol (18% and 13%) in AB-E and AB-G were also observed. In contrast, highly significant decrease in the formate concentration (85% and 83%) were respectively detected in AB-E and AB-G, as compared with the control strain AB-C (Table 5). Additionally, the glucose uptake was improved by 8% & 7%, and the cell densities were also improved by 9% and 6% in AB-E and AB-G, respectively.

The mechanism for the improved hydrogen production by the wild and mutant strains was investigated through metabolic flux analysis. It was observed that most of the formate was converted into H_2 and CO_2 during anaerobic batch fermentation by mutants of E. aerogenes AB91102 (shown in **Table 5**). Hydrogen production from formate pathway can be calculated as: *Ethanol + acetate = H_2 (FHL) + Formate*. The hydrogen through NADH pathway was calculated by the following equation:

Hydrogen from NADH = total hydrogen - hydrogen from FHL.

Hence, we can positively correlate the hydrogen yield from formate pathway with the production of ethanol and in contrast with over stimulation of hydrogenase, which plays positive role in the consumption of formic acid, which is the key molecule for the metabolism of hydrogen production from formate pathway (**Fig. 5**).Over activation of hyc operon results in strengthened metabolic flux from formate to hydrogen, rapid consumption of formate should lead to increasing activation of PFL that catalyses pyruvate degradation to formate and accumulate more acetate and ethanol to facilitate hydrogen production from formate pathway.

3.6 NADH dependent Specific enzyme activities

NADH dependent specific enzyme activities of lactate dehydrogenase (LDH) of AB-E and AB-G was increased by 31 and 29%, respectively (see**Fig. 6**). At the same time, NADH mediated hydrogenase activity was sharply decreased up to 90% and 86% from AB-E and AB-G, respectively, as compared with the control strain. On the other hand, NADH dependent specific enzymes like lactate dehydrogenase (LDH) and 2,3-butanediol dehydrogenase (BDDH) showed no any remarkable change in their activity. There accompanied with a significant decrease in the concentration of formate as low as 0.85 mmol/L and 0.94 mmol/L in AB-E and AB-G respectively, as compared with the control strain AB-C which was 5.58 mmol/L. This change in the pattern of carbon balance and enzymatic activities lead to an increase in the total hydrogen yield of 16% (0.988 mol-H_2/mol-glucose) and 13% (0.959 mol-H_2/mol-glucose), the H_2 yield was mainly increased by formate pathway, and partly through NADH pathway as compared with the wild strain (see **Fig. 5**). Over-expression of *hycE* and *hycG* in AB-E and AB-G showed uppermost alcohol dehydrogenase (ADH) activity [**Fig. 6(C)]**. ADH contributes in metabolism of a wide variety of alcohols and aldehydes and initializes the first step of alcoholic oxidation using NAD^+ as a cofactor and producing NADH and the corresponding carbonyl compounds. Various studies have suggested that ADH enzyme activity is a major rate-limiting factor in ethanol metabolism [20].

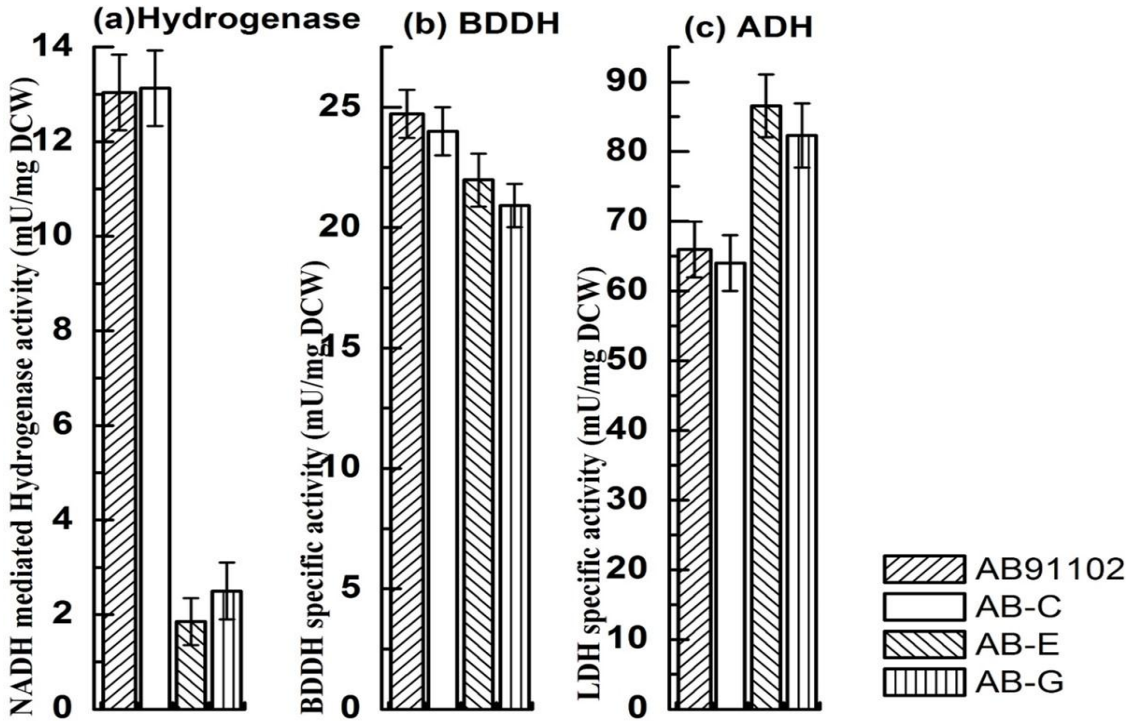

Fig. 6 Comparison of NADH dependent specific enzyme activities; (a) NADH mediated Hydrogenase (b) BDDH and (c) ADH, among wild strain AB91102, control strain AB-C, mutants AB-E, and AB-G.

4. CONCLUSION

In conclusion, we successfully constructed two recombinants of *E. aerogenes* AB91102, namely, AB-E for over-expression of large subunit of hydrogenase, AB-G for over-expression of small subunit of hyc3 operon. In this study, we demonstrated that over-expression of large and small subunits of hycABCDEFGHI operon efficiently enhance H_2 production in the dark-fermentation by mutants of strain *E. aerogenes* AB91102. Relative to the wild strain, the activity of hydrogenase enzymes, hydrogen production rate and yield of engineered strains were meaningfully enhanced. The yield of AB-E and AB-G reached 0.988 and 0.959 mol-H_2/mol-glucose, respectively, as compared with the wild strain which was only 0.850. Analysis of metabolic flux when correlated with NADH mediated hydrogenase activity, showed that hydrogenase subunits may also catalyze H_2 production via an NADH-dependent pathway. Formate was highly catabolized to CO_2 and H_2 and ultimately increased the production of ethanol, which subsequently affected the redox level and energy balance inside the cell that would lead to enhanced H_2 production from hyc3 complex.

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