Isolation and Characterization of *Clostridia* from the Feces of Wild Rabbit and Swine for Hemicellulosic Acetone-Butanol-Ethanol (ABE) Production

L. Veeranjaneya Reddy,[a,b] A. Sreeveda,[b] In-Hye Park,[a] and Young-Jung Wee [a,*]

Isolation and characterization of solventogenic bacteria from animal feces were carried out. Ten samples were collected continually for 5 d from the feces of wild rabbit and wild swine in Jawaharlal Nehru Zoological Park, Hyderabad, India. Ten acetone-positive strains were selected for evaluation of their phenotypic and physiological characteristics. Two potential solvent-producing cultures were selected for 16S rRNA gene analysis. The culture isolated from the wild rabbit feces exhibited 97.3% similarity with *Clostridium beijerinckii* 8052, and the culture isolated from the wild swine feces exhibited 93.8% similarity with *Clostridium saccharoperbutylacetonicum* NI-4(HMT). The isolated strains utilized a wide range of carbohydrate substrates including glucose, fructose, maltose, xylose, arabinose, and glycerol. The major fermentation products from glucose, xylose, and arabinose were acetone, butanol, and ethanol (ABE). The total ABE concentration produced by strain YVU1 was 13.1 g/L from arabinose, 16.3 g/L from xylose, and 20.6 g/L from glucose. Strain YVU2 produced a total ABE concentration of 16.5 g/L, 18.3 g/L, and 22.4 g/L from arabinose, xylose, and glucose, respectively.

Keywords: Animal feces; Solventogenic bacteria; Clostridia; Biomass hydrolytic sugars; Biobutanol production

*Contact information: a: Department of Food science and Technology, Yeungnam University, Gyeongsan, Gyeongbuk 38541, Republic of Korea; b: Department of Microbiology, Yogi Vemana University, Kadapa (A.P.) 516 003, India; *Corresponding author: yjwee@ynu.ac.kr*

INTRODUCTION

Liquid transport fuels derived from renewable lignocellulosic resources are an attractive alternative to traditional fossil fuels. Among lignocellulosic fuels, butanol is one of the most promising candidates for practical uses (Margeot *et al.* 2009). As a biofuel, butanol is less hygroscopic and has a higher caloric content than ethanol (Wallner *et al.* 2009). The commercial production of butanol was initiated during World War I on an industrial scale by the Weizmann process, which is characterized by acetone-butanol-ethanol (ABE) fermentation. However, the Weizmann process is presently not profitable in industrialized countries because of the high cost of substrate (Dürre 2007). The increasing demand for renewable and carbon-neutral liquid transportation fuels has intensified the study of their production through the microbial fermentation of inexpensive lignocellulosic feedstock (Hellier *et al.* 2015). Biological processes have potential use for the industrial-scale production of bulk chemicals and fuels. Inexpensive substrates such as waste biomass could be metabolized by high-performance bacterial isolates to produce valuable solvents. This development was stimulated in recent years with progress in process technology, strain improvement, and the use of an unlimited supply of alternative feedstock, which made the biotechnological production of ABE solvents economically

competitive again (Qureshi *et al.* 2010).

Solvents such as butanol are produced by a limited number of microbes. Microbes such as the *Clostridium* species are the major anaerobic microorganism used to produce butanol through ABE fermentation. The *Clostridium* sp. are Gram-positive, rod shaped, obligate anaerobe, spore-forming, motile, and solvent-producing bacteria (Keis *et al.* 1995). Some *Clostridium* species such as *C. acetobutylicum*, *C. saccharoperbutyl-acetonicum*, *C. beijerinckii*, and *C. butyricum* can ferment sugars into solvents. Biofuel industries are expressing keen interest in *Clostridia*, as these organisms can produce butanol to be used as a transportation biofuel from fermentable agricultural waste materials. All of the above indicated strains are negatively inhibited by their own products (Lee *et al.* 2008; Ahn *et al.* 2011). This inhibition leads to the production of low concentrations of solvents, which increases the cost of obtaining purified product. The isolation of anaerobic native bacteria *via* catabolism reactions is a prerequisite for the development of novel industrial biotechnology. This unique feature has created a tremendous potential for the isolation of *Clostridium* sp. and for biofuel production as the demand for alternative fuels increases.

Second-generation biofuel production requires the development of economically feasible and sustainable processes that use renewable lignocellulosic biomass as a starting material. For the effective utilization of lignocellulosic biomass, both hexoses and pentoses need to be simultaneously assimilated. For the development of efficient consolidated bioprocesses, the efficient microbial utilization of xylose, which is the principal pentose sugar of the hemicellulose component of lignocellulosic biomass, is important (Gírio *et al.* 2010; Zhang *et al.* 2018; Zhou *et al.* 2018). There are few reports on stains that can ferment both hexoses and pentoses. Rumen fluids and feces of ruminants have been investigated widely earlier, and they still can be regarded as a potential habitat for new carbohydrate-specific novel obligate anaerobes (Varel *et al.* 1995; Sankar *et al.* 2003; Gupta *et al.* 2016).

In view of the above, the simplified system of isolation and cultivation of anaerobic microorganisms from the feces of wild rabbit and wild swine was described in detail. In addition, the utilization of carbohydrates such as hexose (glucose) and pentoses (xylose and arabinose) to produce butanol from the isolated strains was evaluated further.

EXPERIMENTAL

Sample Collection and Processing

The fecal matter of wild rabbit and wild swine droppings was collected at Jawaharlal Nehru Zoological Park, Hyderabad, India. The collected fecal matter was air dried and stored at 4 °C.

Media Preparation

The media chosen for the isolation and cultivation of anaerobes were cooked meat medium (CMM) using 45 g of beef extract, 2 g of dextrose, 20 g of peptone, 5 g of sodium chloride, and a pH of 7.2. Reinforced *Clostridia* medium (RCM) was obtained from HiMedia (Mumbai, India), and T6 medium with 6% (w/v) glucose was obtained from Merck (Mumbai, India). One liter of each medium was prepared using the ingredients described with distilled water.

A resazurin (200 mg/100 mL) (HiMedia, Bangalore, India) was used as an indicator. All the three types of media were prepared under strict anaerobic conditions

using an anaerobic work chamber (Anaerobic System Model 1029; Thermo Forma, Marietta, USA). The heat-stable CMM ingredients were dissolved as per required volume in conical flasks and immediately sparged with nitrogen gas for 15 min to 20 min until the resazurin turned colorless. The CMM was then filled into Pyrex screw capped bottles with the required volume, which were simultaneously sparged with nitrogen gas, sealed with butyl rubber septa (Pharmaseals, Mumbai, India), and clamped. The media prepared as above was finally sterilized by autoclave (Technico, Chennai, India) at 121 °C for 15 min.

Isolation of Solventogenic Bacteria

One gram of dried fecal matter was suspended in a sterile conical flask containing 10 mL of distilled water incubated in a water bath at 80 °C for 10 min to deactivate all the vegetative cells for counter selecting against non-spore formers. One milliliter of the diluted fecal matter was inoculated into the serum bottles with RCM and was incubated for 3 d at 37 °C. After incubation, the pure culture was developed from the turbid and gas producing bottles. A single colony culture was achieved by the roll tube method as described by Hungate (1969). All the single colonies obtained were tested for acetone production by the Nitroprusside test (Montoya *et al.* 2000). The acetone-positive colonies were further purified by plating under strict anaerobiosis conditions. Anaerobic conditions for the agar plates were obtained in an anaerobic chamber with a high-purity grade gas mixture of 85% N_2, 10% H_2, and 5% CO_2. A palladium wafer was used as a catalyst.

Morphological and Physiological Characterization

The colonies were checked for their Gram stain, uniformity in size, shape of cells, presence of spores, and motility of the organism by using a microscope (Leica, Mumbai, India) (Holdeman and Moore 1972; Berezina *et al.* 2009). The physiological characteristics were determined based on several biochemical tests such as oxidase, catalase, motility, triple sugar iron (TSI), hydrogen sulfide (H_2S), gas production, indole, methyl red, Simmon's citrate, and Voges Proskaeur. The results were confirmed with Bergey's Manual (Cato *et al.* 1986).

Identification of Bacterial Isolates

Preliminary identification of the isolates was performed using the morphological and physiological characteristics. The identification and differentiation of solventogenic *Clostridia* among *viz. C. acetobutylicum*, *C. saccharoperbutylacetonicum*, *C. beijerinckii*, and *C. butyricum* was done as described by Johnson *et al.* (1997). For all these tests, *C. acetobutylicum* 2878 was used as a test organism. The freshly grown culture was spread on T6 agar plates containing the rifampicin discs and incubated for 24 h for the rifampicin test. For the remaining tests, the activated cultures were inoculated into their respective media present in serum vials. The molecular characterization and identification was carried out using fully grown culture DNA which was isolated by the protocol a HiMedia Bacterial Gram Positive DNA Isolation kit (Mumbai, India). The isolated DNA was investigated for gene sequencing analysis at Xcleris Labs, Ahmadabad, India. The primers used were bacterial 16S rRNA gene primers for the amplification of DNA fragments (8F AGAGTTTGATCCTGGCTCAG and 1492R ACGGCTACCTTGTTACGACTT) and for sequencing the primers (704F GTAGCGGTGAAATGCGTAGA and 907R CCGTCAATTCCTTTGAGTTT). BLAST, Clustal W, and DNASIS/PROSIS for Windows (Hitachi Software Engineering) software packages were used for sequence data

analysis. The phylogenetic tree was constructed by the methods applied and implemented in the Mega 7 software.

Fermentation

C. beijerinckii YVU1 and *C. saccharoperbutylacetonicum* YVU2 were stored as spore suspensions and cultivated as described by López-Contreras *et al.* (2000). For the preparation of the inoculum, the spores were heat-shocked and placed into an RCM medium overnight. The solvent production media was prepared anaerobically in 120 mL serum bottles containing 50 mL of media. As carbon sources, the stock solutions of glucose, xylose, and arabinose or their mixtures were sterilized separately and added to the medium at the indicated concentrations. The initial pH of the cultures was adjusted to 6.0 to 6.4 with 1 M NaOH. All the experiments were performed at least three times.

Analyses

The cell growth was monitored by measuring the optical density at 600 nm using a spectrophotometer (1800; Shimadzu, Kyoto, Japan). During fermentation, 2 mL of the sample was withdrawn aseptically with a syringe every 24 h and centrifuged at 10,000 rpm for 10 min at 4 °C. This sample was used for all the analytical experiments. The amount of reducing sugars in the initial and final stages of the fermentation (both the hexose and pentose) was determined by the dinitrosalicylic acid (DNS) method (Miller 1972). The ABE supernatant was analyzed through the head space method of gas chromatography (HCPL-7850; Agilent, Santa Clara, CA, USA) using a 5% phenyl polyethylene column (DB5; Agilent J & W, Santa Clara, CA, USA) and a flame ionization detector (Agilent J & W, Santa Clara, USA) with nitrogen as the carrier gas.

RESULTS AND DISCUSSION

For the sample preparation, 10 samples were collected during a 5 d period from both feces of wild rabbit and wild swine. Since the fermentable carbohydrates are required for animal growth, many investigators have selected animal intestinal tract juice or fecal matter for the isolation of their solventogenic and cellulolytic microorganisms (Varel *et al.* 1995; Sankar *et al.* 2003; Gupta *et al.* 2016). Through a four-step procedure in this study, the solvent-producing bacteria was isolated and confirmed by using *C. acetobutylicum* 2878 as a reference organism. In the first step, 55 colonies were observed on roll tubes. In the second step, 32 Gram positive rods bacilli were isolated from the 10 samples that were confirmed by endospore staining. Sporulation in *Clostridia* is reported to be associated with the triggering factors involved in the switch to solventogenesis from the acetogenesis phase (Woods and Jones 1986; Sauer *et al.* 1995). In concordance with these reports, the new isolates are with abundant spores and the solvent production. In the third step, single creamy white colonies were once again inoculated in the RCM, where the purified organisms were checked for their turbidity and gas production. In the final step, the plating technique was used to confirm the purity of the organism and the acetone production. The colonies which formed a red-violet ring confirmed the solvent producing bacteria, because presence of acetone will produce red-violet ring with nitroprusside. From the above procedure, 10 acetone positive bacteria, five from each animal, were isolated and studied for their phenotypic and physiological characters.

Table 1 and Fig. 1 show the results of the morphological and biochemical tests of

the isolated strains. According to the results, the 10 isolates were mesophilic and saccharolytic strains, and consequently attributed to the genus *Clostridium*. Physiological tests have been used to distinguish between the four different groups of solventogenic *Clostridia* described by Johnson *et al.* (1997). According to this criterion, it was identified that the 10 isolates belonged to three different groups: 1) four strains as *C. acetobutylicum*, 2) three strains as *C. saccharoperbutylacetonicum*, and 3) three strains as *C. beijerinckii* (Table 2). However, this classification is tentative and will have to be verified and confirmed using molecular methods.

Table 1. Morphological and Physiological Characteristics of the Isolated Strains

Strain	Colony Morphology	Gram Stain	Endospore Stain	Motility	Curd Formation	Riboflavin Production	Rifampicin
C. acetobutylicum 2878 (Test)	Circular, dull beige and rods	+	+	+	24 h	+	S
Clostridium sp. YVU1	Slightly irregular, bright and rods	+	+	+	24 h	-	R
Clostridium sp. YVU2	Circular, dull beige and rods	+	+	+	48 h	-	R
Clostridium sp. YVU3	Circular, dull beige and rods	+	+	+	24 h	+	S
Clostridium sp. YVU4	Circular, whole, raised and rods	+	+	+	24 h	+	S
Clostridium sp. YVU5	Circular, dull beige and rods	+	+	+	24 h	+	S
Clostridium sp. YVU6	Slightly irregular, bright and rods	+	+	+	48 h	-	R
Clostridium sp. YVU7	Slightly irregular, bright and rods	+	+	+	48 h	-	R
Clostridium sp. YVU8	Circular, whole, raised and rods	+	+	+	24 h	-	R
Clostridium sp. YVU9	Slightly irregular, bright and rods	+	+	+	24 h	+	S
Clostridium sp. YVU10	Slightly irregular, bright and rods	+	+	+	24 h	-	R

The isolated cultures were evaluated for their potential use in the conversion of the major sugars associated with the hydrolysis of biomass (glucose, xylose, and L-arabinose) and polysaccharides to solvent. The confirmation of the utilization of various carbon sources was positive when the culture turbidity was 1.0 after 24 h of incubation at 37 °C, whereas it was negative when it did not exceed 0.15 (Table 3) (Keis *et al.* 2001). All 10 of the cultures were tested for their solvent production capacity and it was observed that *Clostridium* sp. YVU1 and *Clostridium* sp. YVU2 had a considerable amount of butanol when compared to the remaining eight strains (Table 4). Two of the top solvent producing *Clostridium* strains (YVU1 and YVU2) were selected for further characterization.

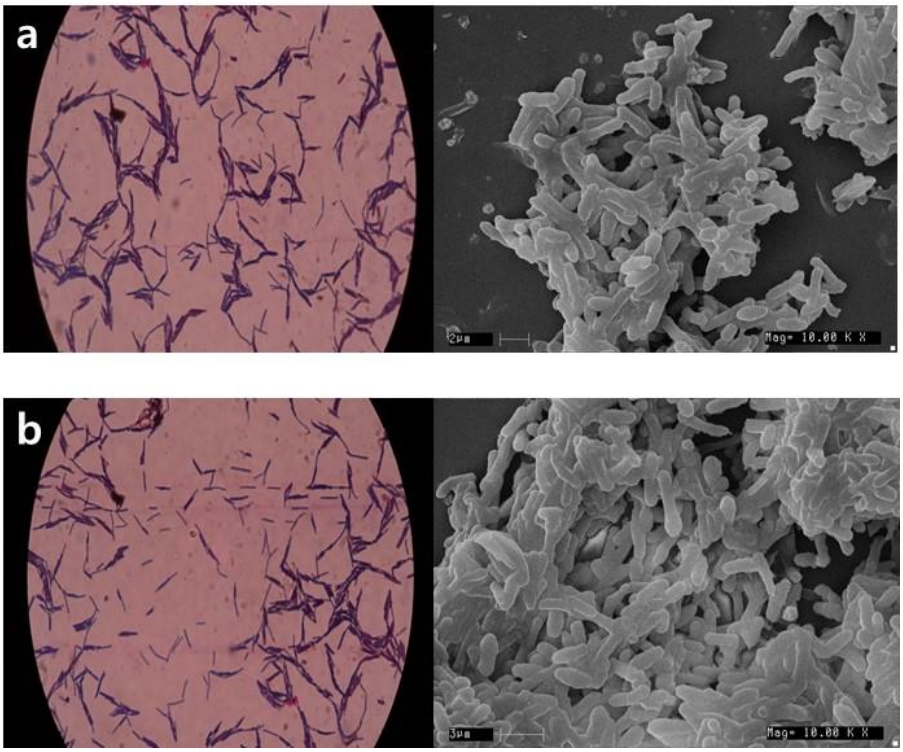

Fig. 1. The fluorescent microscopic and scanning electron microscopic view of the isolated strains: a) strain YVU1; b) strain YVU 2

Table 2. Biochemical Characteristics of the Isolated Strains

Strain	Glycerol	Gelatin	Indole	Catalase	Ribose	Vogues Proskauer
C. acetobutylicum 2878 (Test)	-	-	+	-	-	+
Clostridium sp. YVU1	+	-	-	-	+	-
Clostridium sp. YVU2	+	-	-	-	+	-
Clostridium sp. YVU3	+	-	-	-	-	-
Clostridium sp. YVU4	+	-	-	-	-	+
Clostridium sp. YVU5	-	+	+	+	-	+
Clostridium sp. YVU6	-	-	+	+	+	+
Clostridium sp. YVU7	+	-	-	-	+	-
Clostridium sp. YVU8	-	-	+	-	+	-
Clostridium sp. YVU9	+	-	-	-	+	-
Clostridium sp. YVU10	+	-	+	-	-	+

Table 3. Mono and Polysaccharide Utilization Characteristics of the Isolated Strains

Strain	Glucose	Xylose	Arabinose	Sorbitol	Mannose	Starch	Pectin	Xylan	CMC
C. acetobutylicum 2878 (Test)	+	-	-	-	-	+	-	-	-
Clostridium sp. YVU1	+	+	+	-	+	+	+	-	-
Clostridium sp. YVU2	+	+	+	-	+	+	+	-	-
Clostridium sp. YVU3	+	-	-	-	-	-	-		-
Clostridium sp. YVU4	+	-	-	-	-	+	-	+	-
Clostridium sp. YVU5	+	-	+		-	-		-	+
Clostridium sp. YVU6	+	-	-		+	+	+	+	-
Clostridium sp. YVU7	+	+	+		+	+	-	-	-
Clostridium sp. YVU8	+	+	-		+	+	-	-	-
Clostridium sp. YVU9	+	-	-		+	-	-	-	-
Clostridium sp. YVU10	+	-	-		-	-	+	-	-

Table 4. Solvent Production by Isolated Strains, Compared to the *C. acetobutylicum* NCIM 2878

Strain	Acetone (g/L)	Butanol (g/L)	Ethanol (g/L)	Total Solvent (g/L)
C. acetobutylicum 2878 (Test)	5.4	9.4	1.8	16.6
Clostridium sp. YVU1	4.0	14.5	2.1	20.6
Clostridium sp. YVU2	3.2	16.2	3.0	22.4
Clostridium sp. YVU3	1.3	2.6	0.8	4.7
Clostridium sp. YVU4	1.8	2.5	1.0	5.3
Clostridium sp. YVU5	2.4	1.2	1.3	4.9
Clostridium sp. YVU6	3.2	1.4	1.0	5.6
Clostridium sp. YVU7	1.0	1.8	0.5	3.3
Clostridium sp. YVU8	2.8	4.2	1.1	7.1
Clostridium sp. YVU9	2.5	5.4	1.0	8.9
Clostridium sp. YVU10	3.2	6.0	1.4	10.6

The selected *Clostridia* cultures were subjected to 16S rRNA gene sequencing analysis. The obtained sequences were analyzed by a comparison with the Gene Bank database (https://www.ncbi.nlm.nih.gov/genbank/). During the blast analysis, the culture isolated from wild rabbit showed 97.3% similarity with *C. beijerinckii* 8052 and the culture isolated from wild swine showed 93.8% similarity with *C. saccharoperbutylacetonicum* NI-4(HMT). The phylogenetic tree analysis is depicted in Fig. 2. The sequence was submitted to Gene Bank database, and the accession numbers are KP334151 for *Clostridium* sp. YVU1 and KP334152 for *Clostridium* sp. YVU2.

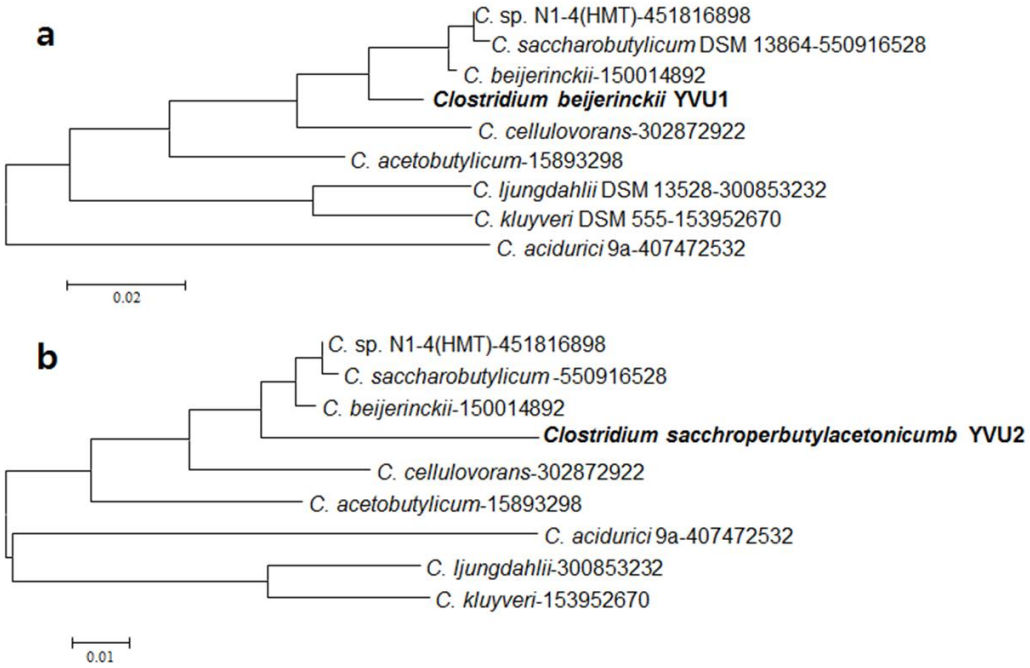

Fig. 2. Unrooted dendrogram based on the 16S rRNA gene sequence data indicating the phylogenetic positions of the isolated strains. The bar indicates 1 and 2 substitutions per 100 nucleotides, respectively. a) strain YVU1; b) strain YVU2

Both bacterial strains, YVU1 and YVU2, had comparable specific growth rates on glucose (0.17 h^{-1} and 0.15 h^{-1}, respectively). It is notable that both strains showed good growth on xylose (0.12 h^{-1} and 0.08 h^{-1}, respectively). The changes in the pH and growth profile of the bacteria are depicted in Fig. 3. At the end of the fermentation time, the butanol yield was approximately 20.6 g/L, 16.3 g/L, and 13.1 g/L (YVU1) and 22.4 g/L, 18.3 g/L, and 16.5 g/L (YVU2) for glucose, xylose, and arabinose substrates, respectively. The utilization of the glucose, xylose, and arabinose and the product formation profiles by the two isolated strains are depicted in the Fig. 4. After entering the exponential growth phase, the sugars were well utilized and remained stationary. These results were comparable with previous investigations where immobilized cells and suspended cells were used for the fermentation of xylose for butanol production (Fond *et al.* 1986; Chen *et al.* 2013). The present results were also compared with the studies of Xin *et al.* (2014), where newly isolated *Clostridium* sp. BOH3 produced 14.9 g/L and 14.5 g/L of butanol from glucose and xylose, respectively. *C. acetobutylicum* ATCC 824 normally ferments glucose to butanol, acetone, and ethanol with a 6:3:1 ratio that is 0.56, 0.22, and 0.07 M/M glucose. This proportion fluctuates with the fermentation conditions and is not constant among the solventogenic *Clostridium* strains, although the solvent production yield usually does not exceed 1 M of each solvent per mole of glucose fermented (Andreesen *et al.* 1989).

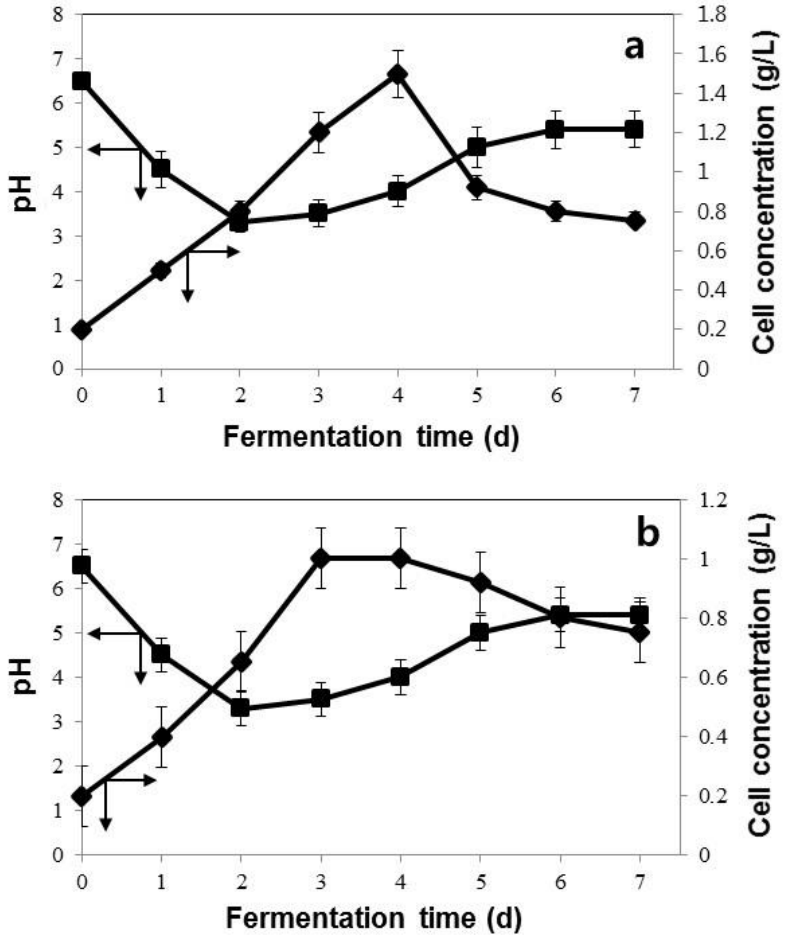

Fig. 3. The growth and pH profiles of the isolated strains. a) strain YVU1; b) strain YVU 2; -■-, pH; -◆-, cell concentration

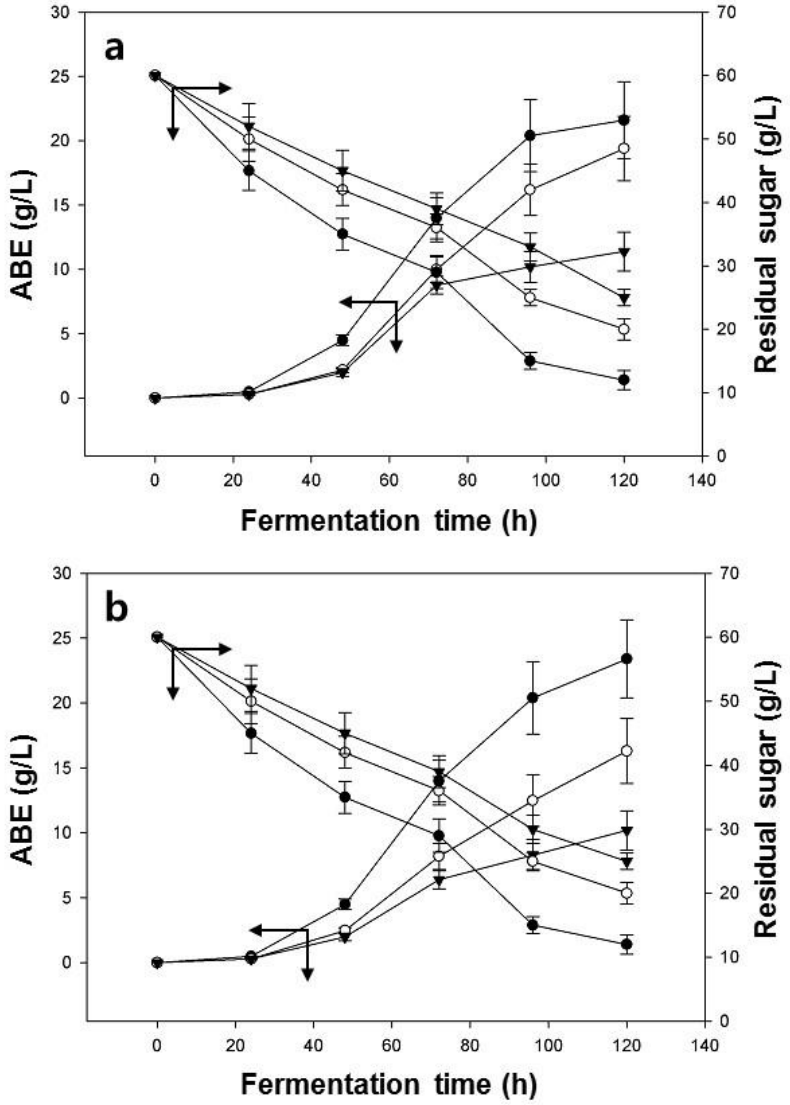

Fig. 4. The ABE production profile from three different sugars by isolated strains. a) strain YVU1; b) strain YVU2; -●-, glucose; -○-, xylose; -▲-, arabinose

It was also interesting to note that the isolated cultures (YVU1 and YVU2) utilized polysaccharides starch and pectin with high growth rates. This characteristic may be used for the assimilation of low-cost complex substrates produced by the food and other agro-industries. Solventogenic *Clostridia* can utilize a wide range of carbon sources such as starch, sucrose, glucose, fructose, galactose, cellobiose, xylose, arabinose, glycerol, and syngas as fermentation substrates for the ABE production (Ranjan and Moholkar 2012). In general, fermentation using *Clostridium* sp. resulted in the ABE production of around 15 g/L to 25 g/L with a yield of 0.25 g to 0.4 g ABE/g sugar (Cheng *et al.* 2012). However, a maximum production level of 32.6 g/L solvents using *C. acetobutylicum* was also reported, hyper-producing the mutant of strain NCIMB 8052 under rigorously optimized conditions (Chen and Blaschek 1999). This clearly suggests that the optimization of culture conditions and selection of hyper-producing strains through mutation is essential for high yields of butanol.

CONCLUSIONS

1. The solventogenic bacteria for production of hemicellulosic ABE were successfully isolated from feces of wild swine and rabbit. The isolated cultures utilized a variety of carbon sources for their growth, especially starch and pectin.
2. Molecular characterization concluded that the top solvent-producing cultures belonged to *C. beijerinckii* and *C. saccharoperbutylacetonicum*. It is interesting that both isolated strains utilized xylose and arabinose, which shows a potential use for the complete degradation of biomass derived sugars from hemicellulose. The isolated cultures, YVU1 and YVU2 were able to produce 16.3 and 18.3 g/L ABE from xylose, respectively.
3. The feces of wild swine and rabbit are good sources for the isolation of solventogenic bacteria. The optimization of the fermentation conditions is expected to further enhance the total solvent productivity by the isolated cultures.

ACKNOWLEDGMENTS

The authors would like to acknowledge the Department of Science and Technology (DST) and the Council of Scientific and Industrial Research (CSIR), Government of India for the financial support given in the form of the research projects entitled 'Biotechnological production of acetone-butanol-ethanol (ABE) from agricultural biomass using solventogenic bacteria' (Ref No: SR/FT/LS-79/2009) and 'Studies on rapid and enhanced production of ethanol through very high gravity (VHG) fermentation' (Ref No: 38 (1310)/11/EMR-II).

REFERENCES CITED

Ahn, J. H., Sang, B. I., and Um, Y. S. (2011). "Butanol production from thin stillage using *Clostridium pasteurianum*," *Bioresource Technol.* 102(7), 4934-4937. DOI: 10.1016/j.biortech.2011.01.046

Andreesen, J. R., Bahl, H., and Gottschalk, G. (1989). "Introduction to the physiology and biochemistry of the genus *Clostridium*," in: *Biotechnology Handbooks: Clostridia*, N. P. Minton and D. J. Clark (eds.), Plenum Press, New York, NY, pp. 27-34.

Berezina, O. V., Brandt, A., Yarotsky, S., Schwarz, W. H., and Zverlov, V. V. (2009). "Isolation of a new butanol-producing *Clostridium* strain: High level of hemicellulosic activity and structure of solventogenesis genes of a new *Clostridium saccharobutylicum* isolate," *Syst. Appl. Microbiol.* 32(7), 449-459. DOI: 10.1016/j.syapm.2009.07.005.

Cato, E. P., George, W. L., and Finegold, S. M. (1986). "Genus *Clostridium*," in: *Bergey's Manual of Systematic Bacteriology*, P. H. A. Mair, N. S. Sharpe, and J. G. Holt (eds.), Williams & Wilkins, Baltimore, MD. pp. 1141-1200.

Chen, C.-K., and Blaschek, H. P. (1999). "Acetate enhances solvent production and prevents degeneration in *Clostridium beijerinckii* BA101," *Appl. Microbiol. Biotechnol.* 52(2), 170-173. DOI: 10.1007/s002530051504

Chen, Y., Zhou, T., Liu, D., Li, A., Xu, S., Liu, Q., Li, B., and Ying, H. (2013).

"Production of butanol from glucose and xylose with immobilized cells of *Clostridium acetobutylicum*," *Biotechnol. Bioprocess Eng.* 18(2), 234-241. DOI: 10.1007/s12257-012-0573-5

Cheng, C.-L., Che, P.-Y., Chen, B.-Y., Lee, W.-J., Chien, L.-J., and Chang, J.-S. (2012). "High yield bio-butanol production by solvent-producing bacterial microflora," *Bioresource Technol.* 113, 58-64. DOI: 10.1016/j.biortech.2011.12.133

Dürre, P. (2007). "Biobutanol: An attractive biofuel," *Biotechnol. J.* 2(12), 1525-1534. DOI: 10.1002/biot.200700168

Fond, O., Engasser, J.-M., Matta-El-Amouri, G., and Petitdemange, H. (1986). "The acetone butanol fermentation on glucose and xylose. I. Regulation and kinetics in batch cultures," *Biotechnol. Bioeng.* 28(2), 160-166. DOI: 10.1002/bit.260280203

Gírio, F. M., Fonseca, C., Carvalheiro, F., Duarte, L. C., Marques, S., Bogel-Łukasik, R. (2010). "Hemicelluloses for fuel ethanol: A review," *Bioresource Technol.* 101(13), 4775-4800. DOI: 10.1016/j.biortech.2010.01.088

Gupta, K. K., Aneja, K. R., and Rana, D. (2016). "Current status of cow dung as a bioresource for sustainable development," *Bioresour. Bioprocess.* 3, 28. DOI 10.1186/s40643-016-0105-9

Hellier, P., Purton, S., and Ladommatos, N. (2015). "Molecular structure of photosynthetic microbial biofuels for improved engine combustion and emissions characteristics," *Front. Bioeng. Biotechnol.* 3(49), 1-12. DOI: 10.3389/fbioe.2015.00049

Holdeman, L. V., and Moore, W. E. C. (1972). "Roll-tube techniques for anaerobic bacteria," *Am. J. Clin. Nutr.* 25(12), 1314-1317. DOI: 10.1093/ajcn/25.12.1314

Hungate, R. E. (1969). "A roll tube method for cultivation of strict anaerobes," in: *Methods in Microbiology*, J. R. Norris and D. W. Ribbons (eds.), Academic Press, Cambridge, MA, pp. 117-132.

Johnson, J. L., Toth, J., Santiwatanakul, S., and Chen, J.-S. (1997). "Cultures of *Clostridium acetobutylicum* from various collections comprise *Clostridium acetobutylicum, Clostridium beijerinckii*, and two other distinct types based on DNA-DNA reassociation," *Int. J. Syst. Bacteriol.* 47(2), 420-424. DOI: 10.1099/00207713-47-2-420

Keis, S., Bennett, C. F., Ward, V. K., and Jones, D. T. (1995). "Taxonomy and phylogeny of industrial solvent-producing Clostridia," *Int. J. Syst. Bacteriol.* 45(4), 693-705. DOI: 10.1099/00207713-45-4-693

Keis, S., Shaheen, R., and Jones, D. T. (2001). "Emended descriptions of *Clostridium acetobutylicum* and *Clostridium beijerinckii*, and descriptions of *Clostridium saccharoperbutylacetonicum* sp. nov. and *Clostridium saccharobutylicum* sp. nov.," *Int. J. Syst. Evol. Microbiol.* 51(6), 2095-2103. DOI: 10.1099/00207713-51-6-2095

Lee, S. Y., Park, J. H., Jang, S. H., Nielsen, L. K., Kim, J., and Jung, K. S. (2008). "Fermentative butanol production by *Clostridia*," *Biotechnol. Bioeng.* 101(2), 209-228. DOI: 10.1002/bit.22003

López-Contreras, A. M., Claassen, P. A. M., Mooibroek, H., and De Vos, W. M. (2000). "Utilisation of saccharides in extruded domestic organic waste by *Clostridium acetobutylicum* ATCC 824 for production of acetone, butanol and ethanol," *Appl. Microbiol. Biotechnol.* 54(2), 162-167. DOI: 10.1007/s002530000374

Margeot, A., Hahn-Hägerdal, B., Edlund, M., Slade, R., and Monot, F. (2009). "New improvements for lignocellulosic ethanol," *Curr. Opin. Biotechnol.* 20(3), 372-380. DOI: 10.1016/j.copbio.2009.05.009

Miller, G. L. (1972). "Use of dinitrosalicylic acid reagent for determination of reducing sugars," *Anal. Chem.* 31(3), 426-428. DOI: 10.1021/ac60147a030

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

Qureshi, N., Saha, B. C., Dien, B., Hector, R. E., and Cotta, M. A. (2010). "Production of butanol (a biofuel) from agricultural residues: Part I - Use of barley straw hydrolysate," *Biomass Bioener.* 34(4), 559-565. DOI: 10.1016/j.biombioe.2009.12.024

Ranjan, A., and Moholkar, V. S. (2012). "Biobutanol: Science, engineering, and economics," *Int. J. Energ. Res.* 36(3), 277-323. DOI: 10.1002/er.1948|

Sankar, M., Delgado O., and Mattiasso, B. (2003). "Isolation and characterization of solventogenic, cellulase-free xylanolytic *Clostridia* from cow rumen," *Water Sci. Technol.* 48(4), 185-188. DOI: 10.1186/s40643-016-0105-9

Sauer, U., Santangelo, A. T., Treuner, A., Buchholz, M., and Durre, P. (1995). "Sigma factor and sporulation genes in *Clostridium*," *FEMS Microbiol. Rev.* 17(3), 331-340. DOI: 10.1111/j.1574-6976.1995.tb00216.x

Varel, V. H., Tannerr, R. S., and Woese, C. R. (1995). "*Clostridium herbivoruns* sp. nov., a cellulolytic anaerobe from the pig intestine," *Int. J. Syst. Bacteriol.* 45(3), 490-494. DOI: 10.1099/00207713-45-3-490

Wallner, T., Miers, S. A., and McConnell, S. (2009). "A comparison of ethanol and butanol as oxygenates using a direct-injection, spark-ignition engine," *J. Eng. Gas Turb. Power* 131(3), 032802-032810. DOI: 10.1115/1.3043810

Woods, D. R. I., and Jones, D. T. (1986). "Physiological response of *Bacteroides* and *Clostridium* strains to environmental stress factors," *Adv. Microb. Physiol.* 28, 1-64. DOI: 10.1016/S0065-2911(08)60236-2

Xin, F., Wu, Y.-R., and He, J. (2014). "Simultaneous fermentation of glucose and xylose to butanol by *Clostridium* sp. strain BOH3," *Appl. Environ. Microbiol.* 80(15), 4771-4778. DOI: 10.1128/AEM.00337-14

Zhang, X., Feng, X., Zhang, H., and Wei, Y. (2018). "Utilization of steam-exploded corn straw to produce biofuel butanol *via* fermentation with a newly selected strain of *Clostridium acetobutylicum*," *BioResources* 13(3), 5805-5817. DOI: 10.15376/biores.13.3.5805-5817

Zhou, Q., Zheng, H., and Yuan, W. (2018). "Modeling butanol synthesis in xylose by *Clostridium saccharoperbutylacetonicum*," *BioResources* 13(4), 7270-7280. DOI: 10.15376/biores.13.4.7270-7280

Article submitted: March 24, 2019; Peer review completed: May 24, 2019; Revised version received: May 29, 2019; Accepted: May 31, 2019; Published: June 10, 2019.
DOI: 10.15376/biores.14.3.5832-5844