World J Microbiol Biotechnol (2013) 29:1059–1065
DOI 10.1007/s11274-013-1269-5

ORIGINAL PAPER

Isolation, characterization and evaluation of hyper 2-propanol producing bacteria from Singapore environment

Zhi Rui Ng · Katsuyuki Takahashi · Zhibin Liu

Received: 25 September 2012 / Accepted: 21 January 2013 / Published online: 30 January 2013

Abstract Three hyper 2-propanol producing strains were isolated from Singapore environment using an enrichment step and a high through-put screening step. The analysis of the amplified 16S rDNA revealed that the isolates belonged to *Clostridium* species and they were named as *Clostridium* sp. BT10-1, *Clostridium* sp. M10-1 and *Clostridium* sp. PU31-4. At 1 L scale, the 2-propanol titer of these positive strains was 1.6–2.1 times of that of *Clostridium beijerinckii* NRRL B593, which is so far the most efficient natural 2-propanol producer. The highest 2-propanol titer was achieved by isolate BT10-6 and it was 5.26 g/L (87.5 mM). These three positive strains BT10-6, M10-1 and PU31-4 consumed glucose almost completely in 40–48 h and gave 2-propanol productivity at 0.132, 0.118 and 0.087 g/L/h, respectively, which is 3.0–4.6 times of 0.029 g/L/h given by *C. beijerinckii* NRRL B593. Butanol was also produced by these positive strains with a slightly lower butanol titer and higher butanol productivity, compared to butanol control strain *C. beijerinckii* NCIMB 8052.

Keywords Isolation · 2-Propanol · Fermentation · *Clostridium* · Singapore

Z. R. Ng · Z. Liu (✉)
Institute of Chemical and Engineering Sciences, 1 Pesek Road, Jurong Island, Singapore 627833, Singapore
e-mail: liu_zhibin@ices.a-star.edu.sg

Present Address:
Z. R. Ng
Abbott Laboratories Singapore, Neuros #03-01, 8 Biomedical Grove, Singapore 138665, Singapore

K. Takahashi
Mitsui Chemical Singapore R&D Centre, Pte. Ltd., #06-08/11 The Kendall, 50 Science Park Road, Singapore Science Park II, Singapore 117406, Singapore

Introduction

2-Propanol is currently produced by chemical routes that use petrochemical-derived precursors and it is widely used as solvent and chemical intermediates (Papa 2005). 2-Propanol can be also used as biofuel to partially replace gasoline and used instead of methanol to esterify fat and oil to produce biodiesel (Lee et al. 1995). Finally, it has its largest potential application that it can be dehydrated to yield propylene (Kibby and Hall 1972). Propylene is currently produced as a by-product from petroleum and natural gas industry and used as monomer for producing polypropylene. As the global demand for 2-propanol and propylene continues to increase and 2-propanol is one of the secondary alcohols that can be produced by fermentation using microorganisms (Osburn et al. 1937; Chen and Hiu 1986), an alternative to the petro-based production process is the production of 2-propanol by fermentation from biomass-derived sugars.

Several species have been reported for 2-propanol production, including solvents producing *Clostridium beijerinckii* strains (Osburn et al. 1937; Chen and Hiu 1986). The metabolic pathway of *C. beijerinckii* has been discovered. By Embden-Meyerhof route, 2-propanol is produced from hexose and pentose via pyruvate, which is a central element and usually converted into acetyl-CoA (Kasap 2002; Thauer et al. 1977). Acetyl-CoA is the branch point for solvents production and it can be further converted into 2-propanol by isopropanol dehydrogenase or butanol by butanol dehydrogenase.

The greatest limitations to biological production of 2-propanol are the very low 2-propanol titer and productivity of the existing *Clostridium* species. The reported maximum 2-propanol concentration achieved by natural *Clostridium* strains was 36 mM (Andreesen et al. 1989).

Some technologies have been made to improve the 2-propanol productivity. This includes continuous production using immobilized natural *Clostridium* or aggregate-forming *Clostridium* variant (Krouwel et al. 1983; Matsumura et al. 1992; Zoutberg et al. 1989) and reducing product inhibition by adding a polymer resin to adsorb products (Groot and Luyben 1986). Other in situ product recovery techniques, such as pervaporation (Groot et al. 1984; Qureshi et al. 1992; Qureshi and Blaschek 1999), perstraction (Qureshi et al. 1992), reverse osmosis (Garcia et al. 1986), liquid–liquid extraction (Evans and Wang 1988) and gas stripping (Ezeji et al. 2004, 2005), could be also used for improving solvent productivity. However, the challenges of low solvents titers and product inhibition are still existing even with these techiniques (Ezeji et al. 2010). The engineered *Escherichia coli*, which had the 2-propanol synthetic pathway by introducing key genes from natural 2-propanol producers, was also investigated for 2-propanol production. Only 2.76 g/L (46 mM) of 2-propanol in batch fermentation and 14 g/L (227 mM) of 2-propanol in continuous fed-batch fermentation were achieved (Hanai et al. 2007; Jojima et al. 2008).

The 2-propanol fermentation process must be economically feasible before it can replace the petrochemical processes, and the existing 2-propanol fermentation technologies using natural or engineered strains still could not achieve this goal. In this study, we describe the isolation and characterization of three hyper 2-propanol producing *Clostridium* strains from Singapore environment. The solvent production including 2-propanol and butanol of these three isolates were first tested in flasks and then scaled up at 1 L scale in 2 L fermenters for comparing the production titer, productivity and substrate consumption.

Materials and methods

Soil samples collection and pre-treatment

Soil samples were collected from nature reserves, agricultural areas and islands located in Singapore. A shovel was used to take samples from 10 to 15 cm of depth. Each sample was packed in a perfectly clean plastic bag and labeled to provide all technical information (i.e. time and location). Soil samples were finely ground and air-dried at room temperature before using.

Cultural enrichment of 2-propanol producing bacteria

3 g of pre-treated soil sample was suspended in 20 mL of potato medium in a sterile 50 mL conical plastic tube and heated at 70 °C for 10 min. It was then cooled down and cultivated in an anaerobic chamber (Forma Scientific 1025) with a high purity 85 % N_2, 10 % H_2 and 5 % CO_2 gas mixture. Palladium wafer was used as catalyst to maintain strict anaerobiosis. Gas production and turbidity were carefully checked each day. After 24–48 h incubation at 30 °C, samples with strong gas production and increased turbidity were selected for isolating 2-propanol producing bacteria. The Potato medium consisted of 1 L potato broth, 5 g sucrose, 1 g NH_4SO_4, 3 g $CaCO_3$ and 1 mL mineral solution (2.4 g/L $Na_2MoO_4{\cdot}2H_2O$, 0.24 g/L $CoCl_2{\cdot}6H_2O$, 1.5 g/L $CaCl_2{\cdot}2H_2O$, 27 g/L $FeCl_3{\cdot}6H_2O$, 0.25 g/L $CuSO_4{\cdot}5H_2O$, 0.29 g/L $ZnSO_4{\cdot}7H_2O$, 1.7 g/L $MnSO_4{\cdot}H_2O$, 12 g/L $MgSO_4$ and 20 mL/L H_2SO_4). To prepare potato broth, 500 g of potatoes were diced and placed in 1 L of deionized water and boiled for 30 min. The liquid named as potato broth was then passed through four layers of cheese cloth, centrifuged for 5 min at 3,000×*g* and stored at −20 °C until used.

Isolation of 2-propanol producing bacteria

The initial screening was started by serial dilution of the selected samples in sterile 0.85 % NaCl. The diluted samples were plated on potato agar medium (prepared by adding 20 g/L agar to potato medium before autoclaving) and incubated at 30 °C in anaerobic chamber for 48–72 h till single colonies formed. Colonies (based on morphology) were inoculated to 2 mL of T6 broth medium in 24-well deep plates. T6 broth medium consisted of 0.5 g/L KH_2PO_4, 0.3 g/L $MgSO_4{\cdot}7H_2O$, 0.01 g/L $FeSO_4{\cdot}7H_2O$, 3.0 g/L ammonium acetate, 2.0 g/L Yeast extract, 6.0 g/L Tryptone, 0.5 g/L cysteine HCl and 60 g/L glucose, pH 6.5. Butanol producing *C. beijerinckii* NCIMB 8052 and 2-propanol producing *C. beijerinckii* NRRL B593 were also inoculated to the same medium to be used as controls. After 96 h incubation at 30 °C in anaerobic chamber, the cells were spun down to collect the supernatant for analyzing the acetone production. 50 µL of supernatant was mixed with 100 µL of 0.1 M iodine solution and 75 µL of 2 M NaOH, yellow precipitates formed in the presence of acetone. 0.1 M iodine solution was prepared by dissolving 1.87 g of potassium iodide and 0.95 g of iodine in 37.5 mL of distilled water and stirring the mixture until it is homogeneous. The supernatants giving visible yellow precipitates were analyzed to determine the solvents producing profile using HPLC method. The isolates, which gave 50 % higher titers of 2-propanol than *C. beijerinckii* NRRL B593, were selected for strain purification and re-screening.

The 2-propanol positive strains were purified on potato agar medium and single colonies were cultivated in 20 mL of potato medium at anaerobic conditions. After 72 h incubation at 30 °C, samples were withdrawn to confirm the 2-propanol production and cultivation was continued

for another 2–3 days for spore formation. The cells with higher 2-propanol titer were finally collected by centrifugation at 4,000 rpm for 10 min and washed with sterile 0.85 % NaCl. Cell pellet was suspended in sterile 10 % (v/v) glycerol to prepare spore suspension which can be kept at 4 °C. To confirm the solvent production of the purified 2-propanol positives, 200 μL of spore suspension were heated at 70 °C for 10 min and then inoculated in 20 mL of potato medium. After 48 h cultivation at 30 °C in anaerobic chamber, 1 mL pre-culture was transferred to 20 mL of T6 broth medium for producing solvents including 2-propanol. Three replicates were performed for each positive and the cultures were incubated at 30 °C for 72 h.

Identification of the hyper 2-propanol producing isolates

The genomic DNA were extracted using Wizard® Genomic DNA Purification Kit (Promega, TM050). Forward primer (5′-AGAGTTTGATCCTGGCTCAG-3′) and reverse primer (5′-TACCTTGTTACGACTT-3′) (AITbiotech, Singapore) were used to amplify the 16S rDNA in 100 μL reaction (Lane 1991): 100 ng of genomic DNA, 2 μL of 20 mM forward primer, 2 μL of 20 mM reverse primer, 10 μL of 2 mM dNTP mix, 20 μL of 5× HF phusion reaction buffer, 0.6 μL Phusion high fidelity DNA polymerase (Finnzymes), H_2O was added to get a total volume of 100 μL. Temperature program was set up as: 98 °C for 30 s; (98 °C for 10 s; 60 °C for 20 s; 72 °C for 1 min) × 30 cycles; 72 °C for 7 min. PCR products were purified using the Qiagen PCR Purification Kit (Qiagen) and sequenced (AITbiotech, Singapore). The 16S rDNA sequences of the 2-propanol positives were aligned in Ribosomal Database Project II (http://rdp.cme.msu.edu) and Genebank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for strain identification. A neighbor-joining phylogenetic tree was constructed with aligned 16S rRNA gene sequences using 1,000 bootstrap replications on Mega 4.0 software (Tamura et al. 2007).

Evaluation of the hyper 2-propanol producing isolates in 2 L fermenter

The pre-culture was initiated by inoculating 100 μL of heated spore suspension (70 °C for 10 min) in 100 mL of potato medium and it was then cultivated at 30 °C for 48 h in anaerobic chamber. 50 mL pre-culture was transferred to 1 L of T6 broth medium in a 2 L fermenter and the fermentations were performed at 30 °C and 200 rpm with 0.1 L/min nitrogen gas and without pH control. Three separate fermentations were conducted for each strain using the same conditions. Samples were withdrawn for HPLC analysis at different time points. The optical density at 600 nm (OD_{600}) was also measured to determine the cell growth. The solvents productivities (g/L/h) were calculated as maximum solvents titer achieved per unit time.

Analytical method

The liquid samples were spun down at 4 °C, 13,000 rpm for 5 min and then filtered. The supernatants were analyzed by an Angilent 1100 HPLC equipped with an Aminex HPX-87H column, an Agilent diode array detector (DAD) and an Agilent refractive index detector. Samples were eluted by 12 mM H_2SO_4 at 0.65 mL/min and 50 °C for 50 min. The retention times for glucose, ethanol, 2-propanol, acetone (DAD at 260 nm), butyric acid (DAD at 210 nm) and butanol are 8.39, 18.94, 21.26, 19.56, 18.76 and 33.37 min, respectively.

Results

Isolation and characterization of hyper 2-propanol producing strains from Singapore environment

Total 525 soil samples were collected from different area of Singapore and the isolation of potent 2-propanol producing strains was performed by using an initial enrichment step in starch-containing potato medium, and a subsequent high through-put cultivation step in 24-well deep plates followed by the detection of the 2-propanol precursor-acetone. Referred to the two control strains *C. beijerinckii* NRRL B593 and *C. beijerinckii* NCIMB 8052, 96 h of cultivation time was used for the isolates to consume most of the glucose and achieve their highest 2-propanol or butanol titer at the high through-put cultivation step. Several isolated strains gave higher 2-propanol titer than *C. beijerinckii* NRRL B593, which is the most efficient natural 2-propanol producer so far. Among them, only three isolates met our criteria, which means 50 % higher titers of 2-propanol than *C. beijerinckii* NRRL B593. These 2-propanol positive strains PU31-4, M10-1 and BT10-6 were purified on potato agar medium and re-screened in 20 mL of T6 broth medium in shaking flasks, their solvents production profile including 2-propanol is shown in Fig. 1. PU31-4, M10-1 and BT10-6 produced 4.29 g/L, 4.10 g/L and 4.08 g/L of 2-propanol, respectively, which is about 1.7–1.8 times of the 2-propanol titer of *C. beijerinckii* NRRL B593. Ethanol, acetone and butanol (up to 12.8 g/L) were also produced by these three isolates. Based on the BLAST search analysis and alignment of the amplified 16S DNA gene sequence, these three positive isolates PU31-4, M10-1 and BT10-6 showed >99 % sequence identity in 16S rRNA with the genus *Clostridium* and they were thus characterized as *Clostridium* strains and named as *Clostridium* sp. BT10-6, *Clostridium* sp. M10-1

and *Clostridium* sp. PU31-4. Figure 2 exhibits the phylogenic tree constructed by neighbor-joining method based on 16S rRNA gene sequences of the isolated strains.

Evaluation of the hyper 2-propanol producing isolates in 2 L fermenter

The solvents production including 2-propanol of these three positive isolates PU31-4, M10-1 and BT10-6 were also evaluated in 1 L of T6 broth medium in 2 L fermenter. To keep a strictly anaerobic condition, the oxygen was removed by flushing nitrogen gas before inoculation and 0.1 L/min of nitrogen gas was continuously sparged during fermentations. All these three positives produced 2-propanol in much higher concentration than control strain *C. beijerinckii* NRRL B593 and the achieved 2-propanol titer of different isolates is shown in Fig. 3. Surprisingly, strain BT10-6 worked much better in fermenter than in shaking flasks and produced 5.26 g/L of 2-propanol in about 40 h, compared to *C. beijerinckii* NRRL B593 giving only 2.56 g/L of 2-propanol in 88 h. It is also worthy mentioning that 0.132, 0.118 and 0.087 g/L/h of 2-propanol productivity given by BT10-6, M10-1 and PU31-4 is 3.0–4.6 times of that given by the control strain, which is only 0.029 g/L/h.

The 2-propanol positives could also produce butanol. Their butanol titer was slightly less than the butanol control strain, but the butanol productivity was higher. The butanol productivity of BT10-6, M10-1 and PU31-4 was 0.253, 0.239 and 0.212 g/L/h, respectively, which is 1.4–1.7 times of 0.146 g/L/h given by butanol control strain (Fig. 4). All the 2-propanol positive strains consumed almost 100 % of the glucose in 40–48 h, compared to the 2-propanol and butanol control strains which gave incomplete utilization of glucose at a much slower rate (Fig. 5). The OD_{600} measurements also indicated that the 2-propanol positives gave higher growth rate than the control strains (data not shown), however, finally they could reach the similar cell densities, which were 12.10 ± 1.14, 12.83 ± 0.36, 12.19 ± 1.30, 13.54 ± 0.10 and 11.93 ± 0.35 for BT10-6, M10-1, PU31-4 and control strain *C. beijerinckii* NRRL B593, *C. beijerinckii* NCIMB 8052, respectively.

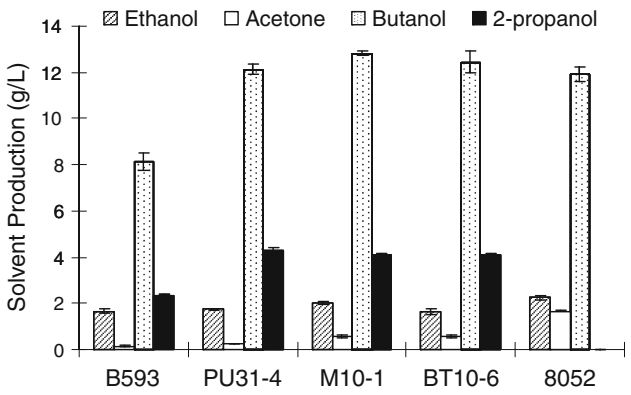

Fig. 1 Solvents production profile of the hyper 2-propanol producing isolates PU31-4, M10-1 and BT10-6. B593: 2-propanol producing control strain *C. beijerinckii* NRRL B593; 8052: butanol producing control strain *C. beijerinckii* NCIMB 8052

Discussion

In this work, we collected large quantities of soil samples from Singapore environment and set up a criteria to isolate hyper 2-propanol producing strains. Since polysaccharolytic enzymes' potential has enforced the isolation and characterization of a large number of anaerobic and spore-forming solvent producing genus *Clostridia* (Annous and Blaschek 1991; Montoya et al. 2000), the soil samples were first enriched in starch-containing medium. Since acetone is a by-product of solvents production and the precursor of 2-propanol (Chen and Hiu 1986; Kasap 2002), it was used as an indicator for isolating microorganisms having potential 2-propanol producing pathway. Acetone can react with iodine solution to produce visible yellow precipitates and this reaction can be performed in high through-put format. Using the above designed high through-put screening technique, three hyper 2-propanol producing strains BT10-6, M10-1 and PU31-4 were successfully isolated and met the criteria, which is 50 % higher titers of 2-propanol than *C. beijerinckii* NRRL B593.

These three 2-propanol positive isolates were characterized as *Clostridium* species, their fermentation process also occurred in two phases (acids production and solvents production) when pH was not controlled, which is similar to the traditional solvent producing *Clostridia* (Shi and Blaschek 2008). This was also confirmed by analyzing the butyric acid production (data not shown). Based on the evaluation performed in 2 L fermenter, the 2-propanol titers of BT10-6, M10-1 and PU31-4 are 1.6–2.1 times of that of *C. beijerinckii* NRRL B593. The maximum 5.26 g/L of 2-propanol was achieved by isolate BT10-6. The reached highest OD_{600} of both isolated strains and the control strain were similar and this revealed that the higher 2-propanol titer of these isolates was not caused by the differences of microbial growth. Moreover, these 2-propanol positive isolates consumed glucose almost completely in 40–48 h. This resulted in their higher growth rate (data not shown) and their higher 2-propanol productivity at 0.132, 0.118 and 0.087 g/L/h for BT10-6, M10-1 and PU31-4, respectively, compared to 0.029 g/L/h given by *C. beijerinckii* NRRL B593.

The butanol titers (up to 10.93 g/L) of the 2-propanol isolates are always higher than their 2-propanol titers. As suggested by the octanol–water partition coefficient (log-K_{ow}) values i.e. 0.05 for 2-propanol and 0.84 for butanol (Sangster 1989; Heipieper et al. 1994; Vermue et al.

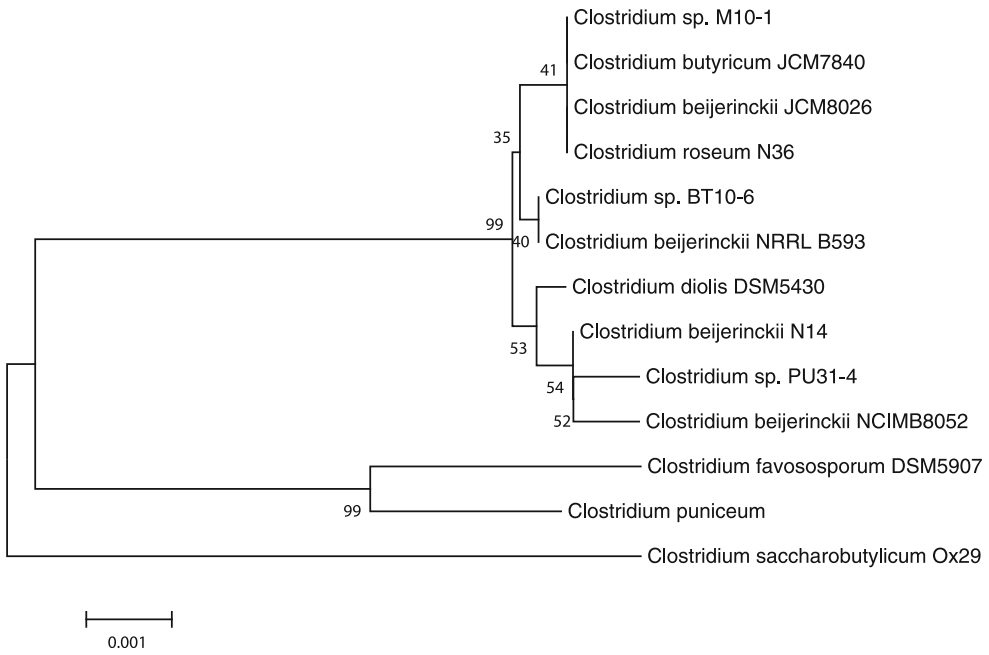

Fig. 2 Phylogenetic tree of three isolated hyper 2-propanol producing strains based on their 16S rRNA gene sequences and closest relatives. *Bar* represents 0.001 nucleotide substitutions

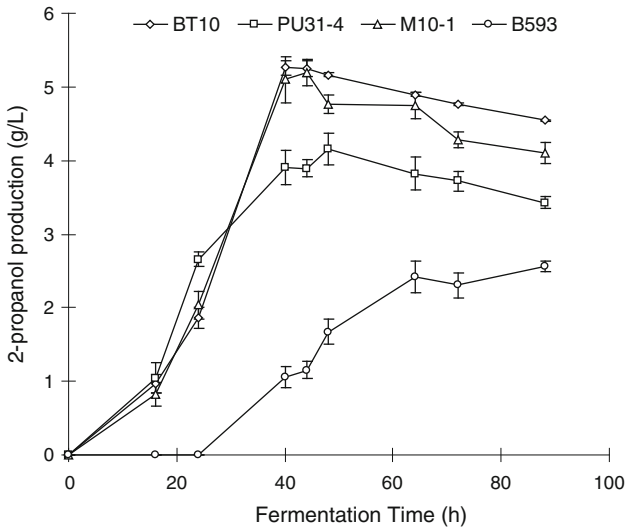

Fig. 3 2-Propanol production of the hyper 2-propanol producing isolates at 1 L scale in 2 L fermenter. B593: 2-propanol producing *C. beijerinckii* NRRL B593

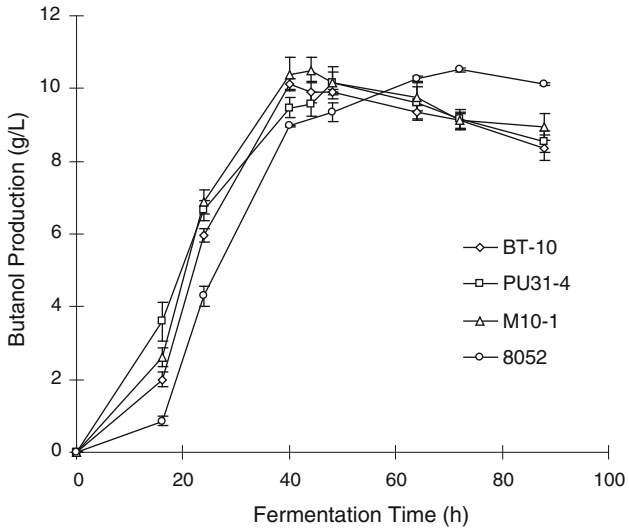

Fig. 4 Butanol production of the hyper 2-propanol producing isolates at 1 L scale in 2 L fermenter. 8052: butanol producing *C. beijerinckii* NCIMB 8052

1993), butanol is more toxic than 2-propanol and thus butanol was most inhibiting the fermentation of these isolates. This was also confirmed by growing the isolates under different butanol or 2-propanol concentrations (data not shown). Using these isolates as starting points, further improvement of the 2-propanol productivity could be achieved by using high cell-density fermentation and continuous fed-batch fermentation technologies combined with the in situ product recovery techniques described previously. It is also possible to use these potent isolates as starting points to improve their 2-propanol titer or even make 2-propanol as the main product, either by deleting the side reactions (such as ethanol and butanol pathways), or by identifying the more efficient 2-propanol producing pathways of these 2-propanol isolates for constructing engineering strains.

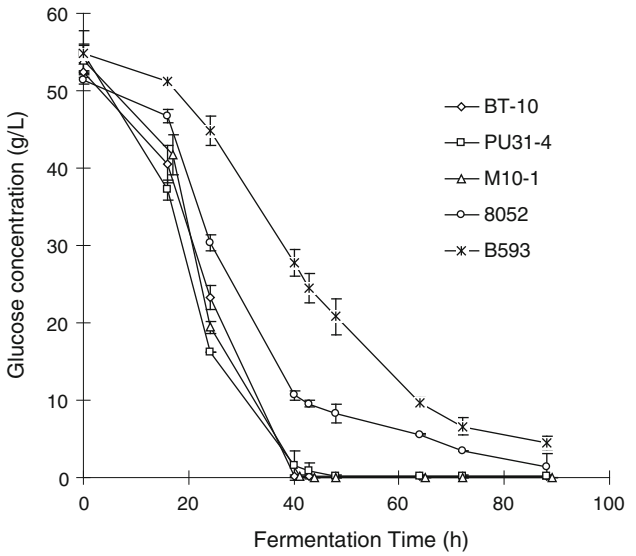

Fig. 5 Glucose consumption of the hyper 2-propanol producing isolates at 1 L scale fermentation in 2 L fermenter. B593: 2-propanol producing *C. beijerinckii* NRRL B593; 8052: butanol producing *C. beijerinckii* NCIMB 8052

Acknowledgments This work was supported by the Agency for Science, Technology and Research (A*star), Singapore (ICES/08-374B01) and was collaborated with Mitsui Chemical Singapore R&D Centre, Pte Ltd.

References

Andreesen JR, Bahl H, Gottschalk G (1989) Introduction to the physiology and biochemistry of the genus *Clostridium*. In: Minton NP, Clarke DJ (eds) Biotechnology handbooks, vol 3., *Clostridia*Plenum press, New York, pp 27–62

Annous BA, Blaschek HP (1991) Isolation and characterization of *Clostridium acetobutylicum* mutants with enhanced amylolytic activity. Appl Environ Microb 57:2544–2548

Chen JS, Hiu SF (1986) Acetone-butanol-isopropanol production by *Clostridium beijerinckii* (synonym, *Clostridium butylicum*). Biotechnol Lett 8:371–376. doi:10.1007/BF01040869

Evans PJ, Wang HW (1988) Enhancement of butanol formation by *Clostridium acetobutylicum* in the presence of decanol-oleyl alcohol mixed extractants. Appl Environ Microbiol 54:1662–1667

Ezeji TC, Qureshi N, Blaschek HP (2004) Acetone-butanol-ethanol (ABE) production from concentrated substrate: reduction in substrate inhibition by fed-batch technique and product inhibition by gas stripping. Appl Microbiol Biotechnol 63:653–658. doi:10.1007/s00253-003-1400-x

Ezeji TC, Qureshi N, Karcher PM, Blaschek HP (2005) Improving the performance of a gas stripping-based recovery system to remove butanol from *Clostridium beijerinckii* fermentation. Bioprocess Biosyst Eng 27:207–214. doi:10.1007/s00449-005-0403-7

Ezeji TC, Milne C, Price ND, Blaschek HP (2010) Achievements and perspectives to overcome the poor solvent resistance in acetone and butanol-producing microorganisms. Appl Microbiol Biotechnol 85:1697–1712. doi:10.1007/s00253-009-2390-0

Garcia A, Iannotti EL, Fischer JL (1986) Butanol fermentation liquor production and separation by reverse osmosis. Biotechnol Bioeng 28:785–791. doi:10.1002/bit.260280603

Groot WJ, Luyben KChAM (1986) In situ product recovery by adsorption in the butanol/isopropanol batch fermentation. Appl Microbiol Biotechnol 25:29–31. doi:10.1007/BF00252508

Groot WJ, van den Oever CE, Kossen NWF (1984) Pervaporation for simultaneous product recovery in the butanol/isopropanol batch fermentation. Biotechnol Lett 6:709–714. doi:10.1007/BF00133061

Hanai T, Atsumi S, Liao JC (2007) Engineered synthetic pathway for isopropanol production in *Escherichia coli*. Appl Environ Microb 73:7814–7818. doi:10.1128/AEM.01140-07

Heipieper HJ, Weber FJ, Sikkema J, Keweloh H, Debont JAM (1994) Mechanisms of resistance of whole cells to toxic organic-solvents. Trends Biotechnol 12:409–415. doi:10.1016/0167-7799(94)90029-9

Jojima T, Inui M, Yukawa H (2008) Production of isopropanol by metabolically engineered *Escherichia coli*. Appl Microbiol Biotechnol 77:1219–1224. doi:10.1007/s00253-007-1246-8

Kasap M (2002) Nitrogen metabolism and solvent production in *Clostridium beijerinckii* NRRL B593. Virginia Polytechnic Institute and State University, Blacksburg Dissertation

Kibby CL, Hall WK (1972) Studies of acid catalyzed reactions. XII. Alcohol decomposition over hydroxyapatite catalysts. J Catal 29:144–159. doi:10.1016/0021-9517(73)90213-3

Krouwel PG, Groot WJ, Kossen NWF, van der Laan WFM (1983) Continuous isopropanol-butanol-ethanol fermentation by immobilized *Clostridium beijerinckii* cells in a packed bed fermenter. Enzym Microb Technol 5:46–54. doi:10.1016/0141-0229(83)90064-9

Lane DJ (1991) 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M (eds) Nucleic acid techniques in bacterial systematics. Wiley, Chichester, pp 115–175

Lee I, Johnson LA, Hammond EG (1995) Use of branched chain esters to reduce the crystallization temperature of biodiesel. J Am Oil Chem Soc 72:1155–1160. doi:10.1007/BF02540982

Matsumura M, Takehara S, Kataoka H (1992) Continuous butanol/isopropanol fermentation in down-flow column reactor coupled with pervaporation using supported liquid membrane. Biotechnol Bioeng 39:148–156. doi:10.1002/bit.260390205

Montoya D, Spitia S, Silva E, Schwarz WH (2000) Isolation of mesophilic solvent-producing Clostridia from Colombian sources: physiological characterization, solvent production and polysaccharide hydrolysis. J Biotechnol 79:117–126. doi:10.1016/S0168-1656(00)00218-2

Osburn OL, Brown RW, Werkman CH (1937) The butyl alcohol-isoppropyl alcohol fermentation. J Biol Chem 121:685–695

Papa AJ (2005) Propanols in: Ullmann's encyclopedia of industrial chemistry. Wiley-VCH, Weinheim. doi:10.1002/14356007.a22_173

Qureshi N, Blaschek HP (1999) Butanol recovery from model solution/fermentation broth by pervaporation: evaluation of membrane performance. Biomass Bioenergy 17:175–184. doi:10.1016/S0961-9534(99)00030-6

Qureshi N, Maddox IS, Friedl A (1992) Application of continuous substrate feeding to the ABE fermentation: relief of product inhibition using extraction, perstraction, stripping and pervaporation. Biotechnol Prog 8:382–390. doi:10.1021/bp00017a002

Sangster J (1989) Octanol-water partition coefficient of simple organic compounds. J Phys Chem Ref Data 18:1111–1227. doi:10.1063/1.555833

Shi Z, Blaschek HP (2008) Transcriptional analysis of *Clostridium beijerinckii* NCIMB 8052 and the hyper-butanol-producing mutant BA101 during the shift from acidogenesis to solventogenesis. Appl Environ Microb 74:7709–7714. doi:10.1128/AEM.01948-08

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Bio Evol 24:1596–1599. doi:10.1093/molbev/msm092

Thauer RK, Jungermann K, Decker K (1977) Energy conservation in thermotropic anaerobic bacteria. Bacteriol Rev 41:100–180

Vermue M, Sikkema J, Verheul A, Bakker R, Tramper J (1993) Toxicity of homologous series of organic-solvents for the gram positive bacteria *Arthrobacter* and *Nocardia* sp. and the gram negative bacteria *Acinetobacter* and *Pseudomonas* sp. Biotechnol Bioeng 42:747–758. doi:10.1002/bit.260420610

Zoutberg GR, Willemsberg R, Smit G, Teixeira de Mattos MJ, Neijssel OM (1989) Solvent production by an aggregate-forming variant of *Clostridium butyricum*. Appl Microbiol Biotechnol 32:22–26. doi:10.1007/BF00164817