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Characterization of a butanol–acetone-producing *Clostridium* strain and identification of its solventogenic genes

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HIGHLIGHTS

- Isolation and characterization of a wild-type *Clostridium* species strain G117.
- G117 is capable to produce 13.50 g/L butanol when fed with 60 g/L glucose.
- G117 also distinguishes itself by generating negligible amount of ethanol.
- G117 butanol dehydrogenase was found to be a putative gene for butanol production.

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ABSTRACT

A unique *Clostridium* species strain G117 was obtained in this study to be capable of producing dominant butanol from glucose. Butanol of 13.50 g/L was produced when culture G117 was fed with 60 g/L glucose, which is ~20% higher than previously reported butanol production by wild-type *Clostridium acetobutylicum* ATCC 824 under similar conditions. Strain G117 also distinguishes itself by generating negligible amount of ethanol, but producing butanol and acetone as biosolvent end-products. A butanol dehydrogenase gene (*bdh* gene) was identified in strain G117, which demonstrated a ~200-fold increase in transcription level measured by quantitative real-time PCR after 10 h of culture growth. The high transcription suggests that this *bdh* gene could be a putative gene involved in butanol production. In all, *Clostridium* sp. strain G117 serves as a potential candidate for industrial biobutanol production while the absence of ethanol ensures an economic-efficient separation and purification of butanol.

1. Introduction

The global energy crisis has renewed interest in producing alternative liquid fuels (e.g., generation of biofuels via biological processes) from clean, renewable sources (Gheshlaghi et al., 2009; Lee et al., 2009). Strict anaerobic, spore-forming bacteria such as *Clostridium* have been reported to produce biosolvents including acetone, butanol, and ethanol (Bramono et al., 2011; Chen, 1995) by fermenting a wide range of monosaccharides and hydrolysate of oligosaccharides (Nair et al., 1994). Comparing with ethanol, butanol shows advantage as an alternative liquid fuel since it possesses higher energy content and lower volatility (Adsul et al., 2011; Bramono et al., 2011; Cheng et al., 2012; Lee et al., 2009; Shi and Blaschek, 2008). However, among all the solvent-producing microorganisms, only a few strains belonging to the genus of *Clostridium* produce butanol as a major fermentation product (Chen, 1995). For example, *Clostridium acetobutylicum* ATCC 824 and *Clostridium beijerinckii* NCIMB 8052 are the two most well-known butanol-producing species (Gu et al., 2009; Shi and Blaschek, 2008), the wild-type of which were reported to produce ~9–12 g/L butanol in a batch culture fed with 40–60 g/L of glucose (Formanek et al., 1997; Monot et al., 1982). A recently reported *Clostridium* sp. strain BOH3 was also able to generate 7.05 g/L butanol after consuming 30 g/L glucose (Bramono et al., 2011). Usually, accumulation of >7.4 g/L butanol inhibits *Clostridium* growth and sugar uptake rate (Lee et al., 2008); hence causing fermentation to abort untimely and resulting in low butanol yield. In turn, this boosts up costs associated with butanol purification and makes large-scale butanol production infeasible (Ezeji et al., 2007). The search for novel wild-type microbes with enhanced butanol production and resistance is thus of great importance for the biofuel industry.

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During the fermentation process, butanol generation usually occurs in the solventogensis phase (the production of acetone, butanol, and ethanol) right after the acidogenesis phase (the production of acetic and butyric acids) (Adsul et al., 2011; Cheng et al., 2012; Dürre et al., 1995; Nair et al., 1994; Shi and Blaschek, 2008; Thormann et al., 2002; Wilkinson et al., 1995). The formation of butanol during solventogenesis is always carried out by aldehyde dehydrogenase (ALDH; EC 1.2.1.10), butanol dehydrogenase (BDH; EC 1.1.1.x), and alcohol dehydrogenase (ADH; EC 1.1.1.1, 1.1.1.2, 1.1.1.71). ALDH catalyzes the production of the respective aldehydes from butyryl-CoA and acetyl-CoA. Butyraldehyde is then converted to butanol by (BDH) or ADH. Functional BDH enzymes are primarily NAD(P)H-dependent and exist as homodimers with each subunit averaging 42kD in molecular mass (Welch et al., 1989). Given that the final step of butanol production is catalyzed by butanol dehydrogenase (Gheshlaghi et al., 2009; Nair et al., 1994; Yan et al., 1988), the presence and characterization of the *bdh* gene in *Clostridium* bacteria become important. For the renowned butanol-producing wild-type *Clostridium* such as *C. acetobutylicum* ATCC 824 and *C. beijerinckii* NCIMB 8052, their *bdh* genes have been discovered and characterized in their genomes (Dürre et al., 1995; Fontaine et al., 2002; Shi and Blaschek, 2008; Thormann et al., 2002).

The aim of this study is to cultivate and characterize a new butanol–acetone-generating microorganism, *Clostridium* species strain G117, which is able to ferment glucose via AB (acetone–butanol) process while exhibiting high butanol yield. A putative *bdh* gene in strain G117 was also identified, the transcription level of which was measured by quantitative real-time PCR.

2. Methods

2.1. Culture isolation and characterization

Soil samples from grassland in Singapore (the tropical climate) were used as inocula in order to screen bacteria capable of producing biofuels. The soil samples were added into anaerobic and autoclaved bottles containing 10 ml of RCM (Reinforced Clostridial Media, Oxoid). After 48-h incubation at 35 °C and a shaking speed of 125 rpm, 0.5 ml culture was spread on a Petri dish containing RCA (Reinforced Clostridial Agar, Oxoid) medium, which was conducted in an anaerobic chamber. One small drop of 5% w/v sodium nitroprusiate and 25% w/v ammonium hydroxide was used to evaluate for acetone production. Acetone-positive colonies forming a violet ring were picked and inoculated into 10 ml RCM to select for acetone/butanol-producing bacteria (Montoya et al., 2000). Eventually, a butanol-producing *Clostridium* bacterium named G117 was obtained.

Genomic DNA of *Clostridium* sp. strain G117 was extracted and purified by using the DNeasy Tissue Kit (QIAGEN GmbH, Germany). The 16SrRNA genes were amplified using a pair of universal primers, 8F (5′-AGA GTT TGA TCC TGG-3′) and 1541R (5′-AAG GAG GTG ATC CAG CCG CA-3′). Amplicons were purified using a commercial DNA purification kit (QIAGEN GmbH, Germany) and sequenced by 1st Base, Singapore (BigDye® Terminator v3.1 cycle sequencing kit and Applied Biosystems Genetic Analyzer). The 16SrRNA gene sequence was aligned using the BLAST algorithm and was deposited in the GenBank database with an accession number JX091678.

2.2. Growth conditions of culture G117

For culture G117, time-course studies were performed in a defined medium as described below. A reduced mineral salts medium (35 mL) (He et al., 2007) was prepared in a 160 mL serum bottle and supplemented with Wolin solution. Trace amount of reducing agents was added to remove any trace amount of oxygen. Glucose (30 or 60 g/L) was added as a carbon source. Active culture G117 (10% v/v) was inoculated into triplicate bottles for each substrate, and incubated at 35 °C with a shaking speed of 125 rpm. Samples were drawn once per day for five days. The pH was adjusted daily with the addition of 5 M NaOH. Growth of culture G117 was tested between pH 5.0 and pH 7.2. In one set of study, three pHs of 5.5, 6.0 and 6.5 were set up to investigate the best solventogenic pH for culture G117. The defined medium was also supplemented with 0%, 0.1%, 0.4%, or 1.0% yeast extract (w/v) to investigate its effect on solvents production.

2.3. Chemical analysis

Samples were extracted from batch cultures for identification and quantification of final products. Volatile fatty acids (VFAs, i.e., acetic and butyric acids) and biosolvents (i.e., acetone, butanol, and ethanol) were measured with a Agilent Technologies 7890A Gas Chromatography (GC) equipped with a Flame Ionization Detector (FID) and a Durabond (DB)-WAXetr column (30 m × 0.25 mm × 0.25 μm, model 123–7334; J&W, USA). The oven temperature was first held at 60 °C for 2 min, followed by a gradual increase of temperature at 15 °C/min to 230 °C, and held for 1.7 min. Helium was used as the carrier gas with a column flow rate of 1.5 mL/min. Five-point standard curves were obtained by running standard solutions containing acetone, butanol, ethanol, acetic acid, and butyric acid.

The residual glucose concentration in the medium was measured on a YSI 2700 SELECT Biochemistry Analyzer (YSI Life Sciences, Yellow Springs, OH, U.S.A.) by using a Glucose Membrane Kit (part No. YSI 2365).

2.4. Identification of putative butanol dehydrogenase (bdh) gene

One pair of degenerate primers, forward primer (5′-GCA TGG CMG WTT TAC WTT ACC-3′) and reverse primer (5′-GCT TAR AAR TYR ACW TYW BYT CC-3′) were designed based on the known sequences of *bdh* genes of *Clostridium* to target the possible *bdh* genes present in isolate G117. The purified PCR products were subsequently cloned with a pCR2.1-TOPO TA cloning kit (Invitrogen, Carlsbad, CA, U.S.A.). Clones with plasmids containing inserts were extracted with a Qiaprep Spin Miniprep kit (QIAGEN, GmbH, Germany) and sent for sequencing with primers M13F (5′-GTAAAACGACGGCCAGT-3′) and M13R (5′-GCGGATAACAATTTC-ACACAGG-3′). The *bdh* gene sequence was aligned using the BLAST algorithm and was deposited in the GenBank database with an accession number JX091679.

2.5. Quantitative real-time PCR

A quantitative real-time PCR (qPCR) (ABI 7500 Fast real-time PCR system; ABI, Foster City, CA) assay was performed using QuantiTect SYBR Green (QIAGEN, GmbH, Germany) assays to detect and quantify the transcription levels of *bdh* gene in strain G117. The *bdh* gene-specific primers used for qPCR were designed based on the *bdh* gene sequence of culture G117, with the following forward primer (5′-CGA ATT AAT TAA TGG ATT AAA TGA TAA GTT AGA-3′) and reverse primer (5′-TTA CAA ATT AAC TTT AGT TCC ATA GTA TGT-3′). Another set of primers were also designed with the following sequence: 338F primer (5′-ACTCCTACGGGAGGC-3′) and 518R primer (5′-ATTACCGCGGCTGG-3′) to target the 16SrRNA gene. A standard curve spanned a range of 10^3–10^7 gene copies per μL of template DNA was obtained by using G117 genomic DNA. Amplification of the *bdh* genes in culture G117 was performed at an annealing temperature of 54 °C for all assays. The 9600 Emulation PCR cycle parameters were as follows: 2 min at 50 °C and 10 min at

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95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 54 °C. The reported *bdh* transcripts were values after being normalized to cell numbers (at each sampling point) of culture G117 as measured by the 16S rRNA gene copies.

3. Results and discussion

3.1. Isolation and phylogenetic analysis of Clostridium sp. strain G117

In order to screen butanol-generating microbes, microcosms were set up with inocula from soil samples collected from grasslands in Singapore. After a 24-h of incubation, serial dilution of the supernatant was carried out. The diluted supernatant of 0.1 ml was spread-plated to RCA agar plates and incubated in an anaerobic chamber for 48 h in order to obtain individual colonies. Since acetone–butanol generating colonies would show a violet color in the presence of 5% w/v sodium nitroprusiate and 25% w/v ammonium hydroxide, violet colonies were picked and inoculated separately to RCM medium spiked with glucose (30 g/L). Among the cultures, the one with the highest butanol generation as measured by GC was selected to conduct three more transfers on agar plates to ensure its purity. After transferring one of the colonies back to RCM medium, the culture still showed similar amount of butanol generation as its parent culture. Therefore, it is designated culture G117 which could produce decent amount of butanol. Under light microscopy, culture G117 showed uniform rod-shaped morphology, indicating the purity of the culture.

The 16S rRNA genes amplified from genomic DNA of culture G117 showed 99% identity to *C. beijerinckii* NCIMB 8052 (Table 1) when blasted with the bacterial sequences in the GenBank database. This isolate is designated *Clostridium* species strain G117. *Clostridium* species have been reported to be a group of endospores forming, motile, rod-shaped, obligate anaerobic prokaryotes that produce acids and solvents. Most of the wild-type *Clostridium* strains are known to produce little or no butanol, however, only a few of *Clostridium* strains produce high amount of butanol as the major fermentation product (Chen, 1995). Therefore, the newly isolated wild-type solvent-producing *Clostridium* sp. strain G117 broadens our knowledge and adds to the pool of known butanol-producing microbes.

3.2. Butanol production from glucose by culture G117

In order to optimize butanol production by culture G117, batch experiments in triplicates were conducted in mineral salts medium spiked with glucose (30 g/L) at a constant pH of 5.5, 6.0, or 6.5. After 5 days of incubation, culture G117 produced similar amount of butanol at pH 6.0 (6.45 g/L) and pH 5.5 (5.93 g/L), while lower butanol (5.61 g/L) was produced at pH 6.5 (Fig. 1). Accompanying with butanol generation, 3.22 g/L acetone, 0.81 g/L acetate, and 0.81 g/L butyrate also appeared as the fermentation products (Fig. 1). Interestingly, negligible amount of ethanol was observed in the entire fermentation process of culture G117, demonstrating a different solvent production profile of AB (acetone–butanol) process comparing with its phylogenetically closest strain – *C. beijerinckii* NCIMB 8052 with an ABE (acetone–butanol–ethanol) fermentation process by producing 4.4, 9.2 and 0.9 g/L, respectively when fed with 60 g/L glucose (Shi and Blaschek, 2008). Although the wild-type NCIMB 8052 produces ethanol, the ratio of ethanol to total solvents (acetone, butanol, and ethanol) is lower than *C. acetobutylicum* ATCC 824 that usually produces acetone, butanol and ethanol at a ratio of 3:6:1 (Formanek et al., 1997). With the absence of ethanol in the fermentation broth, culture G117 shows a neat solvent production profile with acetone and butanol, indicating an advantage in simplifying the following on separation process.

Though culture G117 proliferates between pH 5.0 and pH 7.2, high butanol production of culture G117 was observed between pH 5.5 and pH 6.0. Similar to *C. acetobutylicum* ATCC 824 and *C. beijerinckii* NCIMB 8052, solvent production was enhanced when G117's growth was initiated at slightly acidic pH (Fontaine et al., 2002). Just as most butanol-generating *Clostridium* bacteria, formation of solvents by G117 initiates at the end of log growth phase, being triggered by the production of organic acids during its initial growth phase (Adsul et al., 2011; Cheng et al., 2012; Dürre et al., 1995; Gheshlaghi et al., 2009). Notably, maintaining pH at 5.5–6.0 for culture G117 was found to be critical for high butanol production. We speculated that pH 5.5–6 provides the best metabolic state for solvent production by culture G117, as it allows initial robust growth without creating an early inhibitory acidic environment on cell growth (Gao et al., 2012). Studies have shown that the irreversible onset of sporulation reduces the duration of the solventogenic phase (Awang et al., 1992). For culture G117, butanol production drops as pH decreases and severe sporulation occurs when pH < 4.5, and butanol production cannot be reinstated after the pH is adjusted back to the optimum range. Maintenance of H^+ ion concentration within a physiological range is thus necessary to encourage bacterial growth and to prevent sporulation. Therefore, by preventing sporulation, pH 5.5–6.0 enables butanol generation effectively by directing carbon flow so as to maximize carbon conversion to butanol.

Previous reports indicate that supplementing medium with 0.3–0.5% yeast extract is a common practice in biosolvent (e.g., acetone, butanol) production (Fontaine et al., 2002; Yan et al., 1988; Yu et al., 2011). Bacto tryptone, peptone or hydrolyzed casein has also been used in various quantities in addition to yeast extract (Fontaine et al., 2002; Yan et al., 1988). To investigate the effect of yeast extract on G117's butanol production, three batches of defined medium were supplemented with 0.1%, 0.4% and 1.0% yeast extract in the presence of 30 g/L of glucose at pH 6.0. Butanol production significantly improved to 8.52 and 8.61 g/L when G117 was cultured with 0.4% and 1.0% yeast extract, but not in 0.1% yeast extract (Fig. 2). In the meantime, the butanol/butyrate molar ratio also increased to 13.87, comparing with the ratio of 6.04 without the addition of yeast extract. The butanol yield (gram-butanol/gram-glucose consumed) also increased to 28.56% (g/g) in 0.4% yeast extract from an initial 23.83% without yeast extract (Table 2). Real-time PCR measured the maximum cell numbers in the cultures reaching 2.9×10^8 cells/ml. Therefore, different from previous studies requiring a mixture of complex nutrients such as bacto tryptone, peptone or hydrolyzed casein, yeast extract alone could exert a strong influence on culture G117's butanol production and end product composition, presumably by maximizing cell growth (Li et al., 2011; Lilit et al., 2012); in return, the high microbial population was able to completely utilize glucose during solventogenesis and to efficiently convert carbon to butanol. Overall, yeast extract of 0.4% was selected to be added to the medium in following on experiments. This finding is noteworthy because the butanol yield of culture G117 (28.56%) is the highest among other solvent-producing *Clostridium* strains under similar

Table 1
Top matches of the 16S rRNA gene sequence of isolate G117 against known bacterial sequences in the Genbank database (BLAST, NCBI).

Microorganism	Identity (%)
Clostridium beijerinckii NCIMB 8052	99
Clostridium sp. DL-VIII CDLVIII scaffold_1_Cont1	99
Clostridium sp. DL-VIII CDLVIII scaffold_1_Cont2	99
Clostridium butyricum 5521 gcontig_1106103650310	98
Clostridium butyricum E4 strain 'BoNT E BL5262' CLP.Contig71	98
Clostridium acetobutylicum ATCC824	88

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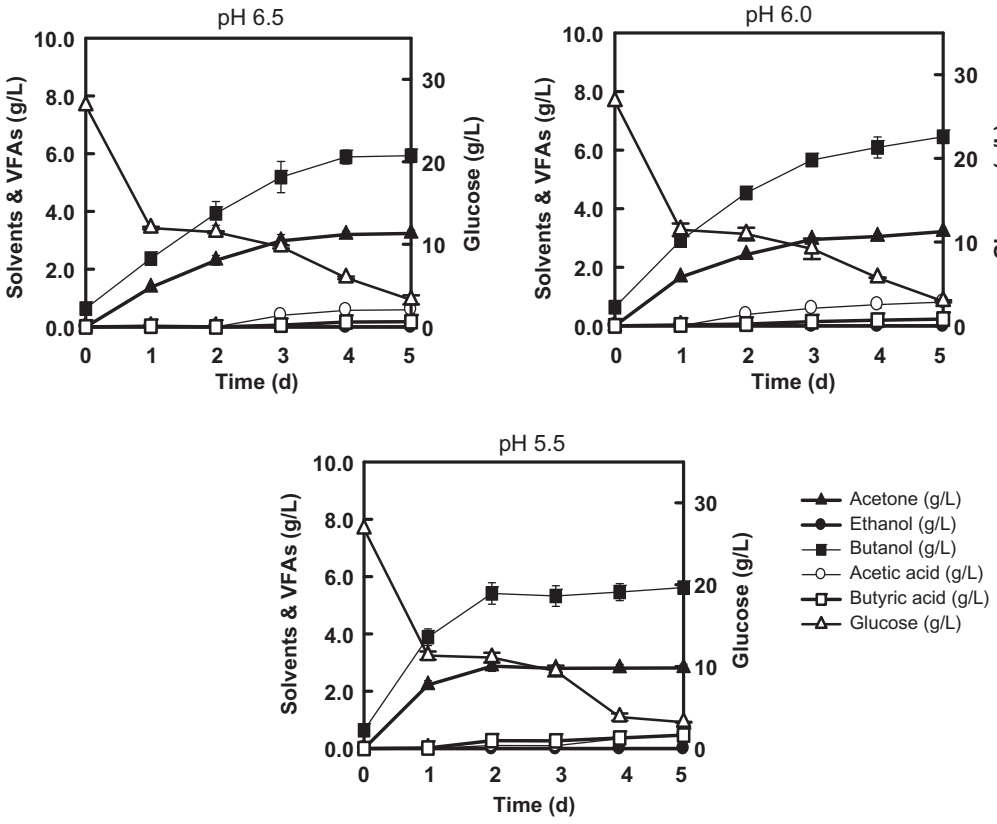

Fig. 1. Biosolvents and VFAs production by *Clostridium* sp. strain G117 fed with glucose (30 g/L) at a pH of (a) 6.5, (b) 6.0 and (c) 5.5.

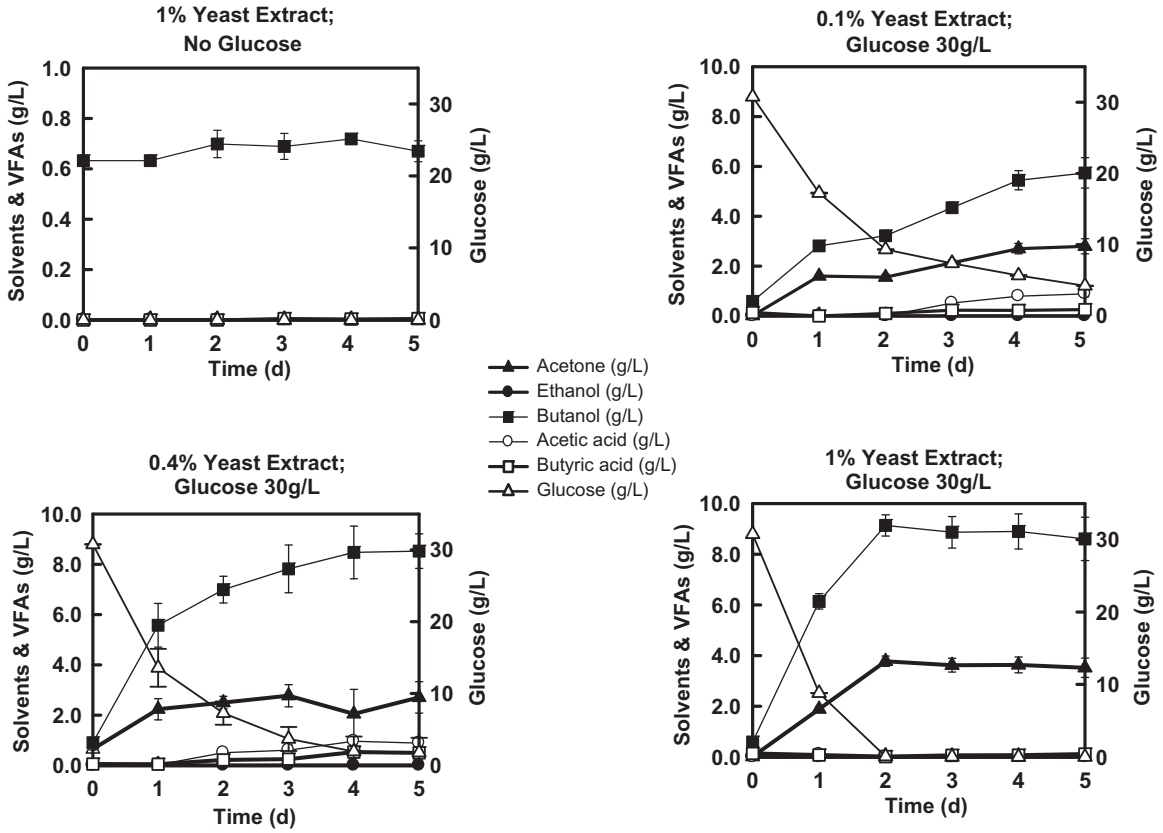

Fig. 2. Biosolvents and VFAs production by *Clostridium* sp. G117 at various amount of yeast extract.

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Table 2
Production and yield of biosolvents and VFAs by *Clostridium* sp. G117 when fed with 30 g/L glucose (1) maintaining pH at 6.5, 6.0, or 5.5, (2) at a constant pH of 6.0 but spiked with various amount of yeast extract of 0.1%, 0.4%, 1.0% in the growth medium.

	pH 5.5, 30 g/L glucose	pH 6.0, 30 g/L glucose	pH 6.5, 30 g/L glucose	pH 6.0, 30 g/L Glucose, 0.1% yeast extract	pH 6.0, 30 g/L Glucose, 0.4% yeast extract	pH 6.0, 30 g/L Glucose, 1% yeast extract	pH 6.0, 60 g/L Glucose 0.4% yeast extract
Butanol (g/L)	5.93	6.45	5.61	5.74	8.52	8.61	13.50
Acetone (g/L)	3.23	3.22	2.81	2.79	2.70	3.63	4.94
Acetate (g/L)	0.59	0.81	1.12	0.89	0.88	0	0.64
Butyrate (g/L)	0.63	0.81	0.71	0.89	1.75	0.23	1.30
Butanol/acetone	1.44	1.57	1.56	1.61	2.47	1.92	2.14
Butanol/butyrate	7.79	6.04	5.97	7.66	5.80	13.87	12.34
Glucose consumed (g/L)	26.79	27.08	26.72	25.77	29.84	30	44.43
Yield (%, gram butanol/ gram glucose consumed)	22.13	23.83	21.01	22.26	28.56	28.68	30.38

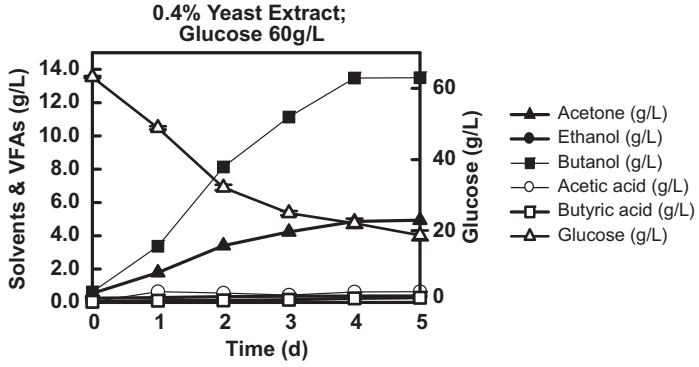

Fig. 3. Biosolvents and VFAs production by *Clostridium* sp. stain G117 fed with 60 g/L Glucose and with 0.4% Yeast Extract.

Table 3
Top matches of G117-*bdh* gene sequence against previous known *bdh* gene and other alcohol dehydrogenase sequences in the Genbank database (BLAST, NCBI).

Microorganism	Identity (%)
Clostridium beijerinckii NCIMB 8052, BDH 1	98
Clostridium beijerinckii NCIMB 8052, BDH 2	90
Clostridium botulinum E1 strain 'BoNT E Beluga' CLO.Contig141	87
Clostridium botulinum B strain Eklund 17BVS	87
Clostridium butyricum E4 strain 'BoNT E BL5262' CLP.Contig70	87
Clostridium butyricum 5521 gcontig_1106103650254 'BoNT E BL5262'	84
Clostridium acetobutylicum ATCC824, ADHE2	44
Clostridium acetobutylicum ATCC824, ADHE	38
Clostridium acetobutylicum ATCC824, BDHA	37
Clostridium acetobutylicum ATCC824, BDHB	36

growth conditions, which can be due to no carbon flowing to ethanol. Most importantly, culture G117 is able to produce 13.50 g/L butanol at a yield of 30.38% when fed with 60 g/L of glucose with 0.4% yeast extract (Fig. 3), which is ~20% higher than previous reported butanol concentration of 11.25 g/L generated by wild-type *C. acetobutylicum* ATCC 824 (Gu et al., 2009; Lee et al., 2008) under similar growth conditions. Therefore, culture G117 is a promising candidate for industrial biobutanol production.

3.3. Sequence analysis of bdh gene in isolate G117

Since the *bdh* gene encodes butanol dehydrogenase responsible for butanol formation, it is important to identify if this gene is present in strain G117 towards understanding its characteristics such as the cofactor binding sites for further optimization of this strain. Degenerate primers were designed based on the *bdh* gene sequences in known butanol-generating *Clostridium* species such as *C. beijerinckii, Clostridium butyricum, and Clostridium botulinum*. The putative *bdh* genes were PCR-amplified by targeting the genomic DNA of culture G117 with the degenerate primers, which were then cloned and sequenced. One putative *bdh* gene was identified in strain G117. Sequence comparison with previous known butanol/alcohol dehydrogenase genes resulted a sequence identity of 98% (*C. beijerinckii* NCIMB 8052_BDH1) to 84% (*C. butyricum* 5521 gcontig_1106103650254 'BoNT E BL5262') (Table 3). But the *bdh* gene in strain G117 shows distant relationship (44–36% identity) with other alcohol dehydrogenase sequences (Table 3). Noteworthy, the *bdh* gene of strain G117 exhibits 98% and 90% sequence identity (Table 3) with its closest two *bdh* genes (designated *bdh1* and *bdh2*) of *C. beijerinckii* NCIMB 8052, which are characterized as the iron-containing alcohol dehydrogenase genes. Since the *adh* (alcohol dehydrogenase) genes and the bifunctional *adhE and adhE2* (acetaldehyde/alcohol dehydrogenase) genes encode a class of enzymes to produce alcohols including butanol

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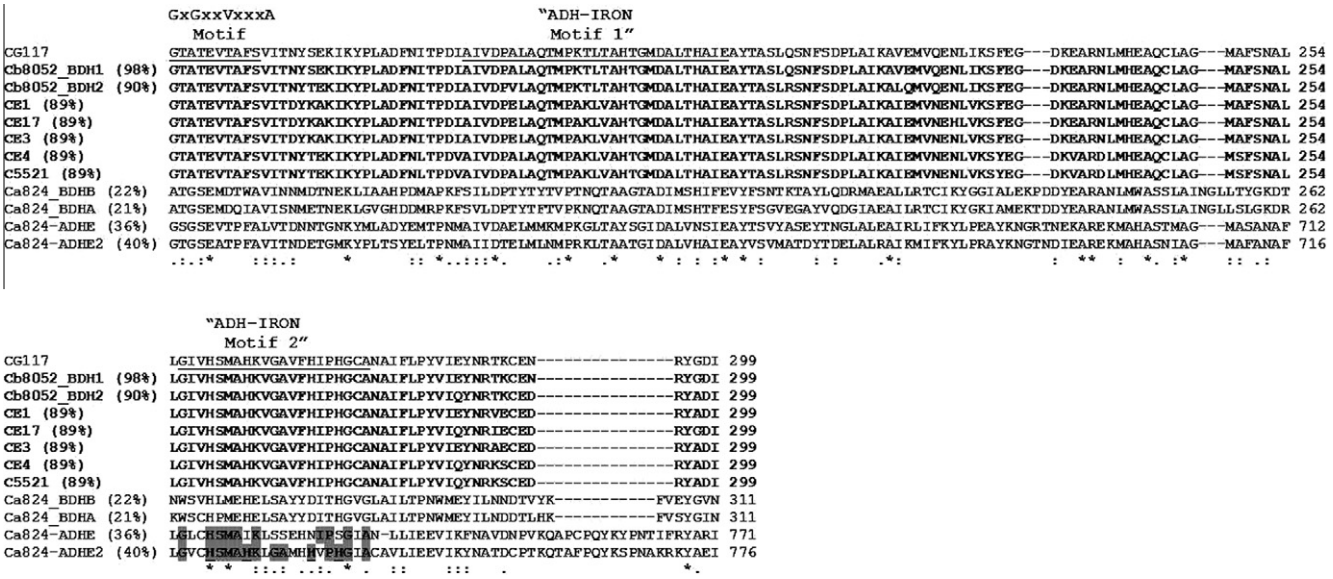

Fig. 4. Sequence Alignment of G117-BDH and its Homologs. Rows 1 to 8: Sequence alignment of *Clostridium* sp. G117 BDH (row 1) with the closest six BDH homologs (black) was performed using ClustalW. Rows 9 to 12: Sequence alignment of *Clostridium* sp. G117 BDH (row 1) with the *Clostridium acetobutylicum* ATCC 824 BDH B, BDH A, ADHE and ADHE2. The amino acids are in one-letter codes; the strictly conserved and semiconserved residues are indicated by asterisk and colon respectively. The GxGxxVxxxA motif is underlined. The two conserved motif: ADH-IRON 1 and 2 (labeled and underlined) of G117 BDH are located at 171 to 199 and 256 to 276 respectively. Motif GIVHSMAHKVGAVFHIPHGCA (ADH-IRON 2) of G117 BDH is underlined. Conserved residues of Ca_ADHE2, Ca_ADHE with CG42 BDH in ADH-IRON motif 2 are highlighted in grey; conserved histidine residues in underlined, bold. CG42: BDH, *Clostridium* sp. G117; Cb8052_BDH1: BDH1, *Clostridium beijerinckii* 8052; Cb8052_BDH2: BDH2, *Clostridium beijerinckii* 8052; CE1: BDH, *Clostridium botulinum* E1 strain 'BoNT E Beluga' CLO.Contig141; CE17: BDH, *Clostridium botulinum* B strain Eklund 17BVS; CE4: BDH, *Clostridium butyricum* E4 strain 'BoNT E BL5262' CLP.Contig70; C5521: BDH, *Clostridium butyricum* 5521 gcontig_1106103650254 'BoNT E BL5262'; Ca824_BDHB: BDHB, *Clostridium acetobutylicum* ATCC 824; Ca824_BDHA: BDHA, *Clostridium acetobutylicum* ATCC 824; Ca_ADHE: ADHE, *Clostridium acetobutylicum* ATCC 824; Ca_ADHE2: ADHE2, Clostridium acetobutylicum ATCC 824.

Table 4
Two conserved motifs of G117-BDH identified by using the "Prosite Pattern" database exhibit Iron-containing alcohol dehydrogenase signature 1 and 2.

Description	Position	Motif
Iron-containing alcohol dehydrogenase signature 1 (ADH-IRON 1)	171–199	[STALIV]-[LIVF]-x-[DE]-x(6,7)-P-x(4)-[ALIV]-x-[GST]-x(2)-D-[TAIVM]-[LIVMF]-x(4)-E.
Iron-containing alcohol dehydrogenase signature 2 (ADH-IRON 2)	256–276	[GSW]-x-[LIVTSACD]-[GH]-x(2)-[GSAE]-[GSHYQ]-x-[LIVTP]-[GAST]-[GAS]-x(3) -[LIVMT]-x-[HNS]-[GA]-x-[GTAC].

(Goodlove et al., 1989), their amino acid sequences were compared with that of G117 BDH. Blast alignment revealed that G117-BDH shares 22% and 21% identity with *C. acetobutylicum* ATCC 824 BDH B and BDH A, respectively (Fig. 4). However, G117 BDH shows greater homology of 36% and 40% sequence identity to the C-terminal alcohol dehydrogenase domains of the bifunctional ADHE and ADHE2 of *C. acetobutylicum* ATCC 824, respectively (Fig. 4).

In order to characterize G117 *bdh* gene, its sequence was also compared with the "Prosite Pattern" database (Sigrist et al., 2010) to identify conserved protein motifs. There are currently 33 and 30 related protein sequences containing the ADH-IRON 1 and 2 motifs in the database. *C. acetobutylicum* ATCC 824 *adhE* and *bdhB* genes were found to contain ADH IRON 1 motif, while that of *bdhA* gene contains both motifs (Fig. 4). As for the closest strain to G117, *C. beijerinckii* NCIMB 8052 *bdh1 and bdh2* genes (not in the "Prosite Pattern" database) were also found containing both motifs (Fig. 4). Not surprisingly, PROSITE analysis (Sigrist et al., 2010) revealed that two conserved Iron-containing alcohol dehydrogenase (ADH) signature 1 and 2 of type III ADHs motifs were present in the G117-*bdh* gene (Table 4). ADH signature 2 motifs of G117 BDH – GIVHSMAHKVGAVFHIPHGCA (labeled and underlined in Fig. 4) is highly conserved with the seven most closely related BDHs (in bold in Fig. 4). In comparison, ADH signature 2 motif of G117 is found to have fourteen conserved residues with that of ADHE2 and ten conserved residues with that of ADHE (conserved residues highlighted in grey in Fig. 4). Among the fourteen conserve residues in the ADH signature 2, there are four histidine residues (underlined bold in Fig. 4) in the motif G117 (Fig. 4). The presence of these conserved histidines suggests the requirement of Fe^{2+}/Zn^{2+} as a cofactor for BDH catalytic activities (Dürre et al., 1995) in *Clostridium* species including strain G117.

In general, all bifunctional acetaldehyde/alcohol dehydrogenases including ADHE2 contain two conserved nucleotide-binding sites (NBS), GVGxG and GCGxxG, located at the N-terminus of the proteins (Dürre et al., 1995). Since BDHs contain only the ADH domain, the two NBSs are absent and therefore suggesting a different localization of the NAD(H) or NADP(H) binding sites for BDHs. The GxGxxVxxxA motif, first reported in *C. acetobutylicum* ATCC 824 ADHE (ADH domain), was found to be highly conserved among ADHs. The GxGxxVxxxA conserved sequence among the bifunctional acetaldehyde/alcohol dehydrogenases is speculated to be implicated in NAD(H) or NADP(H) coenzyme binding (Fontaine et al., 2002; Nair et al., 1994). In place of this ADH signature motif, BDH of G117 and the seven closely related BDHs all possess

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another highly conserved GTATEVTAFS sequence instead (Fig. 4). Although this latter sequence is highly conserved among BDH proteins, it can be partially conserved in the ADH signature motif.

3.4. Expression analysis of bdh gene in strain G117

In order to verify whether the identified *bdh* gene was expressed in strain G117, a qPCR assay was performed to quantify the mRNA copies (as measured as cDNA) transcribed from the G117-*bdh* genes. After starving culture G117 for 72 h, the culture was re-inoculated into a fresh medium containing 30 g/L glucose. Total RNA was extracted from the cultures at different time points, followed by reverse transcription, and subsequent qPCR with primer pairs designed based on the *bdh* gene sequence. At time zero, negligible amounts of *bdh* cDNAs were detected by qPCR. Expression of the *bdh* gene started after 5 h and exhibited highest expression after 10 h of culture growth. The actual gene transcription was normalized to cell numbers of culture G117, which was measured as 16S rRNA gene copies. Notably, the known 14 copies of 16S rRNA gene per genome of *C. beijerinckii* NCIMB 8052 (Wilkinson et al., 1995; Wilkinson and Young, 1995) was also used for strain G117, since G117's 16S rRNA gene sequence shows highest similarity with NCIMB 8052. With the above information, the transcripts of *bdh* gene was calculated to express ~200-fold after 10 h when normalized to the cell numbers of culture G117. The high gene transcription level suggests that the identified *bdh* gene in culture G117 is a putative gene for butanol generation.

4. Conclusions

A wild-type *Clostridium* sp. strain G117 was obtained in this study, which distinguishes itself from other solventogenesis. Strain G117 could produce up to ~13.50 g/L butanol, ~20% higher than previous reported wild type microbes operating at similar conditions. The *bdh* gene characterized from strain G117 was highly transcribed as measured by quantitative real-time PCR, suggesting its role as a putative gene for butanol production. In summary, G117 is promising due to its: (i) capability to produce high concentrations of butanol; (ii) absence of ethanol to ensure following on application of economic-efficient separation of butanol and acetone; and (iii) potential application for industrial biofuel production.

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