AIMS Energy, 4(1): 22-36.
DOI: 10.3934/energy.2016.1.22
Received: 08 October 2015
Accepted: 24 December 2015
Published: 12 January 2016

http://www.aimspress.com/journal/energy

Research article

Novel thermostable clostridial strains through protoplast fusion for enhanced biobutanol production at higher temperature—preliminary study

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Abstract: The objective of this study is to improve the thermal stability of clostridium strains for enhanced biobutanol production. Thermostable clostridia species were developed through protoplast fusion between mesophilic clostridial species (i.e., *Clostridium beijerinckii* and *Clostridium acetobutylicum*) and thermophilic clostridial species (i.e., *Clostridium thermocellum*). Production of biobutanol was examined in the present preliminary study using the clostridium strains and their protoplast fusants using sugar mixture with composition identical to that of wheat straw acid *hydrolysate*. Maximum biobutanol production of 9.4 g/L was achieved by a fused strain at 45 ℃ with total sugar consumption of 66% compared to that at 35 ℃ (i.e., 8.4 g/L production and 64% total sugar consumption). Glucose and xylose uptake rates were generally higher compared to all other individual sugars in the feedstock. In general, average cell concentrations were in close proximity for all parenting and fused strains at 35 ℃; i.e., in the range of 5.12×10^7 to 5.49×10^7 cells/mL. Average cell concentration of fusants between the mesophilic clostridial species and the thermophilic clostridial species slightly increased to ~ 5.62×10^7 cells/mL at a higher temperature of 45 ℃. These results, in addition to the ones obtained for the butanol production, demonstrate enhanced thermal stability of both fusants at a higher temperature (45 ℃).

Keywords: Protoplast fusion; biobutanol; agriculture residues; regeneration medium; clostridial fusants

1. Introduction

Butanol, currently produced from petrochemical route, is an important multi-use feedstock chemical. It has numerous industrial applications in plastic and food industry, and is used as a fuel extender [1,2]. Compared to ethanol, butanol is more hydrophobic and has a higher energy density, which makes it a superior biofuel. Its properties allow the use of existing pipeline infrastructures for transportation and distribution; it can be mixed with gasoline at any ratio. Until around 1950, biological synthesis of butanol served as a major feedstock for this solvent. Eventually, the availability of cheaper raw oil and the increase of substrate prices gave the oil industry a massive economic advantage, which led to the decline in fermentation processes. The increasing cost of fermentation substrates, and the availability of cheaper petrochemical feedstock to synthesize butanol, resulted in the demise of clostridial fermentation process [3]. After the 1974 oil crisis, there was a renewed interest in the biological production of butanol by the clostridia. The current studies are focused on strain improvement, use of economical fermentation substrates, and developing an energy-efficient technology for butanol recovery [4].

Protoplast fusion [5] and transformation [6] have been the primary and industrially important ways to produce genetic recombination in gram-positive bacteria. For its application in pure and applied genetics, protoplast fusion is of current interest. This technology is applied for developing inter and intra specific supra hybrids, possessing higher potentiality compared to their parental strains. Protoplast fusion has proven to be very feasible for interspecific and inter-generic hybridization for stain improvement in edible mushrooms [4]. Induced protoplast fusion has the ability to overcome vegetative incompatibility and produce hybrids with combined properties from both parents [7].

Cellulolytic and solventogenic species like *C. thermocellum*, *C. saccharobutylicum*, *C. cellulolyticum*, and *C. acetobutylicum* are extensively researched biomass-metabolizing bacteria. They have a significant potential for sustainable biofuel production through consolidated bioprocessing [8]. Clostridia are obligate anaerobe, gram-positive, sporulating firmicutes including pathogenic species important to human or animal health and physiology, and non-pathogenic species for commercial conversion of renewable resources into biofuels and commodity chemicals [9]. The most popular strains for butanol fermentation are the mesophiles *Clostridium beijerinckii* and *Clostridium acetobutylicum* [10]. These species possess high industrial potential because of their ability to produce various carbohydrate based solvents via two-stage fermentation. In acidogenic stage, the primary products are acetate and butyrate, while in solventogenic stage, the products are acetone and butanol [11]. Solventogenic *C. beijerinckii* and *C. thermocellum* with acetone, isopropanol, or both [12], produces similar amount of butanol and ethanol. In this stage, propanol and 1, 2 propanediol are also produced by *C. beijerinckii*, grown on certain pentoses [12,13].

It is well known that a less expensive sugar mixture, eligible for huge application in fermentation, can be extracted from agricultural residues like wheat straw (WS), corn fibers (CF), and Distiller's Dried Grains with Solubles (DDGS). Recent advances in the agricultural biomass conversion technology have demonstrated that agricultural wastes, along with their hydrolysate solutions, potentially comprise enough carbon sources for the production of value-added bio-based products [14].

Similar to other bioprocesses, the production of butanol fermentation is limited by high substrate cost, low product yield, and high recovery cost [15]. To overcome these problems, metabolic

engineering and advanced fermentation techniques have been implemented in *Clostridia* [16]. Another active field is exploring potential substrates for butanol fermentation [17]. Production of butanol has been undertaken by a variety of resources like wheat straw, corn stover and corn fiber [18]. Current microorganisms are incapable to efficiently hydrolyze lignocellulosic biomass to produce butanol. Therefore, both physical-chemical pre-treatment [19] and enzymatic hydrolysis [20,21] are required for the production. During pre-treatment, the biomass is exposed to severe conditions including high temperature and reactions with various chemicals. However, this pre-treatment process produces fermentation inhibitors; removal of these inhibitors will add on to the total cost [16,20]. In enzymatic hydrolysis, fewer fermentation inhibitors in the hydrolysate are produced, compared to the other alternative acidic pre-treatment. The challenge facing the enzymatic hydrolysis, during the simultaneous saccharification and fermentation (SSF), is the lower temperature of fermentation (nearly 35 ℃) since the optimum temperature of enzymatic hydrolysis was proven to be 45 ℃. However, the lower efficiency of the enzymes at 35 ℃, especially *cellulase*, prolongs butanol production cycle and increases the fermentation cost [17].

The present work represents results obtained from first attempts to develop a novel thermostable clostridia species through protoplast fusion between mesophilic and thermophilic clostridial species. Therefore, with the use of protoplast manipulation techniques, these methods are appropriate for the genetic engineering of industrial microorganisms. In order to investigate potentials to produce biofuels through utilizing the sustainable and renewable resources of agriculture wastes at higher temperatures, these species were examined in producing butanol at 45 ℃ using sugar mixtures, with composition identical to the acid hydrolysate of wheat straw. More results on the novel fusants in producing biofuels were published recently [22,23].

2. Materials and Methods

2.1. Chemicals and materials

C. beijerinckii (ATCC BA101), *C. acetobutylicum* (ATCC 4259), and *C. thermocellum* (ATCC 27405) were purchased from American Type Culture Collection. Reinforced Clostridial Medium CM0149 [24] for the culture activation of *C. acetobutylicum* ATCC 4259, Cooked Meat Medium MT0350 [25] for *C. beijerinckii* ATCC BA101, and Minimal Defined Medium for *C. thermocellum*, [26] were purchased from Oxoid Ltd. (Basingstoke, Hampshire, England). Analytical grade laboratory media components were obtained from Sigma-Aldrich (USA). Resazurin sodium salt, D-biotin, PABA, thiamine-HCl, $FeSO_4 \cdot 7H_2O$, $MnSO_4 \cdot 4H_2O$, $MgSO_4 \cdot 7H_2O$, $MgCl_2$, $CaCl_2$, K_2HPO_4, agar, yeast extract, casamino acids, L-asparagine, glycine, and other laboratory media components of analytical grade were purchased from Sigma-Aldrich (St. Louis, MO). Gelatin was obtained from BBL (Becton Dickinson, Cockeysville, MD).

2.2. Medium preparation

The initial preparation of cultures was from heat shocked spore suspensions, grown overnight in Clostridium Broth Medium (CBM), under stringent anaerobic conditions [29]. CBM and Regeneration Medium (RM) were prepared by the addition of stock solution to a basal mixture. Stock solution (A) contained D-biotin, 0.1 g; PABA, 0.1 g; thiamine-HCI, 0.01 g; $FeSO_4 \cdot 7H_2O$, 0.1 g;

$MnSO_4 \cdot 4H_2O$, 0.1 g; and $MgSO_4 \cdot 7H_2O$, 2.0 g, all in 100 ml H_2O. The solution was filtered, sterilized, and held in N_2 or CO_2 atmosphere. Stock solution (B) comprised of 25 g glucose in 100 ml of H_2O, while stock solutions (C) and (D) were made from 2.5 M solutions of $MgCl_2$ and $CaCl_2$, respectively. Stock solution (E) contained K_2HPO_4, 7.0 g, and KH_2PO_4, 3.0 g, dissolved in 100 ml H_2O. Stock solutions (B) through (E) were autoclaved separately.

The basal mixture was comprised of gelatin, 50.0 g; agar, 15.0 g; yeast extract, 8.0 g; casamino acids, 2.5 g, and L-asparagine, 1.0 g. The ingredients were mixed with either 930 ml H_2O for RM or 910 ml for CBM; the mixture stirred and was brought to boiling before autoclaving at 121 ℃ for 20 min. 10 ml of stock solution (A) and 40 ml of solution (B) were added to basal ingredients upon cooling. In the preparation of RM, 5 ml of each of solutions (C) and (D) were added, while 15 ml of the solutions was used to prepare CBM. 10 ml of stock solution (E) was finally added for the preparation of both RM and CBM.

2.3. Protoplast formation

Three clostridia species (i.e., Fus1:CbCt, Fus2:CaCb, Fus3:CaCt) were developed through protoplast fusion between mesophilic clostridial species (i.e., *C. beijerinckii* (Cb) and *C. acetobutylicum* (Ca)), and thermophilic clostridial species (i.e., *C. thermocellum* (Ct)). Overnight, cultures of the wild strains were diluted 1:4 with fresh, sterile CBM broth containing 0.4% and 0.8% glycine [28]. As determined by inverted microscopy (Zeiss, Axiovert 200, Germany), when the cultures became nearly 100% motile (45–60 min), adding sucrose up to 0.5 M along with lysozyme (2 mg/mL final concentration) increased the osmotic strength of each culture. After 5 minutes, $MgCl_2$ and $CaCl_2$ were added to final concentrations of 12.5 mM each. Protoplast formation was complete after 60 minutes. The formation of protoplast was observed under inverted microscopy, after incubation at 35 ℃ for 20–30 minutes. The protoplasts were centrifuged at 4500 xg, re-suspended in 4 ml broth media, and after dilution in same buffer, were placed and allowed to regenerate on RM agar. The colonies were checked after 3–5 days of incubation at 35 ℃ under anaerobic conditions (Anaerobic jar-Oxoid). Diluting protoplasts in distilled water and plating on RM agar determined osmotically resistant cells.

2.4. Clostridium morphology

Protoplast suspensions were diluted in CBM broth [28]. The broth was then supplemented with sucrose to 0.5 M to stabilize osmotically, with $MgCl_2$ and $CaCl_2$ added to final concentrations of 12.5 mM. The protoplasts remained intact in this medium for prolonged periods of time. Samples were planted onto CBM and were incubated for 4–5 days at 35 ℃ in anaerobic jar. All results reported were the average values calculated from duplicate experiments.

2.5. Regeneration of protoplasts

For regeneration, large and well-isolated L-colonies were transferred to the regeneration medium (RM). L-colonies were transferred as agar plugs extracted with sterile Pasteur pipettes, and the agar plug extruded, inverted and then pressed onto marked sectors of CBM plates. The CBM plates were incubated for 2–4 days in anaerobic jar until the occurrence of regeneration as shown in Table 2.

2.6. Microorganisms and culture conditions

The stock culture of all clostridium strains were maintained as a cell suspension in 30% (v/v) sterile glycerol at −20 ℃ in screw-capped bottles. All operations involving cells and protoplasts took place in an anaerobic hood at a mean temperature of 35 ± 2 ℃. Dry cell mass (g/L) of clostridial species and its fusants were determined gravimetrically on triplicate samples after growth, by using feedstock containing sugar mixture in time course studies. Monod equation was used in the calculation of specific growth rate (μ; h^{-1}).

2.7. Butanol production

Butanol production studies in fermentation were conducted in 125 mL serum bottles (Bellco Glass, Inc., Vineland, NJ) at two different temperatures. Two mesophilic wild strains of Cb and Ca, one protoplast fusant among both (Fus2:CaCb), in addition to two fusants of these wild strains and thermophilic Ct (Fus1:CbCt and Fus3:CaCt), were all used. Sugar mixture, as feedstock with composition identical to wheat straw hydrolysate obtained earlier by Qureshi, et al. [20], consists of five reducing sugars, was always employed. Total sugar concentration was always 56.9 g/L, which is composed of 48% glucose, 33% xylose, 8.5% arabinose, 5.8% galactose, and 4.7% mannose.

Serum bottles containing different carbohydrates were inoculated with 2% (v/v) CBM-broth culture, and incubated at 35 ℃ & 45 ℃ for one week inside an anaerobic chamber (Terra Universal, Inc. Fullerton, CA). The 5% (w/v) dextrin-1.5% solids glucose-CBM medium in serum bottles were inoculated at the 5% (v/v) level with 5% (w/v) dextrin-1.5% solids glucose-CBM-grown culture, and was continuously spurge with 100 mL/min of sterile N_2. Antifoam 289 (Sigma Chemical Co., St. Louis, MO) was added when necessary, to control foaming in the serum bottles. Samples were withdrawn at intervals for analysis. Lag phase was estimated to take three days; cells then entered the exponential growth phase when the coincident production of butanol started [29].

3. Analytical methods

3.1. Bacterial cell concentration

Flow Cytometer (Guava EasyCyte Mini System, Guava Technologies, Inc., USA) was used according to the manufacturer's guidelines for the analysis of cell concentrations of samples collected during butanol production. The Guava EasyCyte Mini System contained a blue laser (Excitation: 488 nm) that emits 20 mW visible laser radiation, three fluorescent detectors, automated analyzer unit with internal hard drive connected to a computer, single sample tube format, and CytoSoft software with expandable software modules. Using deionized water, the samples were diluted 20-fold. The diluted samples were repeatedly vortexed at high speed for 3 minutes, and were then filtered through 0.45 μm membrane filters (Millipore, USA). 1 ml of each filtered sample was loaded into the sample loader, and was circulated through capillary feeder attached to the system. Guava ViaCount Assay, through the built-in Guava® Express Plus software module, showed the direct absolute cell counts as cells/ml in conjunction with the subpopulation percentages [14]. The average cell proliferation rate was calculated by Flow Cytometer with combination of fluorescent dyes (bis-oxonol/propidium iodide), based on the increasing number of bacterial cells during a

specific period of time, specific growth rate (μ), doubling time (t_d), and proliferation rates [30].

3.2. Sugars and butanol concentrations

Sugars and butanol concentrations were measured using high performance liquid chromatography (HPLC-Perkin Elmer), equipped with a refractive index detector (2414, Waters), and 5 mM H_2SO_4. Two HPLC columns were used separately; Shodex KC811 was used for measuring the concentration of sugars and Shodex SP0810 for measuring the concentration of inhibitors. The samples were centrifuged at 25000 xg for 15 minutes and were then double filtered through 0.2 μm PTFE-filters (Whatman, USA). Each sample was analyzed under a flow rate of 0.6 ml/min and constant pressure. Concentrations were obtained from the area under the correlated peak using previously constructed calibration curves [31].

4. Results and Discussions

4.1. Protoplast formation and regeneration

Morphology studies on CBM agar plates showed that L-colonies normally grew after 4–5 days of incubation at 35 ℃. A few colonies of bacillary also appeared on some CBM plates. The number of these colonies generally correlated with the number of osmo-resistant forms, counted by plating protoplast samples diluted in anaerobic H_2O on non-osmotically reinforced media. Table 1 shows the changes observed in cell viability and L-colonies among the fusants and its wild strains. Results of L-colonies viability, osmo-resistant & % L-colonies demonstrate that fusants Fus1 and Fus2 are more resistant and stable, compared to Fus3 and all other wild strains. Furthermore, protoplasts replicated into colonies of wall-deficient and osmotical1y-sensitive forms, were transferred to the regeneration medium on which they gave rise to the L-colonies form.

Table 1. L-Colony growth of *C. beijerinckii* and fusants among other solventogenic clostridium species.

Strains	Viable Cells [a] (CFU/mL)	Osmo-Resistant [b] (CFU/mL)	L-Colony [c] (CFU/mL)	L- Colonies [d] (%)
Fus1: CbCt	9.6×10^5	5.4×10^4	8.5×10^5	88.0
Fus2: CaCb	7.9×10^6	5.1×10^3	7.6×10^5	9.6
Fus3: CaCt	3.1×10^6	1.2×10^3	2.9×10^5	9.3
Cb	8.1×10^6	4.7×10^4	6.4×10^5	7.9
Ct	4.7×10^5	2.2×10^3	2.2×10^5	46.8
Ca	4.5×10^5	1.4×10^3	2.4×10^5	53.0

CFU: Colony Forming Units.

[a] Viable cells prior to addition of 2 mg/mL lysozyme.

[b] Lysozyme-treated samples, diluted 1:10 in anaerobic H_2O, and plated on CBM agar media plates.

[c] Number of L-colonies appearing after 5 days of incubation on RM agar media plates.

[d] Calculated from: (Number of L-colonies/ total number of viable cells) × 100%.

After growth, in the presence of a partially growth-inhibitory concentration of glycine, clostridium strains became sensitive to lysozyme. Complete growth inhibition was essentially

achieved at 0.8% glycine. Cell growth for around seven cell doublings or more, in the presence of 0.4% glycine, was highly sensitive to lysozyme, and formed stable protoplasts in medium CBM. The numbers of cells that survived this treatment were typically about 1 in $l0^6$ to $l0^4$. Cells of Ca, Cb, and Ct, in the absence of glycine, were grown for only three to four cell doublings in glycine and formed protoplasts very poorly (<90 % conversion in 20 hours with 2 mg lysozyme/mL).

As shown in Table 2, when protoplasts were plated directly onto RM plates, the frequencies of L-colony formation and regeneration were generally lower than the ones that were first plated on CBM. Based on this observation, and after the protoplast cells were grown as L-colonies on CBM, they were transferred to RM. In general, the percentage of L-colonies was highest for Fus1 and Fus2, followed by wild strain Ca. Furthermore, the frequency of regeneration of transferred L-colonies ranged between 6.6% and 11.1%, with average final regeneration frequency of 5.7% for all strains. The wild strain Ct showed the smallest percentage of L-colonies, and had a frequency of regeneration of transferred colonies of 2.4%.

Table 2. L-colony growth and reversion of L-colonies of other solventogenic Clostridium species.

Strains	% L-colonies [a]	% Regeneration [b]
Fus1: CbCt	5.0	11.1
Fus2: CaCb	3.2	7.0
Fus3: CaCt	2.1	3.0
Cb	1.9	4.2
Ct	1.5	2.4
Ca	3.4	6.6

CFU: Colony Forming Units.

[a] *% L-colonies:* Number of L-colonies divided by the number of viable cells before lysozyme addition, minus the osmo-resistant forms times 100%.

[b] *% Regeneration:* Determined by the difference between the number of CFU obtained on the regeneration medium after diluting the protoplast suspensions in CBM or anaerobic water (pH 6.5).

[*]The regeneration frequencies were calculated using the formula: % regeneration = ((CFU on RM agar-osmotically stable cells)/initial cell number).

It was possible to cultivate lysozyme-generated protoplasts of clostridial species and its fusants on CBM as L-colonies. These colonies gave rise to numerous other L-colonies; they continued to grow as L-forms through at least 3 passages when removed as agar plugs, and spread onto fresh CBM plates. However, the colonies transferred from CBM to RM plates continued to gradually grow as L-colonies. This shows the first report of a medium's ability to support solventogenic clostridia as wall-deficient colonies. The colony report of clostridial L-forms and colonies were reported by Heefner, et al. (1984) [32]. Plasmid DNA was used to transform autoplasts and L-phase variants of clostridial species, but only autoplasts could redevelop into rod-shaped cells (clostridium species and its fusants). Species-specific protoplast regeneration has been cited among clostridia [27].

4.2. Biobutanol production

Figure 1 shows the total butanol productions at two different temperatures of 35 and 45 ℃ by wild clostridial and fused strains, using sugar mixture feedstock.

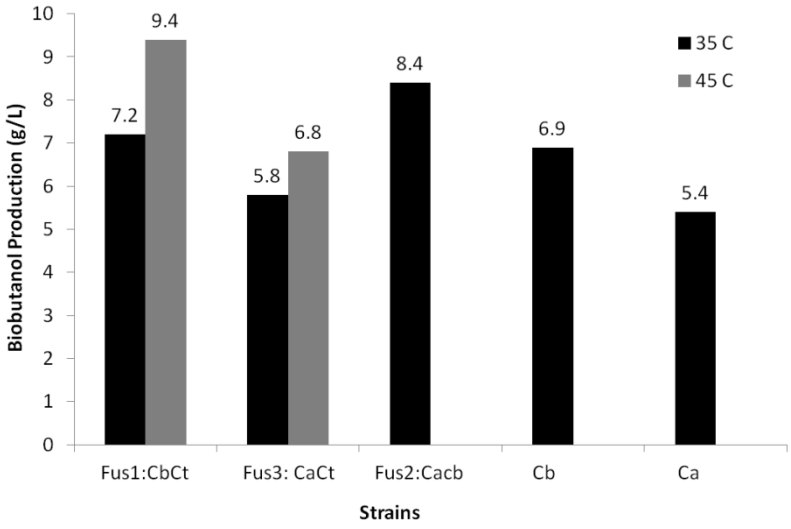

Figure 1. Butanol production at different temperatures by utilizing different clostridium strains and its fusants that contain sugar mixture (total initial sugar concentration was 56.9 g/L).

Examining Figure 1 reveals that at a lower temperature of 35 ℃, higher butanol production was obtained using *C. beijerinckii* compared to *C. acetobutylicum* (Cb and Ca production of 6.9 and 5.4 g/L, respectively). Fused strain, among these two strains (Fus2:CaCb), produced higher production of 8.4 g/L at 35 ℃, compared to the other two fused strains in Table 3. Fus1:CbCt produced 7.2 g/L compared to Fus3:CaCt (i.e., 5.8 g/L). These productions are comparable to the ones obtained by the corresponding mesophilic parenting clostridial species (Ca and Cb). At a higher temperature of 45 ℃, the fused strain of *C. beijerinckii* and *C. thermocellum* (Fus1:CbCt) produced 9.4 g/L butanol, which was the maximum production obtained in this study. This production was higher than the one obtained by the *C. acetobutylicum* and *C. thermocellum* fusant (Fus3:CaCt) at 45 ℃ (i.e., 6.8 g/L). The final pH of the fermentation medium was 6.1–6.5, observed due to the accumulation of different acids like butyric and acetic acids as intermediate [20]. In general, production of butanol reported in the present study in Table 3 (Figure 1) is higher than previous studies, where single sugars were utilized in feedstock [10-12]. This higher production and higher yield of butanol achieved with feedstock of sugar mixture can be attributed to catabolite repression, which high concentrations of single sugar as the main feedstock may cause. This has the ability to inhibit enzymatic reactions involving sensing endogenous levels of sugars, which can definitely be avoided by using sugar mixtures [33]. Figure 2 (a & b) shows the changes in total sugar concentrations respectively, which were documented in the feedstock of sugar mixture.

Table 3. Results calculated for the yield, sugar consumption, and cell proliferation during butanol production at two different temperatures of 35 and 45 ℃ by clostridium strains and its fusants using feedstock containing sugar mixture.

Strain	T (℃)	Butanol Product (g/L)	Rate of Production (g/g h)	Butanol Yield (g/g Sugar)	Sugar Consumed [a] (%)	Av. Cell Conc. (10^7 cells/mL)	Rate of Cell Proliferation [b] (10^7 cells/mL.h)	μ [c] (h^{-1})	t_d [d] (h)
Fus1:CbCt	35	7.2	0.006	0.23	54	5.49	0.098	0.015	46
Fus2:CaCb	35	8.4	0.009	0.21	64	5.45	0.092	0.014	49
Fus3:CaCt	35	5.8	0.006	0.19	46	5.3	0.018	0.010	69
Cb	35	6.9	0.007	0.18	52	5.34	0.017	0.013	53
Ca	35	5.4	0.006	0.2	46	5.12	0.019	0.012	57
Ct	35	-	-	-	43	4.77	0.016	0.009	77
Fus1:CbCt	45	9.4	0.01	0.25	66	5.65	0.054	0.011	63
Fus3:CaCt	45	6.8	0.005	0.21	61	5.58	0.016	0.009	77

[a] Calculated based on the consumption out of total initial sugar concentration of 56.9 g/L.

[b] Calculated based on the hourly increase in cell count.

[c] Specific growth rate calculated from the slope of *ln* dry cell mass (g/L) versus time (h).

[d] Calculated based on specific growth rate ($t_d = \ln(2)/\mu$).

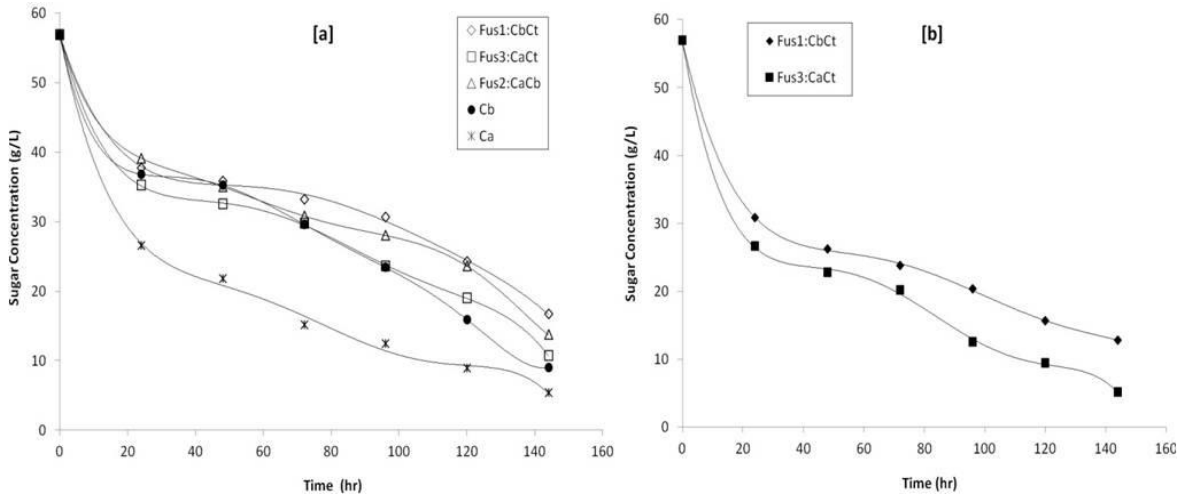

Figure 2. Changes in sugar concentrations during butanol production by clostridium strain and its fusants, using feedstock containing sugar mixture: (a) Clostridium strain and its fusants at 35 ℃; (b) Clostrium fusants at 45 ℃ (total initial sugar concentration was 56.9 g/L).

According to Figure 2, concentration profiles of sugar documented in all media were almost similar, consisting of a sharp drop during fermentation for the first 24 hours. However, alterations in the concentration of sugar, observed in the subsequent days of fermentation, were comparatively much smaller. Table 4 shows the changes observed in the individual sugar consumption in the sugar mixture during biobutanol fermentation at two different temperatures.

Table 4. Results calculated for the individual sugar consumption during butanol production at two different temperatures of 35 and 45 ℃, by clostridium strains and its fusants using feedstock containing sugar mixture.

Strain	Temp	Total [a]	Individual Sugars Consumed (%) [b]				
	(^{o}C)	(%)	Glucose	Xylose	Arabinose	Galactose	Mannose
Fus1:CbCt	35	54	60	58	61	60	65
Fus2:CaCb	35	64	65	68	59	58	63
Fus3:CaCt	35	46	64	60	52	57	59
Cb	35	52	58	67	49	51	51
Ca	35	46	56	52	46	44	49
Fus1:CbCt	45	66	69	70	64	69	77
Fus3:CaCt	45	61	64	60	57	57	66

[a] Based on total amount consumed.

[b] Based on initial individual sugar concentrations.

According to Table 4, individual sugar uptake was generally higher with the fused strains (Fus1, Fus2, and Fus3), compared to the wild clostridial strains at 35 ℃. Further examination of results in Table 4 shows that individual sugar uptakes clearly increased at 45 ℃ with Fus1:CbCt. This, however, was not observed with Fus3:CaCt. The sugar consumption rates are all calculated based on Figure 2. Examining Figure 2a shows that the difference in sugar consumption had close consumption rates of 0.01–0.09 g/L.h for clostridium strain and its fusants at 35 ℃ and 45 ℃ (Figure 2b). Apparently, this lower consumption is due to the complexity in metabolizing sugar [34]. This is presumed to be related to the fact that most glucose is generally metabolized for energy production and cell maintenance [35]. Total consumption of individual sugars in the sugar mixture is also summarized in Table 4. Surprisingly, sugar consumptions clearly increased with clostridial fusant Fus1:CbCt at 45 ℃ after the first day of fermentation. No significant change in individual consumption was observed for the other fused strains at 45 ℃. Based on these observations, it is anticipated that the combination of other sugars may trigger the cell growth at the initial stage of growth and may enhance the biobutanol production. Balancing the nutrients by providing adequate minerals and vitamins may be crucial as they are essential for cell growth and butanol production.

Changes in bacterial cell concentration in clostridium strains and its fusants at two different temperatures of 35 and 45 ℃, that were measured during biobutanol fermentation, in the medium of sugar mixture with composition identical to that of wheat straw acid hydrolysate, are shown in Figure 3 (a and b) respectively. The concentration of bacterial cells increased, reaching high concentration during the first day of fermentation at 35 and 45 ℃. This demonstrates that the larger amounts of metabolized sugars during the first day of fermentation are mainly utilized for cell growth and other secondary metabolites, rather than butanol production. Increase in cell concentrations were slightly low until the first day of fermentation in the clostridial fusants at 35 ℃ (Figure 3a) and 45 ℃ (Figure 3b). As it appears from Figure 3a, bacterial cell concentration increases at 35 ℃ slightly more rapidly for the wild strains Ca, Ct, Cb, and the fusant Fus3:CaCt, compared with that of Fus1:CbCt and Fus2:CaCb. In general, average cell concentrations at 35 ℃ were in close proximity for all parenting and fused strains in Table 3. Results from Table 3 reveal that the average cell concentrations of butanol producing bacteria were in the range of 5.12×10^7 to 5.49×10^7 cells/mL. Specific growth rates were calculated based on dry cell mass (μ) for the different clostridial strains and its fusants with different average cell concentrations.

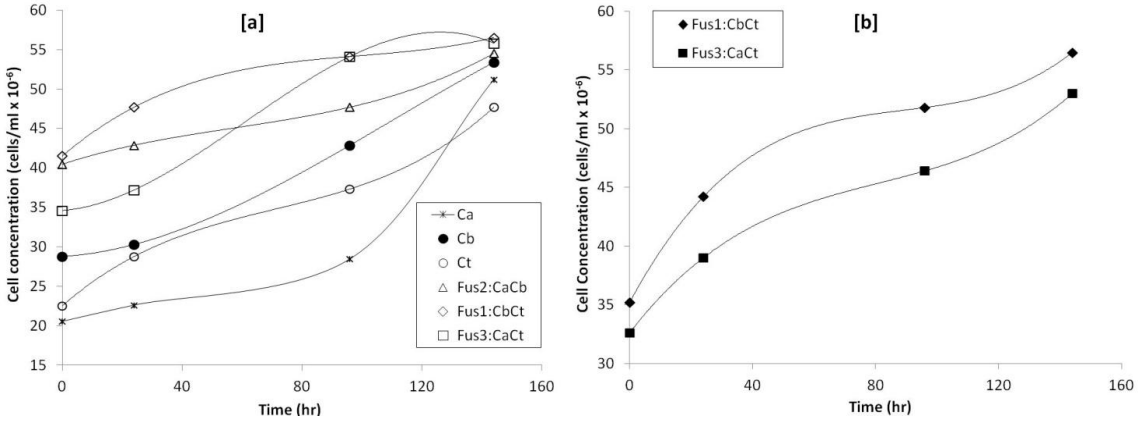

Figure 3. Changes in bacterial cell concentrations during biobutanol production by clostridium strain and its fusants in feedstock containing sugar mixture: (a) Clostridium strain and its fusants at 35 ℃; (b) Clostrium fusants at 45 ℃ (total initial sugar concentration was 56.9 g/L).

Table 3 shows that the higher values of μ, calculated at 35 ℃ for Fus1:CbCt and Fus2:CaCb, represents lower growth rates compared to all other strains. In Figure 3b, the growth in cell concentration at 45 ℃ for Fus1:CbCt increased and had a higher average cell concentration of 5.65×10^7 compared to 5.49×10^7 cells/mL at 35 ℃ (see Table 3). The specific growth rate, calculated based on dry cell mass of Fus3:CaCt, remained similar as shown in Table 3 (i.e., 0.010 and 0.009 h^{-1} at 35 and 45 ℃, respectively). However, the average cell concentrations of both fusants slightly increased at higher temperature (i.e., 5.65×10^7 and 5.58×10^7 cells/ ml for Fus1 and Fus3 at 45 ℃, respectively). This demonstrates enhancement in the thermal stability of both fusants at a higher temperature of 45 ℃. Figure 4 (a & b) shows changes in total sugar concentrations during biobutanol production by Fus1:CbCt and Fus3:CaCt at 35 and 45 ℃.

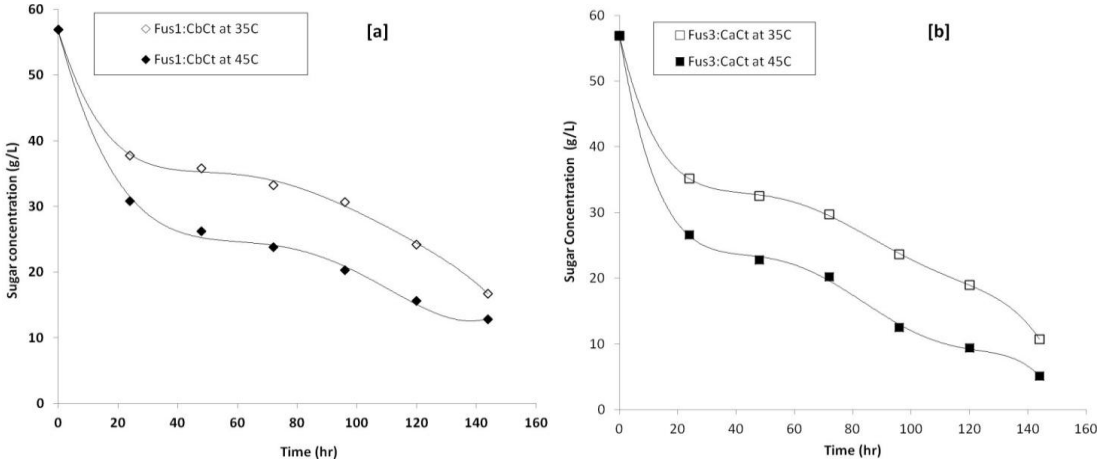

Figure 4. Changes in sugar concentrations during butanol production by clostridium fusants using feedstock containing sugar mixture at 35 ℃ and 45 ℃ (total initial sugar concentration was 56.9 g/L): (a) Fusant 1 (CbCt) and (b) Fusant 3 (CaCt).

Examining Figure 4 for both thermophilic fusants shows that sugar concentrations decreased more rapidly at 45 ℃ compared to 35 ℃, with major consumption observed in the first 24 hours. Total sugar consumptions were 66% and 61% for the two fusants at 45 ℃ respectively, compared to 54% and 46% at 35 ℃ (Table 3). Table 3 shows that sugar consumption of fusants (Fus1:CbCt and Fus3:CaCt) were generally higher than that of the other clostridial wild strains (Ca and Cb), at the two different temperatures of 35 ℃ and 45 ℃. However, lower average cell proliferation rates were calculated at 45 ℃ for both strains. Sugar consumptions of individual sugars were further analyzed and results were summarized in Table 4. Figure 5 (a & b) shows total biobutanol produced with time at 35 ℃ and 45 ℃ for the thermostable fusants Fus1 and Fus3.

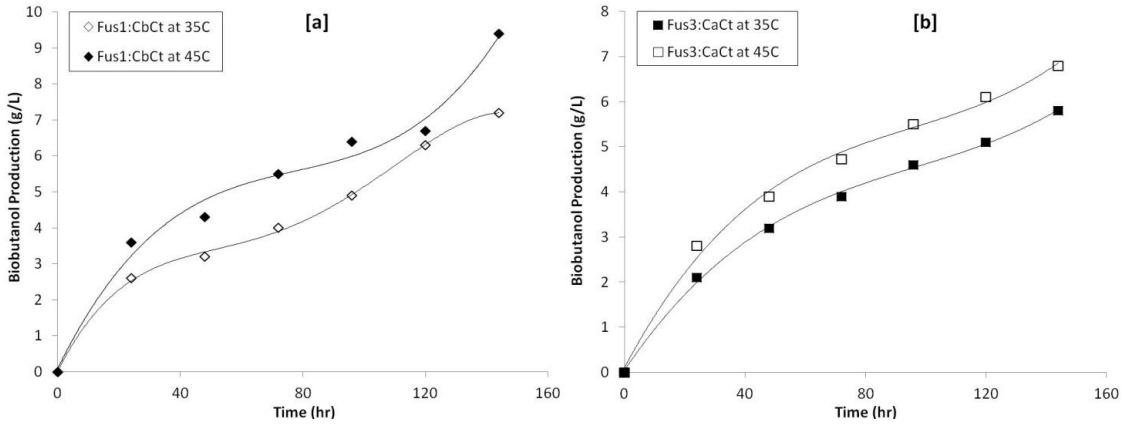

Figure 5. Butanol production by clostridium fusants using feedstock containing sugar mixture at 35 ℃ and 45 ℃: (a) Fusant 1 (CbCt) and (b) Fusant 3 (CaCt).

According to Figure 5, production of biobutanol was obviously higher at higher temperatures for both fusants. Moreover, production was regular with similar increases per day along the fermentation period. As listed in Table 3, total production of 9.4 g/L at 45 ℃ was obtained using Fus1, compared to 7.2 g/L at 35 ℃ (Figure 5a), while Fus3 produced 6.8 g/L at 45 ℃ and 5.8 g/L at 35 ℃ (Figure 5b). The higher butanol production using Fus1 than Fus2 can be related to that of the wild strain Cb, which produced higher butanol than Ca (6.9 and 5.4 g/L respectively; see Figure 1). Results reported in Figure 5 demonstrate thermal stability of the fused strains. Generally, thermal stability of Cb and Ca, that was developed by fusion with Ct (possibly because of chaperon activity), compared with clostridium strains develop for butanol production at 45 ℃ as shown in Figure 5. A recent illustration is the cumulative fusion approach, underlying the engineering of a thermolysin-like protease that combines low temperature activity with thermal stability heat-shock proteins [33,34]. Comparing mesophilic and thermophilic strains, it implies that evolutionary adaptation can maintain corresponding states with respect to conformational flexibility. That way optimizing biological function, under specific physiological conditions, is possible. Clostridium fusants itself showed dual activities, meaning active hydrolysis of sugars in agricultural residues at higher temperatures by producing enzymes during simultaneous hydrolysis and fermentation [34,35].

Results obtained in this study suggest that protoplast fusion can be used in intergeneric transfer of genes in clostridia. This technique provides a method of transferring plasmids from *C. acetobutylicum*, a species for which a protocol of transformation is available, to clostridium strains. Therefore, it facilitates the application of genetic engineering technology to this important group of

bacteria. Further investigations are ongoing in order to find these facts to demonstrate the high potential of producing biobutanol based on renewable agricultural residues. Future objective of this study includes the utilization of fused strains to produce biobutanol production in SSF without the addition of enzymes. Fused strains are expected to grow at a higher temperature of 45 ℃, while producing enzymes (exo-endoglucanase enzymes) for saccharification of agriculture residues.

5. Conclusion

Thermostable clostridia species were developed through protoplast fusion between mesophilic (i.e., *Clostridium beijerinckii* and *Clostridium acetobutylicum*) and thermophilic clostridial species (i.e., *Clostridium thermocellum*). Results obtained in the present study showed that generally, clostridial fused strains achieved higher butanol production compared to their parental strains at 35 ^{o}C. Further enhancement in the production ability of the fused strains was observed clearly at a higher temperature of 45 ^{o}C. The enhancement in production was accompanied with higher sugar consumption rates compared to other strains and at lower temperature. Moreover, an increase in bacterial cell growth rates was observed at a higher temperature of 45 ^{o}C, especially with the fused strains with highest production.

Results in the present study demonstrate the ability to enhance butanol production through utilizing the sustainable agro-industrial wastes as fermentation feedstock. Higher temperatures will allow for the simultaneous saccharification and fermentation of biomass that requires operation at higher temperatures.

Acknowledgements

Authors would like to acknowledge financial support from Agriculture and Agri-Food Canada, the *Natural Sciences and Engineering Research Council* of Canada (*NSERC*), and the Faculty of Engineering and Architectural Science (FEAS) at Ryerson University in Toronto, Canada.

Conflict of interest

All authors declare no conflicts of interest in this paper.

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