Bioresource Technology 155 (2014) 220–228

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Reducing cofactors contribute to the increase of butanol production by a wild-type *Clostridium* sp. strain BOH3

Tinggang Li, Yu Yan, Jianzhong He *

Department of Civil and Environmental Engineering, National University of Singapore, Singapore 117576, Singapore

HIGHLIGHTS

- Improving NAD(P)H availabilities significantly by dosing precursor nicotinic acid.
- Reaching maximal cell growth rate and butanol formation rate by a two-stage pH-shift.
- Achieving 18.7 g/L butanol with a yield of 24.6% and a productivity of 0.26 g/L h.
- Redistributing carbon flux to butanol by manipulating reducing cofactor and pH shift.

ARTICLE INFO

Article history:
Received 24 September 2013
Received in revised form 16 December 2013
Accepted 19 December 2013
Available online 2 January 2014

Keywords:
Butanol
Clostridium sp.
Reducing cofactor
Carbon flux
pH shift

ABSTRACT

Availability of reducing factors (e.g., NADH and NADPH) plays an important role in improving the efficacy of products conversion in cofactor-dependent production systems. In this study, nicotinic acid (NA), the precursor of NADH and NADPH, was supplemented to the growth medium of a wild-type *Clostridium* sp. strain BOH3. Results showed that the addition of precursor NA to the medium led to a significant increase in the levels of NADH and NADPH. Meanwhile, a maximal cell growth rate and butanol generation rate were reached by applying a two-stage pH-shift strategy, achieving 18.7 g/L butanol with a yield of 24.6% and a productivity of 0.26 g/L h. The metabolic patterns were shifted towards more reduced metabolites as reflected by higher butanol-to-acetone ratio (11%) and butanol-to-acid ratio (292%). Redistributing metabolic flux to butanol via manipulations of reducing cofactor and pH shift could become an alternative tool to realize metabolic engineering goals.

Introduction

Butanol is a large-volume, important intermediate chemical and also is considered as a potential superior fuel with favorable physicochemical properties for blending with or directly substituting for fossil gasoline (Dürre, 2007; Kao et al., 2013; Tracy et al., 2012). The current market demand for butanol has been estimated to be 10–12 billion pounds per year, which accounts for 7–8.4 billion dollars with a projected expansion of 3% per year (Donaldson et al., 2007; Kirschner, 2006; Heluane et al., 2011). Due to its attractive market worldwide, the anaerobic production of biobutanol, typically referred to as acetone–butanol–ethanol (ABE) fermentation by solventogenic *Clostridium* spp., has regained its interest as green renewable fuels (Ezeji et al., 2007; Jiang et al., 2009; Li et al., 2013).

Conventional ABE fermentation encounters low butanol productivity, yield and concentration, resulting in a high production cost (Lee et al., 2008). So far, various efforts have been made to improve the butanol concentration, yield and productivity, such as classic chemical mutagenesis (Blaschek et al., 2002), serial enrichment procedures (Baer et al., 1987; Quratulain et al., 1995), overexpression of targeted functional genes in engineered host (Tomas et al., 2003; Yu et al., 2011) for overcoming the butanol toxicity barrier on microorganisms. However, despite technological advances in genetically engineered strains or mutants, unstable butanol production indicates the difficulty and complexity in transferring related pathways to modified strain or a host bacteria due to its inherent instability or inactive expression compared to a wild-type strain (Alsaker et al., 2010; Antoni et al., 2007).

Comparing with the abovementioned strategies, metabolic engineering has the potential to stably enhance process productivity and yield by manipulating the throughput of certain pathways. Recently, cofactors have emerged to be attractive targets to induce changes in metabolism, and thus play an essential role in a large number of biochemical reactions and the production of different fermentation products (San et al., 2002; Yu et al., 2011). The level

* Corresponding author. Address: Department of Civil and Environmental Engineering, National University of Singapore, Block E2-02-13, 1 Engineering Drive 3, Singapore 117576, Singapore. Tel.: +65 6516 3385; fax: +65 6774 4202.
E-mail address: jianzhong.he@nus.edu.sg (J. He).

0960-8524/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biortech.2013.12.089

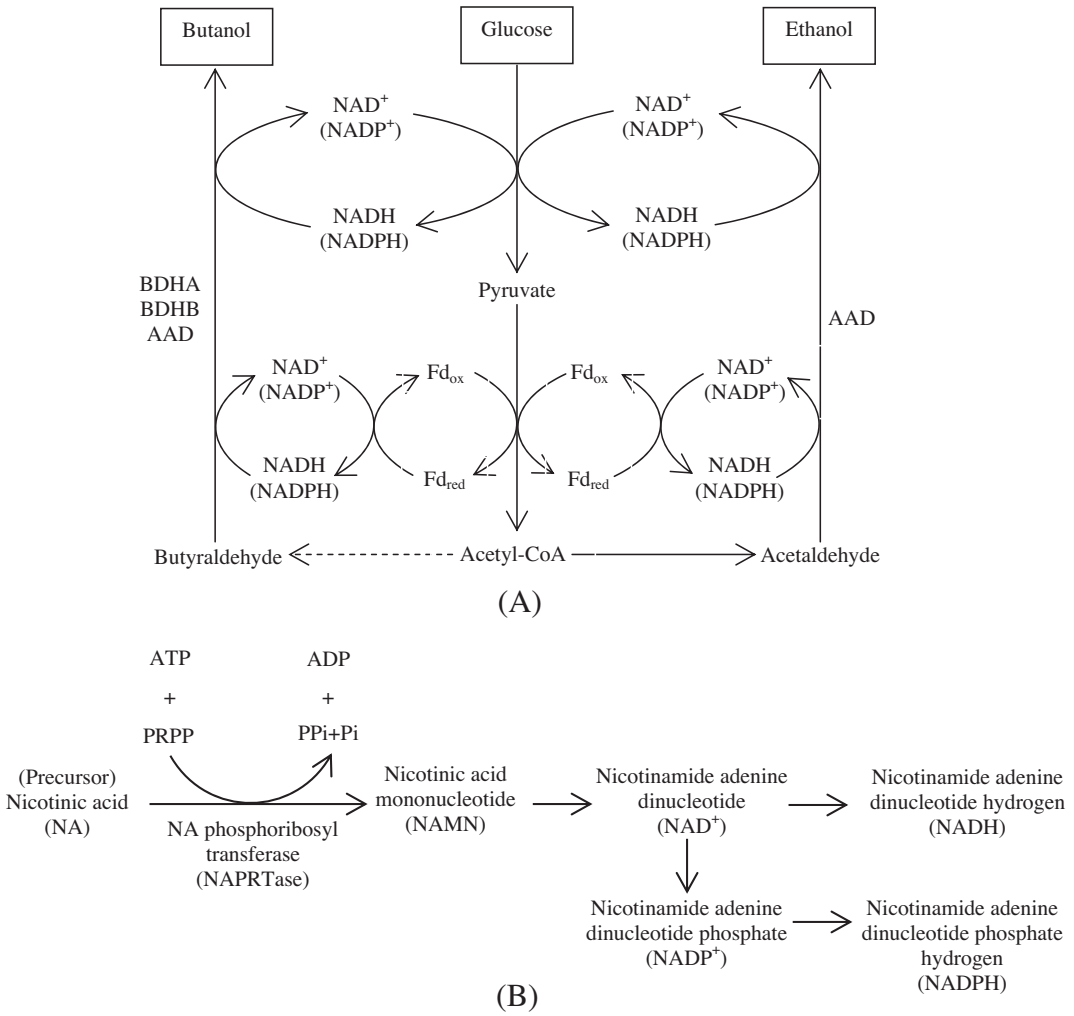

Fig. 1. Relationship of alcohol synthesis and NADH/NADPH regenerations, and salvaging synthesis of NADH and NADPH from precursor nicotinic acid (NA) in the cofactor-dependent system. (A) Alcohol synthesis and NADH/NADPH regenerations; (B) Salvaging synthesis of NADH and NADPH from precursor nicotinic acid (NA). Reducing cofactors (NADH/NADPH)-dependent enzymes are abbreviated as follows: alcohol/aldehyde dehydrogenase (AAD); butanol dehydrogenaseI (BDHA); butanol dehydrogenaseII (BDHB).

and availability of cofactors can become a limiting step in cofactor-dependent production systems (Berríos-Rivera et al., 2002; Knepper et al., 2008), especially the solventogenic *Clostridium* sp. involving NADH- and NADPH-dependent butanol dehydrogenases (Fig. 1A). Consequently, a lack or inefficient regenerations of the cofactor NADH or NADPH would shutdown the butanol dehydrogenase reaction. Cofactor manipulations can potentially become a powerful tool for improvement of overall process yield and productivity. In order to increase the butanol flux, it is crucial to provide constant NADH and NADPH in solventogenic phase to achieve a redox balance for enhanced butanol production.

Therefore, a possible way to achieve high butanol production (defined as amounts greater than the toxicity level of about 10–12 g/L) (Shen et al., 2011) is to direct the flux by triggering the reducing force via increasing the availabilities of NADH and NADPH. In this study, we attempted to increase the yield and productivity of butanol in a wild-type *Clostridium* sp. strain BOH3. To achieve this target, nicotinic acid (NA), the precursor of NADH and NADPH, was supplemented in the medium to increase their availabilities in the cells. To decouple cell growth and solvent production, a two-stage pH-shift strategy in an anaerobic batch bioreactor was developed to improve the fermentation performance of strain BOH3. The activities of butyraldehyde dehydrogenase (BADH) and butanol dehydrogenase (BDH) were further analyzed in the major metabolic flux in the pH-controlled environment. This study proves the significance of reducing cofactors in enhancing anaerobic butanol production in terms of concentration, yield and productivity.

Methods

Culture medium and fermentation experiments

A wild-type *Clostridium* sp. strain BOH3 isolated from a paddy field was used in this study (Bramono et al., 2011). The fermentation medium in the bioreactor consisted of (per liter): glucose 90 g; KH_2PO_4 0.5 g; K_2HPO_4 0.5 g; $MgSO_4$ 0.2 g; CH_3COONH_4 2.2 g; $MnSO_4$ 0.05 g; $FeSO_4{\cdot}7H_2O$ 0.01 g; NaCl 1 g; yeast extract 3 g; plus 1 ml of a trace element solution at a concentration of (per liter): H_3BO_3 0.006 mg, $NiCl_2{\cdot}6H_2O$ 0.024 mg; $ZnCl_2$ 0.1 mg; $CoCl_2{\cdot}6H_2O$ 1.9 mg; $Na_2MoO_4{\cdot}2H_2O$ 0.036 mg; and $CuCl_2{\cdot}2H_2O$ 0.05 mg. All the chemicals in the study were of at least analytical-grade purity and were purchased from Sigma–Aldrich, USA.

For microbial seed cultivation, the medium was first boiled with an addition of 0.25 ml resazurin solution (0.1%) and cooled to room temperature under nitrogen flow. Anaerobic medium (pH 6.5) was

prepared with an addition of 0.0242 g/L of L-cysteine, and 0.048 g/L of $Na_2S{\cdot}6H_2O$, respectively (He et al., 2003). A number of 160-mL serum bottles were filled with 42-mL salt medium solution and then sealed with aluminum caps with butyl stoppers, autoclaved for 30 min, and cooled to room temperature. 5-mL glucose sterile stock solution (300 g/L) and 1-mL yeast extract sterile stock (150 g/L) were added to each bottle. Subsequently, active cells (4%, vol/vol) were inoculated to the bottles and incubated for 24–30 h at 37 °C and stirred at 150 rpm on a rotary shaker.

The bioreactor fermentation was conducted in a 3 L bioreactor (BIOSTAT® B plus, Sartorius, Germany) equipped with probes of redox potential and pH, and with a working volume of 1.5 L at an agitation speed of 150 rpm and at 37 °C. Nicotinic acid (an optimized concentration based on butanol production) was added at a final concentration of 10 mg/L in the medium before fermentation. Seed culture (OD = ~2) was inoculated into the bioreactor (6% [v/v]). A two-stage pH-shift strategy was developed based on the specific cell growth rate and specific butanol formation rate, in which pH 6.0 was maintained for 6 h, then allowed to drop to 5.0 as the culture progressed, and then maintained at 5.0 throughout the rest of the fermentation time. The pH-stat was controlled by automatic addition of 9 M NaOH or 3 M H_2SO_4. Samples were taken at various time intervals. All experiments were done in triplicates.

Analytical methods

The dry cell weight (DCW) was determined at 105 °C (4 h) until constant weight. The concentrations of the main metabolites in the fermentation broth (acetone, butanol, ethanol, acetate and butyrate) were analyzed by gas chromatography (Agilent 7890A, Agilent, USA) equipped with a J & W 123–7334 column (30 m × 320 μm × 0.25 μm) and a flame ionization detector. The injector was kept at 250 °C, the column was programmed from 60 (2 min hold) to 240 °C at 15 °C/min increments (2 min hold), and the detector was set at 250 °C. Helium was used as the carrier gas at 2 mL/min. The sample injection volume was 1 μL with a split rate of 20:1, and the flow rates of hydrogen and air were 30 and 40 mL/min, respectively. Residual glucose concentrations were quantified with a biochemical analyzer (YSI 2700, USA).

Cell extract preparation

Crude cell extracts were prepared from 10 mL of the culture in the bioreactor sampled at various time intervals in order to determine in vitro activity of enzymes in strain BOH3. Anaerobic conditions were maintained throughout the entire procedure. Cells were harvested by centrifugation at 14,000 rpm at 4 °C. The cell pellets were resuspended in 0.5 mL of ice-cold TE buffer (10 mM Tris–HCl, 5 mM EDTA, pH 7.5). Lysis was achieved by ultrasonication on ice for 15 min using a 20 kHz ultrasonicator (VCX 130, Sonics & Materials Inc., CT, USA) with the following conditions: 5 s of sonication with a 10 s interval, set at 50% amplitude. The resulting lysate was then collected and centrifuged at 14,000 rpm at 4 °C for 20 min to remove cell debris. The supernatant was then retrieved in 1 ml solution (10× concentrated) for subsequent enzyme assays. Protein concentration of cell extracts was determined by using the DC protein assay Kit (BioRad, USA). All values of enzyme assay were averaged values of at least three independent extract procedures.

BADH and BDH assay

Unless stated otherwise, all enzyme assays were performed in an anaerobic workstation as abovementioned at 30 °C with 10× concentrated cell extracts. BADH activity was measured by monitoring the reduction of $NADP^+$ (or NAD^+) to NADPH (or NADH) at 340 nm (Dürre et al., 1987; Stim-Herndon et al., 1996). Butyraldehyde was dissolved in methanol. The assay mixture contained (in a volume of 1 mL): 50 mM glycylglycine buffer (pH 9.0), 50 mM butyraldehyde, 0.5 mM butyryl-CoA, 0.3 mM NAD(P), 1.0 mM dithiothreitol, and crude extract (20–600 μg of protein). The extinction coefficient of NAD(P)H is 6.22 mM/cm. The reaction was initiated by the addition of the cell extract. One unit of specific enzyme activity was defined as the amount of enzyme which reduced 1 μmol NAD^+ or $NADP^+$ per minute per milligram of protein at 30 °C under the given conditions.

BDH activity was assayed by monitoring the oxidation of NADPH (or NADH) at 340 nm (Dürre et al., 1987; Stim-Herndon et al., 1996). The NADPH dependent reaction was examined at pH 8.0 under the following conditions: 0.4 mM NADPH, 50 mM butyraldehyde, 35 mM Tris chloride (pH 8.0), and crude extract (60–600 μg of protein). The NADH-dependent reaction was observed by using 2-(N-morpholino)ethanesulfonic acid (MES) buffer (pH 6.0) with other reagents as described above. Final reaction volume was 600 μL. The change in absorbance of blank reactions without butyraldehyde but with the appropriate quantity of methanol was subtracted from the total. The reaction was initiated by the addition of the cell extract. One unit of specific enzyme activity was defined as the amount of enzyme which oxidized 1 μmol NADH or NADPH per minute per milligram of protein at 30 °C under the given conditions. All values of enzyme assay were averaged values of at least three independent extract procedures.

NAD^+/NADH and $NADP^+$/NADPH assay

NAD^+/NADH or $NADP^+$/NADPH levels were measured with Amplite™ Fluorimetric NAD^+/NADH or $NADP^+$/NADPH ratio assay kits (ATT Bioquest, CA). Cells were harvested by centrifugation at 14,000 rpm at 4 °C. The pellets were then resuspended with 0.2 mL PBS buffer (pH 7.4) and 0.2 mL NAD^+/NADH or $NADP^+$/NADPH lysis buffer provided in the assay kits. Lysis was allowed to proceed for 15 min at room temperature until the cell resuspension turned clear. The lysate was then centrifuged at 1,500 rpm for 5 min at 4 °C. The supernatant was retrieved for subsequent NAD^+/NADH or $NADP^+$/NADPH assays. For the measurement of intracellular NAD^+/NADH or $NADP^+$/NADPH levels, 25 μL of cell lysates were treated with or without NADH/NAD^+ or NADPH/$NADP^+$ extraction solution for 15 min, and then neutralized with extraction solutions at room temperature, and incubated the reaction at room temperature in the dark for 30 min after adding 75 μL of NADH or NADPH reaction mixture. The readings were taken by running a 96-well black plate on a fluorescence microplate reader (Infinite 200 pro, Tecan, Switzerland) at Ex/Em = 530–570/590–600 nm (maximum Ex/Em = 540/590 nm). The blank signal was subtracted from the values for those wells with the NADH or NADPH reactions.

Results and discussion

A two-stage pH-shift regulation for improved cell growth and butanol production

In acetone–butanol–ethanol (ABE) fermentation process, pH plays a key role in shifting cell growth phase to solventogenic phase (Lee et al., 2008). Sylvia et al. (2011) suggested the influence of pH on fermentation products: at high pH (pH 5.7) acids are the dominant products while at low pH (pH 4.5) this switches to solvents. However, the pH range for solvent formation appears to vary quite widely, depending on the particular strain and the culture conditions used (Jones and Woods, 1986). Fig. 2 shows different profile of the specific cell growth rate (expressed as cell μ_{max})

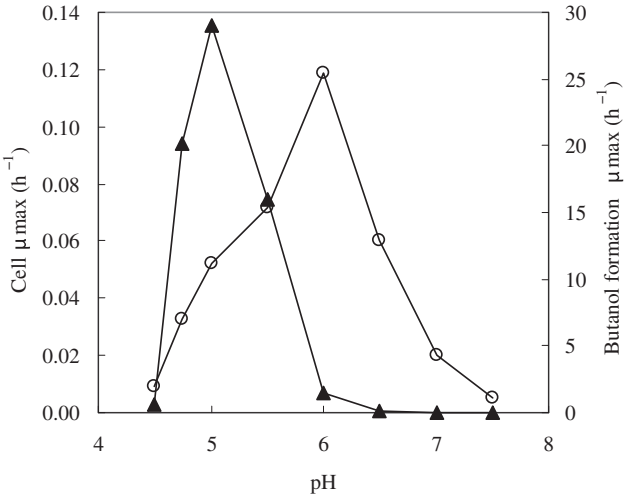

Fig. 2. Effects of pH on specific cell growth rate and specific butanol formation rate. cell μ_{max} (open circle symbol) and butanol μ_{max} (solid triangle symbol).

versus the specific butanol formation rate (in terms of butanol μ_{max}) of culture BOH3 at various pHs. The optimum pH for cell growth was 6.0 and cell μ_{max} reached 0.112 h^{-1} (Fig. 2). Cell growth was severely inhibited at a pH lower than 4.5 or higher than 8.0. On the other hand, butanol μ_{max} increased almost linearly from pH 4.5 to 5.0, reached the highest (~30 h^{-1}) at pH 5, and then gradually decreased. No butanol formation occurred at a pH lower than 4.5 or higher than 6.5, indicating a very narrow pH range (4.8–5.3) suitable for butanol production. Therefore, at early phase of fermentation, it is appropriate to control pH at a high value (e.g., pH 5.5–6.0) to maximize cell growth, while at mid- and later-fermentation phase, a lower pH (e.g., pH 5.0) should be more appropriate in order to maximize butanol μ_{max}. As shown in Fig. 2, an optimal pH-shift strategy can be developed as following: pH is firstly controlled at 6.0 during the first 6 h (excluding lag phase), and then pH is allowed to drop to 5.0 as the culture progressed; subsequently, pH is automatically maintained at 5.0 for onwards reactions.

Effects of nicotinic acid on butanol production and cell growth

To determine the effects of NA on strain BOH3's growth and butanol production, various amount of NA (0–20 mg/L) was dosed to the fermentation medium. The growth of strain BOH3 reached 3.2 g/L DCW and butanol was produced up to 6.7 g/L in the absence of NA (Fig. S-1). However, cell growth and butanol concentration increased along with the increase of NA concentration in the fermentation medium. The highest butanol production (12.3 g/L) and cell growth (4.8 g/L) were achieved in a serum bottle medium spiked with 10 mg/L NA, which is 84% and 50% higher than those in the control bottles (absence of NA), respectively (Fig. S-1).

Increases of NADH and NADPH availabilities

The feasibility of increasing NADH and NADPH availabilities by supplementing NA (soluble and transportable), a precursor of NADH or NADPH (Fig. 1B), was investigated as a means to manipulate the intracellular NADH or NADPH level under the defined two-stage pH-shift strategy. More specifically, this study assesses the effect of regenerating NADH and NADPH in the native cofactor-dependent BADH and BDH in strain BOH3 with (10 mg/L) or without the addition of NA. NADH and NADPH are the cofactors of glycolysis and butanol synthesis in strain BOH3. Without them,

Table 1
Variation of cofactors of strain BOH3 grown in a bioreactor.

Parameters	Control[a]	With NA[b]
DCW (g/L)	5.4 ± 0.3	6.6 ± 0.4
Total NADH (μmol/g-DCW)	82.6 ± 5.0	244.1 ± 13.2
Total NAD^+ (μmol/g-DCW)	105.8 ± 5.7	105.6 ± 6.3
Total NADPH (μmol/g-DCW)	17.5 ± 0.9	57.3 ± 3.7
Total $NADP^+$ (μmol/g-DCW)	48.9 ± 2.9	46.5 ± 2.8
Ratio of NADH and NAD^+	0.78 ± 0.06	2.31 ± 0.12
Ratio of NADPH and $NADP^+$	0.36 ± 0.01	1.23 ± 0.07
Sum of NAD(H/+)(μmol/g-DCW)	188.4 ± 9.5	454.1 ± 25.1
Sum of NADP(H/+) (μmol/g-DCW)	66.4 ± 3.8	103.8 ± 6.2

[a] Grown in a two-stage pH-shift bioreactor without addition of NA.
[b] Grown in a two-stage pH-shift bioreactor with addition of NA (10 mg/L).

the butanol-producing pathway and glycolysis would be shut down quickly. Relatively low growth (5.4 g/L DCW) of strain BOH3 was observed in the absence of NA in the fermentation medium under two-stage pH shift strategy (Table 1). Compared to the control (without addition of NA under two-stage pH shift strategy), the total NADH and NADPH levels increased 1.95 and 2.27 times after adding NA to the bioreactor operating with the two-stage pH-shift strategy. Also, the enhanced ratios from 0.78 to 2.31 (NADH/NAD^+) and from 0.36 to 1.23 (NADPH/$NADP^+$) indicate that more reducing equivalents were produced in the forms of NADH and NADPH. However, the NADH/NAD^+ and NADPH/$NADP^+$ ratios did not exhibit a consistent trend compared to the increase of NADH or NADPH level. It may be due to the fact that NADH/NAD^+ ratio is sensitive to growth rate as well as other environmental factors (e.g., pH), where the defined pH-shift strategy simultaneously benefited cell growth (regeneration of NADH) and butanol synthesis (consumption of NADH) (Figs. 1 and 2). In contrast, the NAD^+ or $NADP^+$ level did not increase obviously after the addition of NA. This could be attributed to that the lack of NADH or NADPH in the reducing cofactor-dependent production system stimulated more oxidant state of NAD^+ or $NADP^+$ converting into reducing equivalents at the defined pH-shift conditions (Table 1 and Fig. S-2).

Effect of NA addition on BADH and BDH activities and metabolite flux in acidogenesis and solventogenesis phases

To further assess effects of increasing availabilities of NADH and NADPH after supplementing precursor NA on redox cofactor-dependent enzymes and metabolites in strain BOH3, cell crude extracts harvested at intervals were assayed for BADH and BDH activities in vitro and for changes in metabolites (Table 2). NADH- and NADPH-dependent BDH activities in solventogenic stage were higher in the presence of NA than those in the control (~2 and 1.6 times, respectively), while the BDH activities in acidogenic stage also showed slight enhancement compared to the control (up to 1.6 times). NADH-dependent BADH activity was lower than NADH-dependent BDH activity but with a similar trend (Table 2). No NADPH-dependent BADH activity was detected throughout the culturing stage, even in the presence of NA. Furthermore, overall BDH activities were greater than BADH activities, suggesting that a more selective specificity carries redox cofactors (NADH and NADPH) on BDH in strain BOH3.

Analysis of the metabolites produced by strain BOH3 indicates a superior butanol formation capability in the presence of NA as compared with the control, which was due to the enhanced activities of the NADH- and NADPH-dependent BDH and NADH-dependent BADH (Table 2). In addition, a slight increase of ethanol concentration was observed in both of the above experimental conditions, possibly due to a more affinity of NADH or NADPH-dependent bifunctional enzyme aldehyde/alcohol dehydrogenase (AAD) to butyraldehyde than to aldehyde. Since no attempt was

Table 2
Growth, enzyme activity and metabolic products of *Clostridium* sp. strain BOH3.

Fermentation stage	NA (cofactor precursor)	Enzyme activity (U/mg-protein)				Metabolic products (g/L)				
		BDH		BADH		Biosolvents			Bioacids	
		NADH-dependent	NADPH-dependent	NADH-dependent	NADPH-dependent	Acetone	Butanol	Ethanol	Acetic acid	Butyric acid
Acidogenic[a]	–	0.276 ± 0.017	0.134 ± 0.008	0.102 ± 0.005	ND	0.5 ± 0.02	0.6 ± 0.04	0.4 ± 0.01	1.8 ± 0.09	2.4 ± 0.1
	+	0.402 ± 0.021	0.238 ± 0.015	0.177 ± 0.018	ND	0.6 ± 0.04	1.2 ± 0.07	0.4 ± 0.02	2.6 ± 0.1	3.0 ± 0.2
Solventogenic[b]	–	0.427 ± 0.028	0.052 ± 0.003	0.096 ± 0.005	ND	1.8 ± 0.1	5.6 ± 0.2	0.7 ± 0.03	4.9 ± 0.3	6.1 ± 0.4
	+	0.601 ± 0.037	0.061 ± 0.06	0.121 ± 0.009	ND	2.2 ± 0.1	8.0 ± 0.5	1.8 ± 0.1	2.1 ± 0.1	2.8 ± 0.1

–, control without addition of NA under a two-stage pH-shift strategy; +, with addition of NA under a two-stage pH-shift strategy.
ND, not detected.
[a] Cultures were harvested at 24 h.
[b] Cultures were harvested at 48 h.

made to enhance acetone or bioacids (acetate and butyrate) pathway expression (redox cofactors is not required in the synthesis of acetone, acetate and butyrate), it is meaningful to calculate butanol-to-acetone and biosolvent-to-bioacid ratio. The butanol-to-acetone ratio was doubled in the presence of NA than that in the control. The biosolvent-to-bioacid ratio also showed an enhancement in the solventogenic phase (Table 2), leading to elevated final solvent levels and decreased final acid levels. Therefore, a large quantity of NADH and NADPH synthesized with the aid of precursor NA was crucial for both the enhanced production of butanol and the promotion of glucose consumption and cell growth (Table 2). Based on these findings, we validated the feasibility of increasing the total NADH and NADPH levels (Table 1) by enhancing the salvaging pathway (e.g., adding precursor NA to the fermentation medium).

Enhanced butanol production triggered by elevated availabilities of reducing cofactors

In order to gain more insights into the physiological changes of the wild-type strain BOH3, batch fermentations were carried out with or without NA addition. The two-stage pH-shift strategy was employed to the fermentation process in order to obtain optimized cell μ_{max} and butanol μ_{max}. The addition of NA promoted the cell growth by 11% and reduced their doubling times by ~40%. The wild-type strain BOH3 produced 18.7 g/L butanol and 27.8 g/L ABE bio-solvents, which increased 16% as compared to the control (without NA addition) (Fig. 3A and B) and 179% comparing to without pH-shift control strategy (6.7 g/L of butanol production) (Fig. S-3). Less butyrate was produced (4.3 g/L) in the presence of NA than that of the control (6.3 g/L), although butyrate was reassimilated at the end of fermentation in both runs; more acetic acid was accumulated at the end of fermentation in the control reactor, which was 2.9 times higher than that in the NA-addition reactor, resulting in inhibition on the growth of strain BOH3 (Fig. 3A and B). Compared to the control, the biosolvent-to-bioacid ratio increased ~2.2 times (10.3 to 3.2) with NA addition. The profile of pH shift exhibited a similar trend in reactors with or without NA addition although 2–4 h lag phase was observed in the absence of NA. Notably, NA helped to improve the redox potential of the culture and to maintain constant redox potential balance from 18 h onwards so as to enhance solvent production (Fig. 3C). The glucose consumption rate, the butanol yield, and the butanol productivity were ~9, 7 and 14% higher, respectively, than the corresponding values of the control (without NA) (Fig. 3D–F).

With increased availabilities of NADH and NADPH in a 3-L bioreactor operating under a two-stage pH-shift strategy, the wild-type strain BOH3 achieved 18.7 g/L butanol with a yield of 24.6% and a productivity of 0.26 g/L h, which is at least 25% higher than previous wild-type strains and is comparable to gene-modified strains (Chen et al., 2013; Fontaine et al., 2002; Yu et al., 2011). In order to increase the yield and productivity of butanol, most studies have focused on enhancing butanol production through chemical mutagenesis, genome shuffling (Mao et al., 2010), genetic deletion (Jiang et al., 2009; Shao et al., 2007), and gene overexpression (Sillers et al., 2008; Yu et al., 2011). For example, Mao et al. (2010) obtained 15.3 g/L butanol with ~21% butanol yield and 0.26 g/liter/h butanol productivity by *Clostridium acetobutylicum* mutant Rh8 in a 4-L bioreactor after applying chemical mutagenesis and genome shuffling; and Shen et al. (2011) achieved 15 g/L butanol in flasks through the artificial driving forces created by NADH and acetyl-CoA accumulation system in host *Escherichia coli*. As shown in this study, the increased intracellular levels of NADH and NADPH were attributed to the available precursor NA, which is soluble and transportable to the cells. Butanol production can be enhanced with more available NADH and NADPH – the reducing cofactors in strain BOH3. This study demonstrated that the availabilities of reducing cofactors (e.g., NADH and NADPH) could determine the amount of butanol generation by a wild-type *Clostridium* sp. stain BOH3 – possessing a native cofactor-dependent enzymatic system. To our knowledge, this is the first report on the feasibility of increasing the amount of NADH and NADPH in the cells so as to substantially enhance the salvaging pathway on butanol production with the aid of NA (the precursor of NADH and NADPH) with the aid of precursor compound – NA (Table 1 and Fig. 3). The reducing cofactors NADH and NADPH could also play a key role in enhancing reduced products (e.g., butanol) and maintaining redox potential balance in fermentative bacteria (e.g., strain BOH3). The wild-type strain BOH3 has been maintained to produce stable amount of butanol (>18 g/L) for more than one year.

The improved butanol concentration in the fermentation broth should be attributed to the metabolite flux pattern changes in strain BOH3, as caused by the higher availabilities of NADH and NADPH. In this case, reducing cofactors were regulated by the addition of NA, which increased the level of NADH and NADPH and possibly complemented with the lack of reducing cofactors in the alcohol-dependent pathways of strain BOH3. Consequently, under the defined conditions, a significant shift in the metabolic directions towards the production of more reduced metabolites (e.g. 18.7 g/L butanol) occurred, as evidenced by improved butanol-to-acetone ratio (11%) and butanol-to-bioacid ratio (292%) (no accumulation of acetic acid) at the end of the fermentation (Fig. 3A and B). In contrast, early attempts to redirect metabolite flux have been made to overexpress *aad* (encoding alcohol/aldehyde dehydrogenase, AAD) gene so as to improve the butanol selective production in a *C. acetobutylicum* mutant M5 (Sillers et al., 2008). However, large amounts of acetate (14.9 g/L) and butyrate (7.7 g/L) were accumulated due to the inability of the mutant M5 to re-assimilate acid products, and butanol concentration never exceeded the amount (11.1 g/L) generated by wild-type *C.*

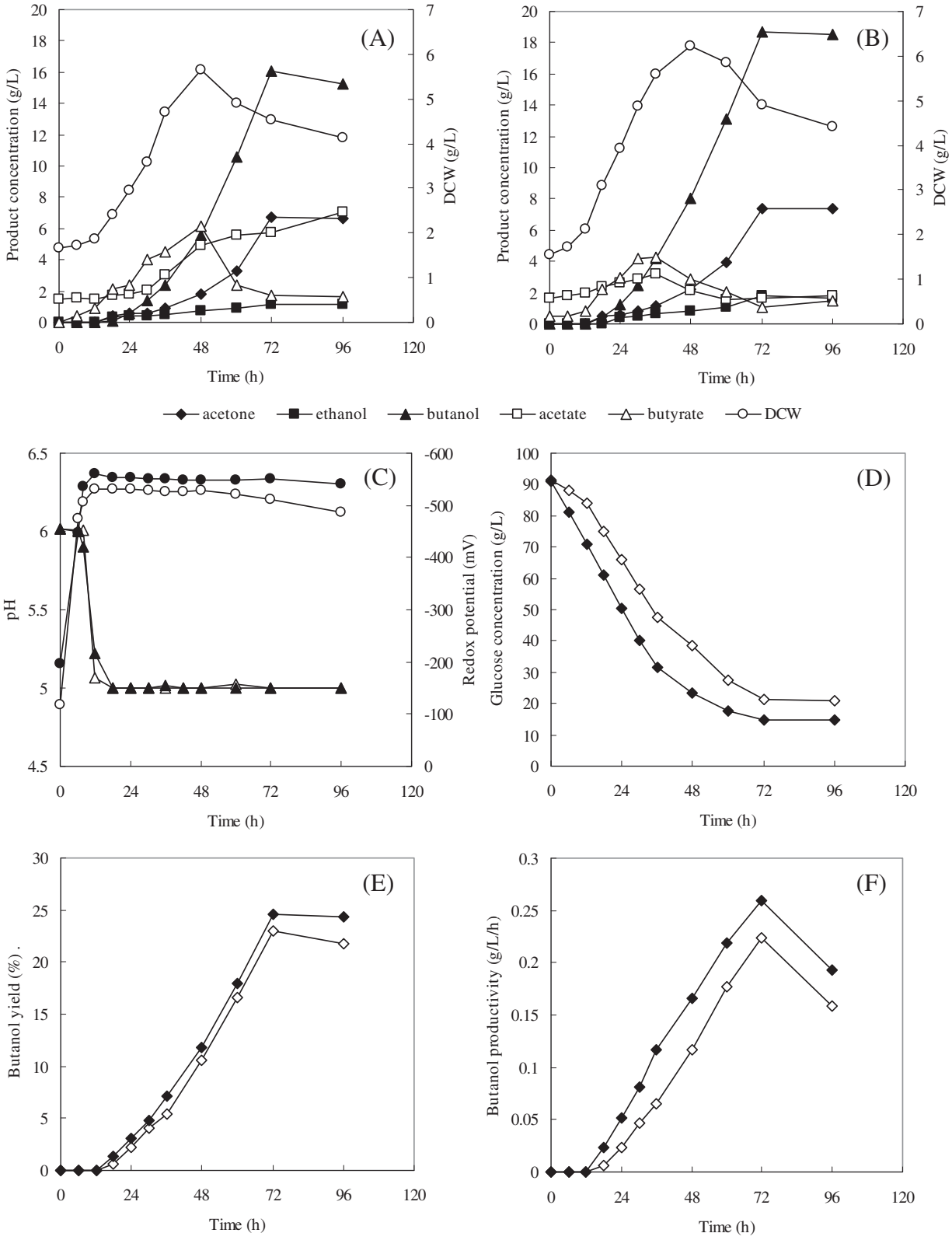

Fig. 3. Time-courses of batch fermentation (A) without NA addition and (B) with NA addition in *Clostridium* sp. strain BOH3 under a two-stage pH-shift strategy. (C) pH profile (circle symbol) and redox profile (triangle symbol); (D) Glucose utilization profiles; (E) average butanol yield profiles; (F) average butanol productivity profiles. With addition of NA (solid symbols) and without addition of NA (open symbols).

acetobutylicum. In our current study, it suggests that adjusting the levels of NADH and NADPH could be an efficient approach in improving butanol production, and enough NADH and NADPH and or higher NADH/NAD^+ and NADPH/$NADP^+$ ratios benefit the cells in accelerating the butanol production and glycolysis (in terms of cell growth) so as to change the final distribution of the metabolites. On the other hand, manipulating cofactors may also provide an additional means to determine cellular metabolism, in

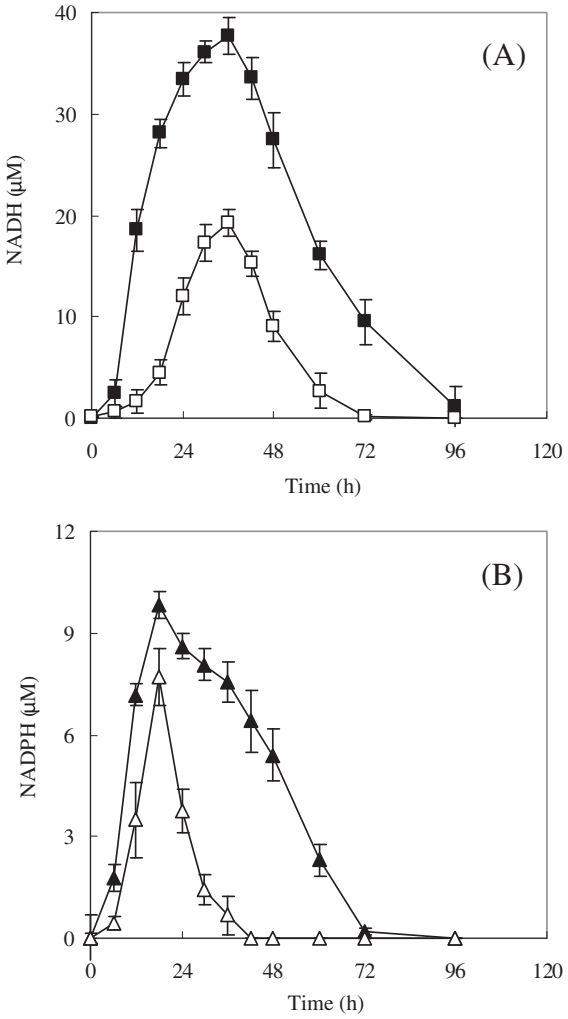

Fig. 4. Physiological profiles of reducing cofactors NADH and NADPH with or without addition of NA under a two-stage pH-shift strategy. (A) NADH profiles; (B) NADPH profiles. With addition of NA (solid symbols) and without addition of NA (open symbols).

particular the interplay between cofactor levels and metabolic fluxes.

In vitro physiological profiles of reducing cofactors and these cofactor-dependent BADH and BDH activities

Redox cofactors such as NADH and NADPH were monitored in the crude cell extracts from cells of the above bioreactor. Both NADPH and NADH exhibited similar patterns with hump-shaped profiles, with the highest level appeared at about one fourth of fermentation stage (Fig. 4). NADPH level was observed to be lower but peaked 18 h earlier than NADH regardless of NA addition or not. However, levels of NADH and NADPH in the extract with NA in the fermentation medium were 96% and 27% higher than those in the extract from the control, respectively (Fig. 4A and B).

It is of interest to examine how butyraldehyde dehydrogenase (BADH) and butanol dehydrogenase (BDH) activities were regulated and actually distributed with respect to their corresponding cofactor specificity in strain BOH3. As shown in Fig. 5A, BADH activity was NADH-dependent in both cell extracts (with or without addition of NA) of strain BOH3, whereas NADPH-dependent BADH activity was either not detected or just above the detection limit. In contrast, BDH activities were both NADH- and NADPH-dependent in both cell extracts (with or without addition of NA)

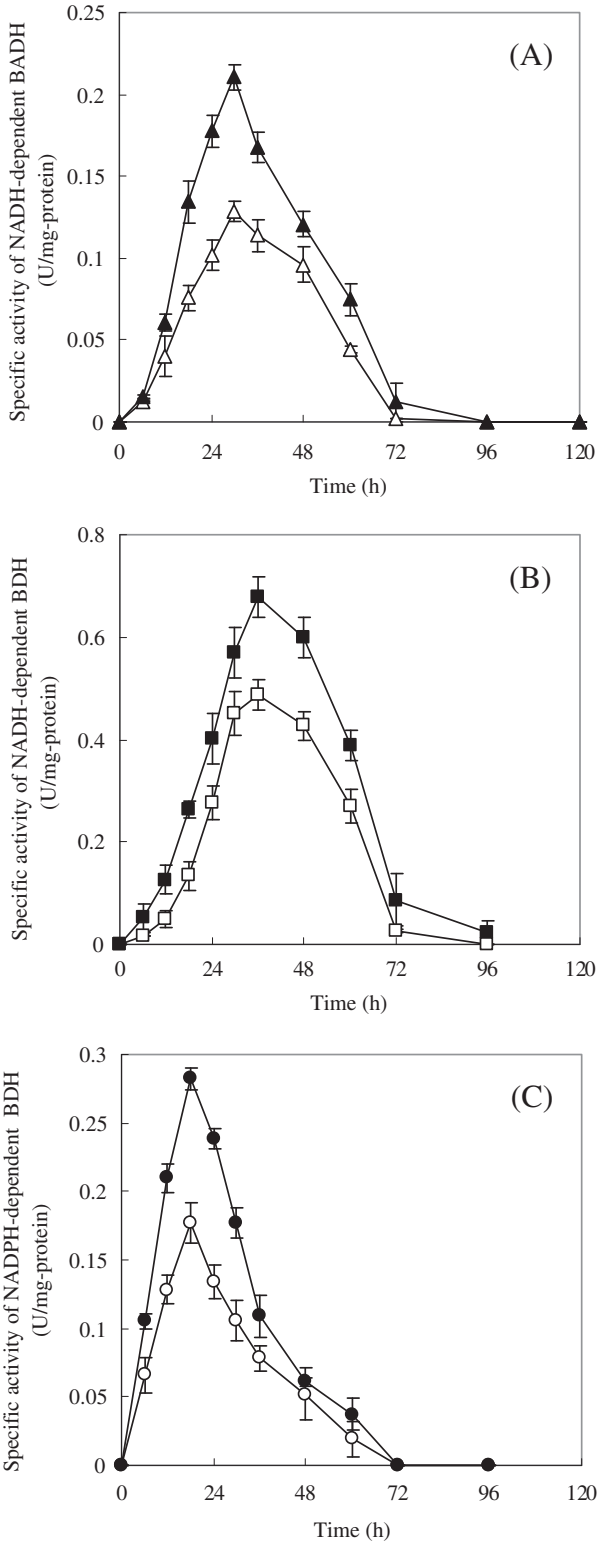

Fig. 5. In vitro physiological profiles of BADH and BDH activities with or without addition of NA under a two-stage pH-shift strategy. (A) NADH-dependent BADH profiles; (B) NADH-dependent BDH profiles; (C) NADPH-dependent BDH profiles. With addition of NA (solid symbols) and without addition of NA (open symbols).

of strain BOH3 (Fig. 5B and C). In vitro enzymatic activity analysis demonstrated that at least three enzymes requiring reducing cofactors were directly involved in butanol production. In the cell extract with NA addition, NADH-dependent BADH, NADH- and NADPH-dependent BDH exhibited relatively high activities for

butanol production with a broad range of variation but a maximum activity of 0.21, 0.68 and 0.28 U/mg-protein at 18, 30 and 36 h, respectively, (Fig. 5). Overall, the activities of NADH-dependent BADH, NADH- and NADPH-dependent BDH increased ~64%, 39%, and 60% in the cell extract with NA addition than those in the cell extract of the control. For both cell extracts, NADPH-dependent BDH activity peaked 12 or 18 h earlier than NADH-dependent BADH or NADH-dependent BDH although less activity than that of the latter (Fig. 5A–C).

It should be noted that the higher NADH and NADPH levels (with the aid of NA) in the entire fermentation course as compared to the control (Fig. 4) helped to enhance BDH and BADH activities and to maintain redox balance in the culture, leading to a higher productivity and yield during the fermentation process. Another interesting observation is that addition of the precursor NA to the medium triggered the accumulation of reducing cofactors (NADH and NADPH), which induced the NADH-dependant BADH and NADH- and NADPH-dependant BDH right before the onset of butanol production (Figs. 4 and 5). This suggests the potential role of reducing cofactors in switching acidogenesis phase to solventogenesis phase in strain BOH3. In comparison, previous studies were trying to improve the butanol dehydrogenase activity by dosing in reduced compounds to the medium. For example, the BDH genes were induced most strongly in the presence of carbon monoxide, followed by methyl viologen, butyric acid and glucose to produce butanol up to 8.9 g/L, though some reduced compounds show toxicity to the cells (e.g., methyl viologen at 25.7 mg/L) (Kim et al., 1984; Tashiro et al., 2007; Vasconcelos et al., 1994). Also, the more reduced substrate (mannitol vs. glucose) could drive metabolic flux toward the more reduced product (16.0 g/L butanol) against the more oxidized acid products (Yu et al., 2011). Therefore, the redirection of the carbon flux could be achieved by complement of the enzyme cofactor so as to increase butanol production. As a precursor for reducing cofactors, NA can effectively trigger increases in butanol production up to ~18.7 g/L and does not exhibit toxicity to the cells. In all, the efficiency of the NADH- and NADPH-dependent butanol production pathway as illustrated in this work suggests a potential application in industry for butanol production by the wild-type strain BOH3.

Conclusion

Availability of reducing factors (NADH and NADPH) offers a potential approach to improve butanol generation in the native cofactor-dependent system of wild-type *Clostridium* sp. stain BOH3. The addition of precursor nicotinic acid to the medium led to a significant increase in levels of NADH and NADPH. Redistributing metabolic flux to butanol via manipulations of reducing cofactor and pH shift could become an alternative approach to realize metabolic engineering goals. Further studies on improvement of butanol yield of strain BOH3 could be conducted on identifying the essential butanol stress-related genes to understand butanol tolerance mechanisms on a basis of adaptive response.

Acknowledgement

This study was supported by the Competitive Research Programme from Singapore National Research Foundation (No. NRF–CRP5–2009–05).

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.biortech.2013.12.089.

References

Alsaker, K.V., Paredes, C., Papoutsakis, E.T., 2010. Metabolite stress and tolerance in the production of biofuels and chemicals: gene expression based systems analysis of butanol, butyrate, and acetate stresses in the anaerobe *Clostridium acetobutylicum*. Biotechnol. Bioeng. 105, 1131–1147.

Antoni, D., Zverlov, V.V., Schwarz, W.H., 2007. Biofuels from microbes. Appl. Microbiol. Biotechnol. 77, 23–35.

Baer, S.H., Blaschek, H.P., Smith, T.L., 1987. Effect of butanol challenge and temperature on lipid composition and membrane fluidity of butanol-tolerant *Clostridium acetobutylicum*. Appl. Environ. Microbiol. 53, 2854–2861.

Berríos-Rivera, S.J., Bennett, G.N., San, K.Y., 2002. The effect of increasing NADH availability on the redistribution of metabolic fluxes in *Escherichia coli* chemostat cultures. Metab. Eng. 4, 230–237.

Blaschek, H.P., Annous, B.A., Formanek, J., Chen, C.K., 2002. Method of producing butanol using a mutant strain of *Clostridium beijerinckii*, U.S. patent 6358717.

Bramono, S.E., Lam, Y.S., Ong, S.L., He, J., 2011. A mesophilic *Clostridium* species that produces butanol from monosaccharides and hydrogen from polysaccharides. Bioresour. Technol. 102, 9558–9563.

Chen, S.K., Chin, W.C., Tsuge, K.J., Huang, C.C., Li, S.Y., 2013. Fermentation approach for enhancing 1-butanol production using engineered butanologenic *Escherichia coli*. Bioresour. Technol. 145, 204–209.

Donaldson, G.K., Huang, L.L., Maggio-Hall, L.A., Nagarajan, V., Nakamura, C.E., Suh, W., 2007. Fermentative production of four carbon alcohols, International Patent WO2007/041269.

Dürre, P., 2007. Biobutanol: an attractive biofuel. Biotechnol. J. 2, 1525–1534.

Dürre, P., Kuhn, A., Gottward, M., Gottschalk, G., 1987. Enzymatic investigations on butanol dehydrogenase and butyraldehyde dehydrogenase in extracts of *Clostridium acetobutylicum*. Appl. Microbiol. Biotechnol. 26, 268–272.

Ezeji, T.C., Qureshi, N., Blaschek, H.P., 2007. Bioproduction of butanol from biomass: from genes to bioreactors. Curr. Opin. Biotechnol. 18, 220–227.

Fontaine, L., Meynial-Salles, I., Girbal, L., Yang, X.H., Croux, C., Soucaille, P., 2002. Molecular characterization and transcriptional analysis of *adhE2*, the gene encoding the NADH-dependent aldehyde/alcohol dehydrogenase responsible for butanol production in alcohologenic cultures of *Clostridium acetobutylicum* ATCC 824. J. Bacteriol. 184, 821–830.

He, J., Ritalahti, K.M., Yang, K.L., Koenigsberg, S.S., Loffler, F.E., 2003. Detoxification of vinyl chloride to ethane coupled to growth of an anaerobic bacterium. Nature 424, 62–65.

Heluane, H., Evans, M.R., Dagher, S.F., Bruno-Bárcena, J.M., 2011. Meta-analysis and functional validation of nutritional requirements of solventogenic clostridia growing under butanol stress conditions and coutilization of D-glucose and D-xylose. Appl. Environ. Microbiol. 77, 4473–4485.

Jiang, Y., Xu, C.M., Dong, F., Yang, Y.L., Jiang, W.H., Yang, S., 2009. Disruption of the acetoacetate decarboxylase gene in solvent-producing *Clostridium acetobutylicum* increases the butanol ratio. Metab. Eng. 11, 284–291.

Jones, D.T., Woods, D.R., 1986. Acetone–butanol fermentation revisited. Microbiol. Rev. 50, 484–524.

Kao, W.C., Lin, D.S., Cheng, C.L., Chen, B.Y., Lin, C.Y., Chang, J.S., 2013. Enhancing butanol production with *Clostridium pasteurianum* CH4 using sequential glucose–glycerol addition and simultaneous dual-substrate cultivation strategies. Bioresour. Technol. 135, 324–330.

Knepper, A., Schleicher, M., Klauke, M., Weuster-Botz, D., 2008. Enhancement of the NAD(P)(H) pool in *Saccharomyces cerevisiae*. Eng. Life Sci. 8, 381–389.

Kim, B.H., Bellows, P., Datta, R., Zeikus, J.G., 1984. Control of carbon and electron flow in *Clostridium acetobutylicum* fermentations: utilization of carbon monoxide to inhibit hydrogen production and to enhance butanol yields. Appl. Environ. Microbiol. 48, 764–770.

Kirschner, M., 2006. n-Butanol. Chem. Market Rep. 269, 42.

Lee, S.Y., Park, J.H., Jang, S.H., Nielsen, L.K., Kim, J., Jung, K.S., 2008. Fermentative butanol production by clostridia. Biotechnol. Bioeng. 101, 209–228.

Li, L., Ai, H.X., Zhang, S.X., Li, S., Liang, Z.X., Wu, Z.Q., Yang, S.T., Wang, J.F., 2013. Enhanced butanol production by coculture of *Clostridium beijerinckii* and *Clostridium tyrobutyricum*. Bioresour. Technol. 143, 397–404.

Mao, S.M., Luo, Y.M., Zhang, T.R., Li, J.S., Bao, G.H., Zhu, Y., Chen, Z.G., Zhang, Y.P., Li, Y., Ma, Y.H., 2010. Proteome reference map and comparative proteomic analysis between a wild type *Clostridium acetobutylicum* DSM 1731 and its mutant with enhanced butanol tolerance and butanol yield. J. Proteome Res. 9, 3046–3061.

Quratulain, S., Qadeer, M.A., Chaudhry, M.Y., Kausar, A.R., 1995. Development and characterization of butanol resistant strain of *Clostridium acetobutylicum* in molasses medium. Folia Microbiol. 40, 467–471.

San, K.Y., Bennett, G.N., Berríos-Rivera, S.J., Vadali, R.V., Yang, Y.T., Horton, E., Rudolph, F.B., Sariyar, B., Blackwood, K., 2002. Metabolic engineering through cofactor manipulation and its effects on metabolic flux redistribution in *Escherichia coli*. Metab. Eng. 4, 182–192.

Shao, L.J., Hu, S.Y., Yang, Y., Gu, Y., Chen, J., Yang, Y.L., Jiang, W.H., Yang, S., 2007. Targeted gene disruption by use of a group II intron (targetron) vector in *Clostridium acetobutylicum*. Cell Res. 17, 963–965.

Shen, C.R., Lan, E.I., Dekishima, Y., Baez, A., Cho, K.M., Liao, J.C., 2011. Driving forces enable high-titer anaerobic 1-butanol synthesis in *Escherichia coli*. Appl. Environ. Microbiol. 77, 2905–2915.

Sillers, R., Chow, A., Tracy, B., Papoutsakis, E.T., 2008. Metabolic engineering of the non-sporulating, non-solventogenic *Clostridium acetobutylicum* strain M5 to produce butanol without acetone demonstrate the robustness of the

acid-formation pathways and the importance of the electron balance. Metab. Eng. 10, 321–332.
Stim-Herndon, K.P., Nair, R., Papoutsakis, E.T., Bennett, G.N., 1996. Analysis of degenerate variants of *Clostridium acetobutylicum* ATCC 824. Anaerobe 2, 11–18.
Sylvia, H., Jabbari, S., Millat, T., Janssen, H., Fischer, R.J., Bahl, H., King, J.R., Wolkenhauer, O., 2011. A systems biology approach to investigate the effect of pH-induced gene regulation on solvent production by *Clostridium acetobutylicum* in continuous culture. BMC Syst. Biol. 5 (10), 1–13.
Tashiro, Y., Shinto, H., Hayashi, M., Baba, S., Kobayashi, G., Sonomoto, K., 2007. Novel high-efficient butanol production from butyrate by non-growing *Clostridium* saccharoperbutylacetonicum N1-4 (ATCC 13564) with methyl viologen. J. Biosci. Bioeng. 104, 238–240.
Tracy, B.P., 2012. Improving butanol fermentation to enter the advanced biofuel market. mBio 3, 1–3.
Tomas, C.A., Welker, N.E., Papoutsakis, E.T., 2003. Overexpression of *groESL* in *Clostridium acetobutylicum* results in increased solvent production and tolerance, prolonged metabolism, and changes in the cell's transcriptional program. Appl. Environ. Microbiol. 69, 4951–4965.
Vasconcelos, I., Girbal, L., Soucailie, P., 1994. Regulation of carbon and electron flow in *Clostridium acetobutylicum* grown in chemostat culture at neutral pH on mixtures of glucose and glycerol. J. Bacteriol. 176, 14431450.
Yu, M.R., Zhang, Y.L., Tang, I.C., Yang, S.T., 2011. Metabolic engineering of *Clostridium tyrobutyricum* for n-butanol production. Metab. Eng. 13, 373–382.