Bioprocess Biosyst Eng (2015) 38:2249–2262
DOI 10.1007/s00449-015-1462-z

ORIGINAL PAPER

# Novel clostridial fusants in comparison with co-cultured counterpart species for enhanced production of biobutanol using green renewable and sustainable feedstock

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Received: 17 February 2015 / Accepted: 17 August 2015 / Published online: 22 September 2015


**Abstract** In this work, biobutanol was produced through simultaneous saccharification and fermentation (SSF) of wheat straw (WS) that traditionally produces acetone, butanol and ethanol solvents (ABE). Thermal stability was imparted to two mesophilic clostridial wild strains (*Clostridium beijerinckii* and *Clostridium acetobutylicum*) through protoplast fusion with that of a corresponding thermophilic clostridial species (*Clostridium thermocellum*). Production was pursued by the fused strains at 45 °C compared to that of the corresponding co-cultures at 35 °C. Results showed that the fused strains generally achieved higher production at 45 °C than that of the corresponding co-cultures at 35 °C. Highest butanol production of 13.82 g/L was recorded with *C. beijerinckii* fusant, with ABE solvents production of 23 g/L (yields of 0.17 and 0.57, respectively). Total sugar consumption of this strain was the highest among all strains and was 84 %. Fused strains also showed immense level of tolerance towards butanol toxicity compared to the wild strains. Filter paper enzyme assay demonstrated that fused strains were able to produce cellulolytic enzymes in the range of 58.73–68.52 FPU/ml. Cellulosome producing *C. thermocellum* and its ability to ferment sugars offers a promising future in biofuels through eliminating the need to add external enzymes. Generally, productions reported in the present study were higher than literature where biobutanol stripping systems were employed to eliminate toxicity during production. This demonstrates a clear potential for improving productivity and yield at a larger-scale facility.

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**Keywords** Biomass · Co-culture · Green · Biofuel · Protoplast fusion · Renewable energy · Simultaneous saccharification and fermentation · Wheat straw

## Introduction

The ever-increasing demand for fuel has increased production from oil fields that are declining at an annual rate of 4–5 %. The need to switch to biofuels was triggered by the global statistics forecast that production of oil and gas is approaching its maximum and the world is now finding one new barrel of oil for every four it consumes [1]. The endless reliance of the global economy and industry on decreasing fossil fuel reserves has generated amplified interest in non-fossil-based energy, primarily derived from fermentation of agricultural residues like wheat straw, bagasse and corn stover. The presence of the largest crude oil deposits in the most antagonistic regions has fostered research on biofuels like ethanol and butanol. Butanol can be prepared from acetone–butanol–ethanol (ABE) fermentation process using agricultural substrates. ABE fermentation is a two-phase process involving acidogenesis (production of acids like acetic and butyric acid) followed by solventogenesis (production of solvents by conversion to acetone, butanol and ethanol) [2]. Production of butanol through fermentation has been around for a while and is one of the oldest fermentation processes that commercialized the production of biofuels. Although there were large commercial operations during world wars (I and II), its production declined with the discovery of oil and inexpensive operating costs associated with crude oil-based petroleum industries [3]. The primary problem is associated with the decline of the commercial fermentation process was the cost of substrates for making butanol [4].

The primary source of substrates until recently was from edible parts of biomass like seeds, starches and crops. The immense demand for food crops not only for consumption, but also for fuel production created a supply shortage thereby boosting the cost of substrates required for biofuel production [5]. Utilizing agricultural wastes left out after harvesting can diminish the economic concerns of higher substrate costs. The biomass substrates undergo thermochemical and biochemical treatments to convert it to biofuels. The last generation of biomass sources such as microbes and microalgae are considered to be viable alternative energy resource. Previous attempts in producing butanol from wheat straw (WS) hydrolysate have been very successful. In these carefully constructed experiments, not only was the fermentation more rapid than the ones in which glucose (as a substrate) was exclusively used, but also there was an absence of inhibition due to salts or other inhibitory products [6].

Butanol as a biofuel has some attractive properties that other biofuels like ethanol do not possess. It is worthwhile to note that butanol's energy content is 30 % more than ethanol and is closer to gasoline. Biobutanol, in its pure form can be blended in any concentration with gasoline unlike ethanol which can only be blended up to 85 % [7]. Introduction of biobutanol into the existing car engines as a combustible fuel requires minimal modification to the engine technology. Its lower vapor pressure makes it safer to handle. Butanol is not hygroscopic which allows blending with gasoline at a refinery well in advance of storage and distribution. This is in stark contrast to ethanol, which requires blending to occur shortly before distribution [7]. Therefore, butanol can be a more practical solution to address current and imminent energy crisis.

In previous works, thermostable clostridial species were developed through protoplast fusion between mesophilic (*Clostridium acetobutylicum* and *Clostridium beijerinckii*) and thermophilic strains (*Clostridium thermocellum*) [8]. Protoplast fusion was employed to produce genetically improved strains for ABE fermentation. In this method cell gene recombination occurs because the DNA of two kinds of non-divided cells co-exists inside a cell during fusion. The method is primarily used for cell function improvement and is well-known process to change the genetic characteristics of microorganisms without the need of complicated engineering techniques [9].

Biobutanol is generally produced from renewable sources using *C. acetobutylicum* or *C. beijerinckii* [10]. The motivation behind using these strains lies in the fact that they can exploit both hexoses and pentoses, which places them in disparity with their yeast (ethanol-producing) counterparts [3]. Furthermore, *C. thermocellum* is an important anaerobic thermophile that is used in industrial conversion of cellulosic biomass into liquid biofuels. Hydrolysis and fermentation are usually performed simultaneously and the process is referred to as simultaneous saccharification and fermentation (SSF). A significant challenge facing enzymatic hydrolysis is the lower temperature during SSF considering the optimal temperature for saccharification is around 45 °C [10]. Therefore, in this study we have used 45 °C as optimal temperature for performing SSF for protoplast fused strains. Since it is a simultaneous process, it is required that both hydrolysis and fermentation occur at the same temperature. However, during the fermentation segment of the SSF, addition of external enzymes to the newly developed fused strains was found to have a negative effect on the butanol production [8]. The reason for the negative effect was that the bacteria were able to produce their enzymes at 45 °C and addition of any supplementary enzymes would compete with the existing ones, thereby reducing the concentration of the final products. Cost reduction of raw materials (including enzymes and substrates) is an important factor in the profitability from industrialization of the ABE process [11]. Studies have revealed that *C. thermocellum* has immense potential of hydrolyzing cellulose by a different mode of action from the classical mechanism involving solubilization by *cellobiohydrolase* [12].

In the present study, to further investigate the effectiveness of the protoplast fusion between a butanol-producing mesophilic strains with a thermophile, it was important to compare the ABE production by the fused strains at 45 °C (optimum SSF temperature) [6] to that of corresponding co-cultures at 35 °C. No external enzymes were added during the SSF and all enzymes were generated after inoculation with the fused strains. Thus, a co-culture was prepared and used to perform SSF of WS and compare solvent production with corresponding experiments with the fused strains.

## Experimental methodology

### Chemicals and materials

Wild strains of *C. beijerinckii* (*Cb*) ATCC BA101, *C. acetobutylicum* (*Ca*) ATCC 4259, and *C. thermocellum* (*Ct*) ATCC 27405 were purchased from American Type Culture Collection.

Reinforced Clostridial Medium (RCM) CM0149 for the culture activation of *C. acetobutylicum*, Cooked Meat Medium (CMM) MT0350 for *C. beijerinckii*, and Minimal Defined Medium (MDM) for *C. thermocellum* were purchased from Oxoid Ltd. (Basingstoke, Hampshire, England). The laboratory media components of analytical grade were purchased from Sigma-Aldrich (St. Louis, MO). Gelatin was obtained from BBL (Becton-Dickinson, Cockeysville, MD).

### Culture conditions and medium preparation

All Clostridium strains were heat shocked for 5 min at 80 °C [13]. Any anaerobic manipulations were carried out in an anaerobic chamber (i.e., Glove Box; Terra Universal, Canada) at a temperature of 25 ± 2 °C under aseptic conditions. CMM were used for culture activation/maintenance of wild and fused strains and were prepared by dissolving 12.5 g, respectively, in 100 mL distilled $H_2O$. Clostridium Broth Medium (CBM) is a medium used to grow the fused clostridium strains and was prepared by adding 2 g glucose, 0.04 g $MgSO_4{\cdot}7H_2O$, 0.002 g of $MnSO_4{\cdot}4H_2O$, 0.002 g of $FeSO_4{\cdot}7H_2O$, 0.0002 g of *para*-amino benzoic acid (PABA), 0.004 g of biotin, 0.00002 g of thiamin-HCl and 0.8 g of casein hydrolysate in 200 ml water [14]. Two basic vitamins required to maintain active growth of the culture were biotin and PABA, with PABA being the limiting factor [15]. Clostridium Growth medium (CGM) was used as alternative medium to CBM for growing Clostridium bacteria for the co-culture process since it had selectivity for butanol [16]. It contained 0.75 g/L $KH_2PO_4$, 0.982 g/L $K_2HPO_4$, 1 g/L NaCl, 0.01 g/L $MnSO_4$, 0.004 g/L *p*-amino benzoic acid, 0.348 g/L $MgSO_4$, 0.01 g/L $FeSO_4$, 2 g/L asparagine, 5 g/L yeast extract, 2 g/L $(NH_4)_2SO_4$ and 40 g/L glucose [17]. Regeneration Medium (RM) was an essential medium for growing bacterial colonies and was prepared by the addition of stock solutions to a basal mixture. Stock solutions A–E were prepared for making RM. Stock solution A contained 1 g/L biotin, 1 g/L PABA, 0.1 g/L thiamin-HCl, 1 g/L $FeSO_4{\cdot}7H_2O$, 1 g/L $MnSO_4{\cdot}4H_2O$ and 20 g/L of $MgSO_4{\cdot}7H_2O$. 100 ml of Stock solution A was prepared and filter sterilized and kept in $N_2/CO_2$ atmosphere. Stock solution B was made by adding 25 g glucose in 100 ml of $H_2O$. Stock solution C comprised 2.5 M solution of $MgCl_2$. Stock D solution consists of 2.5 M solution of $CaCl_2$ and finally stock solution E consists of 7.0 g of $K_2HPO_4$, and 3.0 g of KH2PO4, dissolved in 100 ml $H_2O$. Stock solutions B through E were autoclaved separately. Basal Mixture contained 50 g gelatin, 15 g agar, 8 g yeast extract, 2.5 g casamino acids, 1 g asparagine. These ingredients were mixed in 930 ml $H_2O$ and the mixture was stirred and boiled before autoclaving at 121 °C for 20 min [18]. Upon cooling, 10 ml of stock solution A and 40 ml of solution B was added to the basal mixture. To make the RM medium, 5 ml of each of solutions C and D was added along with 10 ml of stock solution E. This viscous medium was then poured into petri dishes and allowed to set until they form a smooth solid surface for the bacteria to grow. These are agar streak plates and are an essential tool in microbiology. This allowed bacteria and fungi to grow on a semi-solid surface to produce discrete colonies. National Biological Resource Center (NBRC) Medium 979 is used for co-culture and contains 1.3 g/L $(NH_4)_2SO_4$, 2.6 g/L $MgCl_2{\cdot}6H_2O$, 1.43 g/L $KH_2PO_4$, 7.2 g/L $K_2HPO_4{\cdot}3H_2O$, 0.13 g/L $CaCl_2{\cdot}2H_2O$, 6 g/L sodium glycerophosphate, 1.1 mg/L $FeSO_4{\cdot}7H_2O$, 0.25 g/L glutathione, 4.5 g/L yeast extract, 1 mg/L Resazurin and 5 g/L cellobiose. All ingredients except for cellobiose were mixed and autoclaved under $N_2/CO_2$ condition. Filter-sterile 10 % cellobiose solution was added aseptically and anaerobically before inoculation.

### Pre-treatment of wheat straw

The source of the wheat straw (WS) used in our study was from Springridge Farm in Milton, Ontario, and was stored at room temperature. Prior to using it as a substrate for fermentation, the wheat straw was grounded into fine particles using a 1-mm sieve screen in a hammer mill (Retsch GmbH Inc., USA). The moisture content of the wheat straw was reduced through heating in a conventional oven at 105 °C for 10 h until no further weight loss was reported. Acidic pre-treated WS was obtained by suspending 4.5 g (dry) in 50 mL of 1 % dilute sulphuric acid ($H_2SO_4$) in 250 ml Wheaton serum bottles [6]. Dilute sulphuric acid (1 %) solution was prepared by adding 1 ml of 99.99 % sulphuric acid to 99 ml water. The WS–acid solution was autoclaved at 121 °C for 60 min. Any decrease in water content was accommodated for after autoclaving.

The serum bottles were removed from the autoclave and allowed to cool down to room temperature [6]. This pre-treated WS can now be used in the SSF process. Previous studies in the lab have shown that the fused strains developed more rapidly at high temperatures and produced the required enzymes for the saccharification of WS (i.e., *endoglucanase*, *exoglucanase*, and *β-glucosidase*). This eliminated the need to add enzymes required to hydrolyze the agriculture substrates in feedstock and reduced the total cost of operation.

### Butanol production using clostridial fusants and co-culture species

In this work, ABE solvent production was examined in SSF using the two protoplast fused strains that were prepared earlier at 45 °C in comparison with co-culture fermentation of the corresponding species at 35 °C. Two clostridia fusants (*CbCt* and *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*)]. Fused strains were prepared following the general protocol proposed in earlier publication [8]. All strains were stored in an ultra-low freezer at −80 °C.

The co-culture fermentation used in this research was based on co-culture techniques used by the Department of Fermentation Science and Technology at the Tokyo University of Agriculture in Japan [19]. *Ct* (8 %) was inoculated with 100 ml of NBRC medium and incubated at 60 °C in strictly anaerobic environment. *Ca* and *Cb* were cultured anaerobically at 35 °C in CGM in strict nitrogen medium without any outside air contamination. Co-culture experiments were performed in a 250-ml serum bottles. *Ct* cells grown in NBRC medium were collected by centrifugation. Cell pellets were washed and resuspended in the same medium without any added carbon source. Cell suspension solution (6 ml) was then inoculated in 6 ml of NBRC medium containing avicel cellulose and cultured at 60 °C in a serum bottle.

Butanol-producing *Ca* and *Cb* strains were collected after 24 h by centrifugation and were washed and suspended back into CGM medium containing 4 % glucose. 1 ml of this cell suspension was then added to serum bottles containing *Ct* after the incubation temperature was decreased from 60 to 34 °C [19]. The co-culture was then incubated at 35 °C for about 3 days. Samples were collected during the co-culture experiments in Eppendorf tubes with 30 % (v/v) sterile glycerol and stored at −80 °C.

### Batch SSF using co-cultured and fused strains

ABE solvents were produced in batch SSF experiments performed in 250 ml sealed Wheaton serum bottles that used two fused strains and their corresponding co-cultures at two different temperatures of 35 and 45 °C. Profiles for ABE concentration, sugar consumption, pH changes (acid concentrations) and cell growths were thoroughly examined for all SSF experiments. Incubation temperature during fermentation for protoplast fusion strains was maintained at 45 °C and for co-culture strains was maintained at 35 and 45 °C. Before inoculation, 40 ml growth medium (i.e., CBM) was added to provide the strains with nutrients for growth. The pH was adjusted to 6.5 with 10 M NaOH and the serum bottle was transferred to the anaerobic hood. The culture medium and WS was then inoculated with 7–8 ml of actively growing cells (protoplast fused or co-cultured). When dealing with bacteria, manipulations were performed under a constant supply of $N_2$ gas. After the inoculation was complete, the fermentation broth was bubbled with $N_2$ gas for about 5–10 min. The blue neoprene rubber stopper along with a metallic cap was used to seal the bottles using a vial crimper (Cole Palmer Canada). The bottles are brought out of the glove box and transferred to the incubator. The temperature of the incubator was adjusted to the right fermentation temperature (35 and 45 °C for co-culture strains and 45 °C for protoplast fusion strains). Samples were collected every 12 h under aseptic conditions and kept in 2-ml Eppendorf tubes at −80 °C in the ultra-low freezer until analyzed. All samples were analyzed for pH, cell growth, in addition to ABE and sugar concentration using HPLC. Total sugars produced in all SSF experiments were assumed to be equivalent to the maximum concentration reached during the lag phase in the first 24 h of SSF. All results reported were the average reading of triplicate experiments, and the average percentages of relative standard deviations (average RSD %) are listed for all figures in the present work.

### Enzyme assay

The filter paper assay (FPA) was used in this study to quantify the enzyme activity and is adapted from the National Renewable Energy Laboratory in the US department of energy [20]. The assay was performed so that 2 mg of reducing sugar like glucose was released from 50 mg of filter paper (4 % conversion) in 60 min and was assigned as the intercept for calculating Filter Paper Cellulose Units (FPU). Reducing sugar was measured according to DNS assay method [21]. Since the reducing sugar yield was not a direct linear function of the enzyme concentration, the assay procedure therefore involved finding a dilution of the original enzyme stock such that a 0.5-ml aliquot of the dilution that would catalyze 4 % conversion in 60 min. A DNS reagent was prepared by adding 10.6 g of 3,5-dinitrosalicylic acid and 19.8 g of sodium hydroxide in 1416 ml of distilled water. The above solution was dissolved and 306 g of Rochelle salt, 7.6 ml of phenol melt and 8.3 g of sodium metabisulfite was added. 3 ml of this solution was titrated with 0.1 N HCl to the phenolphthalein endpoint and stored for use in filter paper assay experiment.

Three categories of experimental test tubes were prepared as follows; assay mixtures, blanks and controls and glucose standards. The substrate used was 50 mg of Whatman No. 1 filter paper strip. For preparing the enzyme assay tubes, a rolled filter paper strip was placed in test tubes. To these test tubes 1 ml of 0.05 M Na-citrate solution was added such that the buffer saturates the filter paper strip. The buffer and substrate were brought up to equilibrium temperature of 50 °C by keeping in a water bath. To each test tube 0.5 ml of enzyme sample (from SSF bottles) was added. Several dilutions of the original enzyme sample were made and added to the citrate buffer. At least two dilutions are required to make sure that one dilution releases more than 2 mg of glucose and the other less than 2.0 mg of glucose. However, for our enzyme assay, three dilutions were run to target the 2 mg glucose release target. The solutions were incubated at 50 °C for 60 min. The enzyme reaction was then stopped immediately by adding 3 ml DNS reagent.

A 0.05 M citrate buffer solution with pH of 4.8 was prepared by adding 210 g of citric acid monohydrate in 750 ml deionized water. The solution was diluted to 1 L and the pH adjusted to 4.5 using NaOH. A 1.5 ml citrate buffer solution was used as a reagent blank. For each dilution, a separate enzyme control was prepared by adding 1 ml citrate buffer to 0.5 ml enzyme dilution. To account for a substrate control a filter paper strip was added to 1.5 ml citrate buffer.

To prepare glucose standards, a stock solution of anhydrous glucose (10 mg/ml) was made. Dilutions of this stock solution in the ratio of 1:1.5, 1:2, 1:3 and 1:5 were made in citrate buffer. Finally, all the blanks, controls, glucose standards and enzyme assays were incubated in 50 °C water bath for 60 min and were stopped by adding 3 ml of the prepared DNS reagent. The test tubes were then boiled for exactly 5 min in a boiling water bath. All samples including controls, blanks, and glucose standards were boiled together and cooled in ice-water bath. The test tube contents were then diluted in water (0.2 ml of color-developed reaction mixture in 2.5 ml water). Color formation in terms of absorbance was measured using UV spectrophotometer against the reagent blank at 540 nm.

A linear glucose standard curve with concentration units of mg/0.5 ml was plotted against absorbance at 540 nm, $A_{540}$. This standard curve was used to determine the amount of glucose released from each sample. Calculation of FPA from the graph of dilution versus glucose concentration was done using Eq. 1.

$$\text{Filter paper activity} = \frac{0.37}{[\text{enzyme}]\text{releasing } 2\,\text{mg of glucose}} \left(\frac{\text{units}}{\text{mL}}\right) \quad (1)$$

## Analytical techniques

### High-performance liquid chromatography (HPLC)

The Eppendorf tubes that were used to store sample size of 1 ml after each time interval were used for quantitative analysis of ABE (acetone, butanol, and ethanol), acids (acetic acid and butyric acid) sugars (glucose, xylose, arabinose, mannose, galactose) and inhibitor concentrations. These samples were stored at −80 °C until analyzed. Sugar concentrations were measured using high-performance liquid chromatography (HPLC-Perkin Elmer) equipped with an automatic sample injector and a refractive index detector (2414, Waters).

Three HPLC columns were used as follows Shodex KC811 for measuring sugars, Shodex SP0810 to measure inhibitors, and Aminex HPX-87H to measure ABE solvent and acids concentrations. The samples were centrifuged at 15000 g for 15 min and double filtered through 0.2 μm PTFE-filters (Whatman, USA). The solvent (mobile phase, 5 mM $H_2SO4$) was filter sterilized and autoclaved at 121 °C for 15 min. Following that, solvent was degassed using vacuum filtration. A blank sample with only double distilled water was applied in the first sample vial tray of HPLC. This blank was used to increase the flow rate of the solvent from 0 to 0.6 ml/min. The flow rate was maintained at 0.6 ml/min for 1 h while increasing the temperature of the HPLC column from 20 to 60 °C. This also fixes the pressure at a constant value and cancels most of the noise generated during the analysis. Then, each sample vial was arranged in sequence and the automatic sample injector extracted 0.1 μl of sample. Each sample was analyzed through the HPLC for 30 min. Data was processed by the computer software (Turbochrom Navigator). It was important to fill the HPLC testing vials to a minimum headspace to reduce the loss of solvents in vapor phase. The reliability of HPLC column, and testing parameters were confirmed by running solvents, acids, and sugars standards in triplicate.

### Haemocytometer

A Qiujing XB.K.25 haemocytometer with dimensions 0.1 mm × 1/400 $mm^2$ was used for counting cells and reporting cell concentration in number of cells per ml. The haemocytometer was cleaned with 75 % ethanol before use. Diluted samples of 10 μL were injected on both sides of the haemocytometer and were observed under an optical microscope (Zeiss Axio ObserverA1). Each side has four quadrants and therefore cells in a total of 8 quadrants need to be counted. Number of bacterial cells in each of the eight quadrants were counted and recorded. The number of cells in each quadrant divided by the number of quadrants (which is 8) gave the average cell count in each quadrant. This number corresponds to the number of cells present in 0.1 mm × 1/400 $mm^2$ volume of each quadrant. Cell density in cells/ml was then calculated for protoplast fused and co-culture strains of wheat straw fermentation. The only drawback of this technique was that without tagging the bacterial cells, there was no way of quantifying the dead cells and so were assumed to be negligible.

A second, more traditional approach was also employed to count cells for a part of the project. Dilutions in the range of $10^5$ of the sample were prepared and 0.1 ml of the final dilution was plated onto agar plates. The plates were marked with the appropriate strain and the dilution rate. The plates were left overnight for colonies to form. Each colony represents one cell in the original sample. The number of colonies were counted and multiplied by the dilution rate and expressed in cells/ml.

### UV–Vis spectrophotometer

Enzyme activity was measured using pre-calibrated UV/VIS scanning spectrophotometer. A glucose standard calibration curve was generation by measuring the absorbance of different glucose standard solutions (mg/0.5 ml) with a wavelength setup of 540 nm. Enzyme samples were analyzed for absorbance against a reagent blank at the same wavelength. Preceding any analysis, 0.2 ml of color-developed reaction mixture for sample assays, blanks, standards and controls were diluted in 2.5 ml water and were placed in the spectrophotometer cuvette and analyzed for absorbance.

## Results and discussion

### Production of ABE and acids

Final ABE concentration was quantified for the SSF experiments using *CaCt* (fused) and *CbCt* (fused) in comparison to their corresponding co-culture strains. Table 1 summarizes final production of ABE solvents and acids achieved by both classes of strains.

As listed in Table 1, final biobutanol production achieved using *CaCt* (fused) strain was 12.08 g/L, which is comparable to previous research studies where butanol production was examined using WS and clostridium strains with initial sugar concentration of 62 g/L [10]. Several processes were examined in Qureshi et al. [10] study including two that utilized SSF at 35 °C. However, one particular process that employed SSF coupled with gas stripping to remove butanol from the batch system recorded the highest butanol production of 12.7 g/L. *CaCt* (fused) strain used in the current study measured almost same maximum productivity without utilizing gas stripping. Removal of butanol by gas stripping has been reported to be an essential procedure to avoid toxicity that suppresses further butanol production [10]. From Table 1, total ABE solvents production by *CaCt* (fused) was found to be 20.2 g/L. While few papers have reported higher solvent concentrations than the reported by the current study, their main substrate was pure glucose or food grade corn starch, soy molasses and peanuts [4].

Furfural inhibitor production during the SSF was quantified to be in the range of 0.5–0.6 g/L for all strains. These concentrations were significantly lower than the inhibitory levels of 1 g/L, above which furfural activity was known to negatively affect the fermentation [22]. The low concentration of furfural can be explained by the lower temperature of 120 °C at which pre-treatment was performed. Research studies have shown that the concentration of inhibitors increases with increase in pre-treatment temperature [23]. Therefore, this study was focussed on the affect of other inhibitors and toxins like acids and butanol while neglecting the effect of insignificant concentrations of furfural.

Results from Table 1 show that *CaCt* (co-culture) strains produced ~36.8 % of the ABE production obtained using the corresponding *CaCt* (fused) strain. Studies done to examine butanol production from crystalline cellulose by co-culturing *Ct* with another mesophilic strain of *C. saccharoperbutylacetonicum* reported a final concentration of 4.5 g/L after 5 days of fermentation [19]. This result was comparable to the 4.53 g/L of butanol obtained with *CaCt* (co-culture) strains in Table 1. Figure 1 shows the ABE solvents concentration profile during SSF of wheat straw using *CaCt* (fused) strains.

As shown in Fig. 1, the *CaCt* (fused) strains produced 20.13 g/L of total ABE after 120 h of SSF consuming 35 g/L sugars (Table 1). The concentrations of individual solvents such as acetone, butanol and ethanol at the end of fermentation were 6.05, 12.07 and 1.85 g/L, respectively. It can be observed from Fig. 1 that no solvent production was observed during the first 24 h of SSF. Although the bacterial cells were out of their lag phase and well into the exponential phase between 24 and 48 h, enzyme production and utilization was mostly devoted towards breaking down of cellulose and hemicellulose rather than fermentation to solvents. From Fig. 1, the production of solvents was most predominant between 24 and 84 h with the production of 5.73, 10.15 and 1.58 g/L of acetone, butanol and ethanol between those time periods. Solvent toxicity was often a cause of concern while monitoring product

**Table 1** Concentration of ABE and acids, sugars consumed and average cell proliferation rate for all protoplast fused strains at 45 °C and co-culture strains at 35 °C

| Strains | ABE (g/L) | | | Acids (g/L) | | Total sugars consumed (g/L) | Average cell proliferation rate ($\times 10^5$ cells/ml h) |
|---|---|---|---|---|---|---|---|
| | Acetone | Butanol | Ethanol | Acetic | Butyric | | |
| *CaCt* (fused) | 6.05 | 12.07 | 1.85 | 1.78 | 0.92 | 35 | 3.25 |
| *CbCt* (fused) | 6.89 | 13.82 | 2.29 | 1.75 | 0.87 | 40.21 | 3.46 |
| *CaCt* (co-culture) | 2.15 | 4.53 | 0.76 | 2.43 | 1.28 | 26.59 | 2.95 |
| *CbCt* (co-culture) | 2.77 | 5.79 | 0.96 | 2.21 | 1.17 | 28.08 | 3.16 |

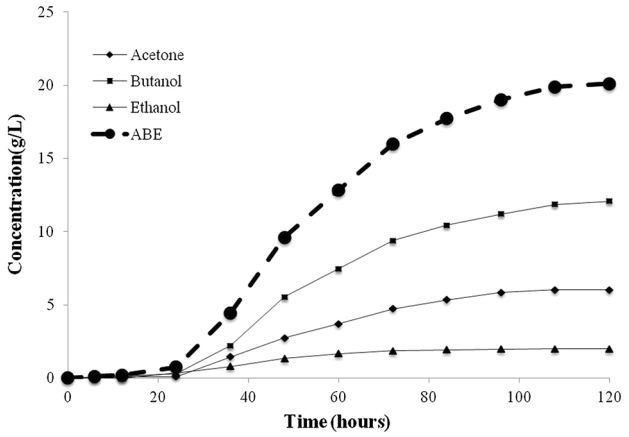


**Fig. 1** ABE solvents concentration profile during SSF at 45 °C using *CaCt* (fused) strains (average RSD = 3 %)

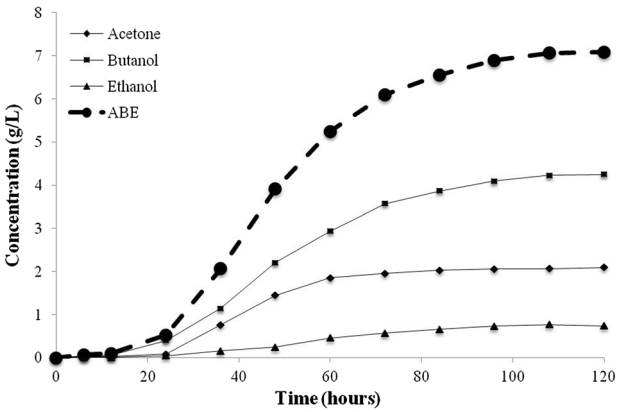


**Fig. 2** ABE solvents concentration profile during SSF at 35 °C using *CaCt* (co-culture) strains (average RSD = 2.2 %)

formation during ABE fermentation. During the final phases of SSF, it was observed that the cell metabolism proceeded until the solvent reached a concentration of 19.8 g/L at 108 h. In the next 12 h, the increase in total solvent concentration was just about 1.5 % (from 19.8 g/L at 108 h to 20.1 g/L at 120 h). Among the three solvents, butanol renders the most toxic affect on cell activity and metabolism [6].

According to previous study by Jones and Woods [24], production of butanol in the range of 7–13 g/L had resulted in cell growth inhibition. It can be seen that butanol concentration increased between 12 and 108 h reaching 11.86 g/L. However, after 108 h, the *CaCt* (fused) strains were still exposed to butanol toxicity due to the hydrophobic nature of butanol. Based on this observation, butanol toxicity was most likely the factor behind sluggish production of ABE solvents after 108 h where the results show that the total change in butanol concentration was just 1.8 % going from 11.86 g/L at 96 h to 12.08 g/L at the end of fermentation.

Acetone and ethanol toxicity were not of a major concern when compared to butanol. Cell growth inhibition was previously observed by other researchers for acetone at 70 g/L and for ethanol at 50–60 g/L [25]. In the current study, ethanol and acetone concentrations did not reach above 10 g/L and therefore do not contribute to solvent toxicity and cell inhibition. However, in the case of butanol, it can be concluded that genetic improvement has provided the strain with some butanol tolerance and has thus resulted in relatively high butanol production (12.08 g/L) with *CaCt* (fused) strain. Still, this strain does experience toxicity by butanol for concentrations above 11.86 g/L (reached at 108 h) as demonstrated in Fig. 1.

SSF was performed at two different temperatures of 35 and 45 °C for *CaCt* (co-culture). Although *Ca* was mesophilic with optimal temperature in the range of 10–65 °C, it was able to produce solvents only in the range of 33–37 °C, unless it was genetically modified to produce solvents at a higher temperature [26]. Figure 2 shows the ABE solvents concentration profile during 120 h of SSF using *CaCt* (co-culture) strains. The first 24 h was characterized by a sluggish growth as bacteria multiply and produce enzymes essential to saccharify polysaccharides into sugars. The concentrations of acetone, butanol and ethanol at the end of fermentation were 2.15, 4.53 and 0.76 g/L, respectively. Butanol toxicity does not seem to be a major concern here, as the concentration of butanol after 120 h of fermentation does not reach the inhibition limit of 7–13 g/L. Inhibition caused by acetone and ethanol production was also not of concern as its levels were far below the solvent toxicity range. According to results in Table 1 and Fig. 1, *CaCt* (fused) strain was able to produce 174 % more butanol than its co-culture counterparts. When the SSF temperature for *CaCt* (co-culture) was increased to 45 °C, the solvent activity of the *Ca* was ineffective and the thermophilic strain did not provide any tolerance towards high temperature to the butanol-producing strains. However, insignificant amount of ethanol was produced due to the ethanogenic nature of *Ct*.

As shown in Table 1, highest biobutanol production of 13.82 g/L was recorded with *CbCt* (fused) strain. This strain recorded an 8.8 % increase in biobutanol production compared to SSF gas stripping procedure conducted earlier by Qureshi et al. [10]. One of the issues associated with industrializing fermentation technology for biobutanol production is the lower solvent production achieved during this process. Parekh and Blaschek [27] used the same strain of *Cb* (BA101) from our study to set up a pilot plant to produce biobutanol from corn steep water. The results obtained in this pilot study reported a solvent concentration of 24 g/L. Comparing this to the ABE solvents production

of around 23 g/L obtained with *CbCt* (fused) in laboratory-scale batch fermentations of the present study demonstrates potential to enhance production levels at pilot plant scale.

The substrate used in the current study is also of significant importance. While butanol concentrations of around 19.6 g/L (from glucose) and 15.8 g/L (from corn) have been reported in earlier studies using *Cb*, the main source of substrate was either added sugars in the form of food source such as corn or soy [4]. However, the biobutanol production from WS in the same study was 9.8 g/L. This level of production is nearly 30 % less than the achieved from *CbCt* (fused) strains in the current study. Figure 3 shows the ABE solvents concentration profile during the 120 h fermentation using *CbCt* (fused) strains.

As shown in Fig. 3, the *CbCt* (fused) strains produced 23 g/L of ABE after 120 h of SSF. Furthermore, concentrations of acetone, butanol and ethanol at the end of fermentation were 6.89, 13.82 and 2.29 g/L, respectively (see Table 1). It can also be observed from Fig. 3 that production of solvents was most predominant between 24 and 96 h with production of 6.75, 13 and 2.2 g/L of, respectively, acetone, butanol and ethanol, which were produced during this time period. During the later SSF stage, it was observed that the cell metabolism proceeded until the solvent reached a concentration of 22.02 g/L at 96 h. For the following 24 h, the increase in total solvent concentration was just about 4 % (from 22.02 g/L at 96 h to 23 g/L at 120 h). This strain had undoubtedly shown the strongest tolerance particularly towards butanol toxicity. From Fig. 3, it can be seen that butanol concentration steadily increased from 24 to 96 h reaching ~13.42 g/L. After 96 h, the total change in butanol concentration was just 2.75 % going from 13.42 g/L at 96 h to 13.8 g/L at the end of fermentation. In our current study with *CbCt* (fused), ethanol and acetone concentrations do not reach above 10 g/L and therefore do not contribute to solvent toxicity and cell inhibition. However, it can be concluded that genetic improvements accomplished through protoplast fusion has provided the strains with higher butanol tolerance and has thus resulted in relatively high biobutanol production of 13.8 g/L with *CbCt* (fused) strains. Still, this strain does experience toxicity from butanol for concentrations above 13.42 g/L reached at 96 h.

Figure 4 shows the ABE solvents concentration profile in SSF using *CbCt* (co-culture) strains. Nakayama et al. [19] reported around 2 g/L of butanol using only crystalline cellulose as substrate. In the current study, *CbCt* (co-culture) strains produced 5.8 g/L of butanol (9.52 g/L of ABE) after 120 h of SSF. The concentrations of acetone, butanol and ethanol at the end of fermentation were 2.77, 5.8 and 0.96 g/L, respectively (see Table 1). Similar to *CaCt* (co-culture), neither butanol toxicity nor acetone, ethanol toxicity was of major concern.

Figures 3 and 4 show that after 120 h of SSF, *CbCt* (fused) strain was able to produce 138 % more butanol than *CbCt* (co-culture) strains. When the SSF temperature of the co-culture was increased to 45 °C, the activity of the *Cb* was ineffective and the thermophilic strain did not provide any tolerance towards high temperature for the butanol-producing strains. However, insignificant amounts of ethanol were produced due to the ethanogenic nature of *Ct*.

## Production of acids and pH changes during SSF

The two acids generated during SSF for all strains were butyric acid and acetic acid. These acids were produced during the acidogenic phase of the ABE metabolic pathway. According to results in Table 1, *CaCt* (co-culture) produced the highest acids of all strains of about 2.43 g/L of acetic acid and 1.28 g/L of butyric acid. Figure 5 shows the change in the pH during batch SSF for all strains used

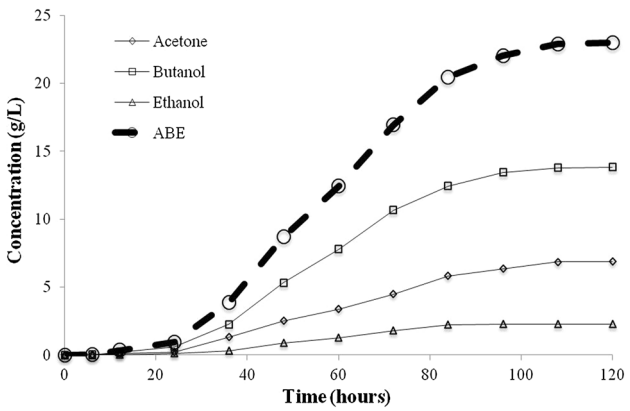


**Fig. 3** ABE solvents concentration profile during SSF at 45 °C using *CbCt* (fused) strains (average RSD = 2.3 %)

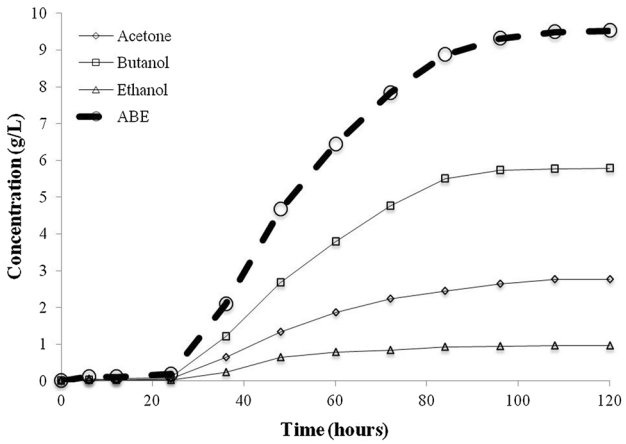


**Fig. 4** ABE solvents concentration profile during SSF at 35 °C using *CbCt* (co-culture) strains (average RSD = 3 %)

in the study. It shows that pH values during SSF for *CaCt* (co-culture) strain decreased the most in accordance with acid generation (pH 4.34 at 24 h). This was followed by *CbCt* (co-culture) strains that produced 2.21 g/L of acetic acid and 1.17 g/L of butyric acid in Table 1. In general, co-culture strains produced more acid than fusion strains (Table 1). Parent *CbCt* (fused) strains produced about 1.75 g/L of acetic acid and 0.87 g/L of butyric acid with the pH dropping from 6.5 to 5.2 in the first 12 h of fermentation before increasing to 5 after 36 h. During the period from 48 to 120 h, pH of the system was in the range of 5–6.3 (Fig. 5). *CaCt* (fused) strain produced around 1.78 g/L of acetic acid and 0.92 g/L of butyric acid that resulted in pH dropping from 6.5 to 4.8 in the first 24 h before returning back to 5.0 after 48 h.

It is known that the pH of the system is an important indicator of the biological synthetic processes since major changes can inhibit production [28]. While the reduction in pH at the beginning of fermentation could be attributed to the production of acids (i.e., acetic and butyric acids), the increase in pH following that was due to the conversion of acids to the ABE solvents (i.e., acetone, butanol, and ethanol) during the solventogenesis stage [29]. The phenomenon of acid crash was discussed earlier and was cited as a concern if acidic concentration in the broth exceeds 60 mmol/L. The highest acidic molar concentration for *CaCt* (fused), *CbCt* (fused), *CaCt* (co-culture) and *CbCt* (co-culture) are 40.4 mmol/L (both acetic and butyric acid), 48.9 mmol/L, 55 mmol/L and 50.1 mmol/L, respectively. Therefore, it can be concluded after examining the total acid concentration in our system that acid crash was not a major cause of concern. However, deviation from the optimum pH impacts production in general, which demonstrates potential to further enhance biobutanol production using the fused strains developed in the present work.

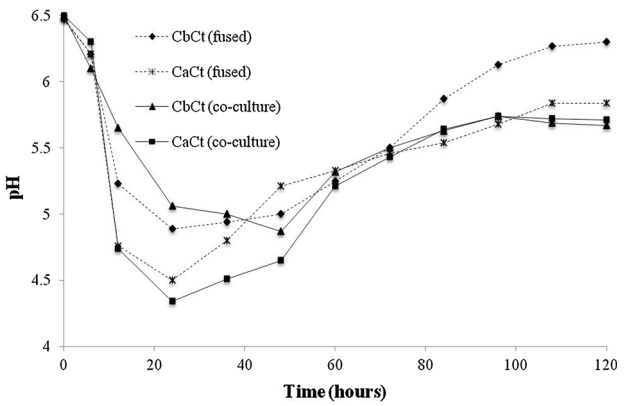


**Fig. 5** Changes in pH during SSF for the fused and co-cultured strains (average RSD = 2.3 %)

## Sugar consumption in batch SSF

Figure 6 shows the corresponding change in the percentages of individual sugars concentrations during SSF with respect to the initial concentration of total sugars at the beginning of fermentation (after pre-treatment) using *CaCt* (fused) strains. Examining Fig. 6 shows that generally sugar concentrations increased in the first day of fermentation. This can obviously be observed for glucose, xylose, and arabinose, while the increase in the concentration of galactose was relatively minor. The increase in the total sugar is a result of saccharification of unhydrolyzed polysaccharides that release monomers like glucose, xylose, mannose, galactose and arabinose by action of the enzymes released from the fused strains (*Ct* in particular). Table 2 shows individual and total sugars concentrations at the end of SSF for all strains. The concentration of total sugars in Table 2 after pre-treatment was 29.8 g/L. However, this concentration increased to about 45.3 g/L at 24 h in accordance with hydrolysis enzymes generated by *CaCt* (fused) strains. This accounted for a 60 % increase in sugar concentration within 24 h of enzymatic saccharification in Fig. 6.

Several researchers have studied the extraordinary capability of cellulolytic *Ct* to convert cellulose and cellobiose to glucose [30]. As reported in Table 1, butanol concentration achieved by *CaCt* (fused) strain was 12 g/L. The increased resistance of the strains towards butanol toxicity has contributed to the increase in sugar consumption. From Fig. 6, glucose formed 60 % of the total sugars at the beginning of fermentation and after pre-treatment. Maximum glucose consumption is noticed between 24 and 36 h where the glucose remaining in the system dropped from 88 % (25.4 g/L) to 47 % (13.46 g/L). At the end of SSF, only 4.7 % (2.61 g/L) of glucose remained. In other studies that examine *Ca* wild strain, it was found that in

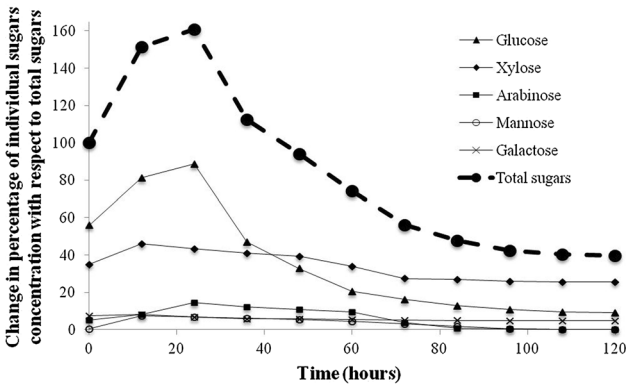


**Fig. 6** Percentage of individual sugar concentration with respect to total sugars during SSF (45 °C) for *CaCt* (fused) strains (average RSD = 1.46 %)

**Table 2** Final concentration of individual and total sugars obtained at the end of SSF for all strains (i.e., fused and co-cultured)

| Strains | Glucose | Xylose | Arabinose | Mannose | Galactose | Total |
|---|---|---|---|---|---|---|
| *CaCt* (fused) | 2.61 | 7.31 | 0 | 0 | 1.40 | 11.32 |
| *CbCt* (fused) | 0.52 | 5.76 | 0 | 0 | 0.87 | 7.15 |
| *CaCt* (co-culture) | 6.85 | 9.32 | 0 | 0 | 2.18 | 18.35 |
| *CbCt* (co-culture) | 5.1 | 8.35 | 0 | 0 | 2 | 15.45 |

either batch or fed-batch cultures, xylose utilization were inhibited at higher glucose concentrations [31]. This phenomenon is evident from Fig. 6. It was noticed that the presence of higher percentages of glucose in the batch between 12 and 36 h has inhibited the utilization of xylose leading to invariable percentages of xylose left during that time period. As the concentration of glucose went significantly below 15 g/L, there was a rapid increase in the consumption of xylose, which decreased from 41 % at 36 h to 27 % at 72 h. Still, xylose is not completely consumed as towards the end of the fermentation nearly 25 % (~7.31 g/L) remained in the system. Furthermore, there were no traces of arabinose or mannose at the end of SSF as they were completely consumed.

Figure 7 shows the change in the percentages of individual sugars concentrations during SSF with respect to the initial concentration of total sugars at the beginning of fermentation (after pre-treatment) using *CaCt* (co-culture) strains. Figure 7 shows that 61 % of the total sugars remained in the system after 120 h. Xylose inhibition can be observed for higher glucose concentrations (above 15 g/L) between 12 and 36 h where remaining xylose decreased from 44 to 41.7 %, which represent preferential consumption of glucose. During the subsequent 24 h, and at lower percentage of glucose in the system, the xylose in the system drops from 41.7 to 27.2 %. Furthermore, arabinose and mannose were completely consumed at 108 and 96 h, respectively. At the end of SSF, total sugar consumed was 59 % with *CaCt* (co-culture) strains registering a 26.3 % decline in sugar consumption compared to their *CaCt* (fused) counterparts.

Figure 8 shows the percent sugars remaining in the system with respect to the initial concentration of total sugars using *CbCt* (fused) strains. Total sugar consumption for this strain was the highest among all strains and was around 84 % (40.21 g/L; see Table 1). This is higher than results reported by Liu et al. [32] with *Cb* and WS as substrate, which shows the effectiveness of the fused strain compared with the wild strain.

Total sugars remaining unconsumed in the system decreased by 36.8 % compared to *CaCt* (fused) strains. Similarly, arabinose and mannose were completely consumed. From Fig. 8, up to 24 h, total sugars in the system increased 60 % in accordance with hydrolysis using enzymes (~17 g/L; see Table 1). A characteristic of *Cb* is that it is more capable than *Ca* in terms of breaking down pentose sugars such as xylose even in high glucose concentrations. Hence a steady decline was seen in remaining xylose even at glucose concentrations that inhibited xylose consumption. Between 24 and 36 h in Fig. 8, glucose is sufficiently high (above 15 g/L); however, the percentage of xylose remaining has also decreased from 44 to 35 % (from 12.48 g/L at 24 h to 10.12 g/L at 36 h). This suggests that this strain might not be affected by xylose inhibition as much as its *CaCt* (fused) strain. Figure 8 also indicates that hemicellulotic pentose sugar such as xylose was not completely consumed at the end of the fermentation. At 120 h, the concentration of xylose was 5.76 g/L,

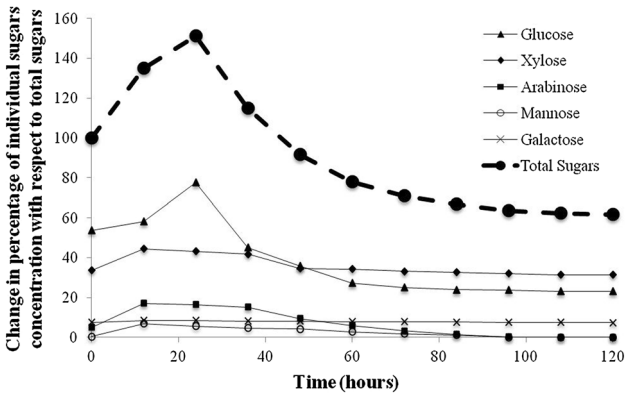


**Fig. 7** Percentage of individual sugar concentration with respect to total sugars during SSF (35 °C) for *CaCt* (co-culture) strains (average RSD = 1.76 %)

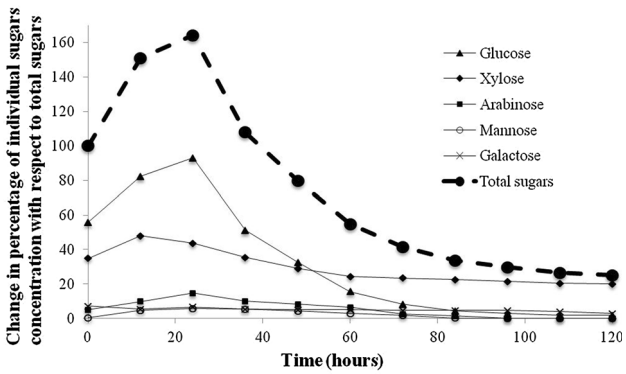


**Fig. 8** Percentage of individual sugar concentration with respect to total sugars during SSF (45 °C) for *CbCt* (fused) strains (average RSD = 2.65 %)

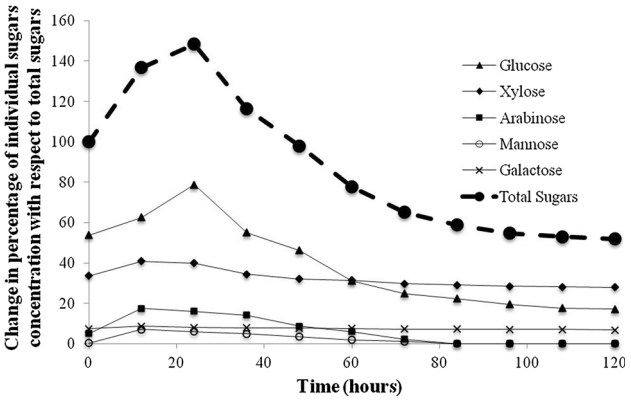


**Fig. 9** Percentages of individual sugar concentration with respect to total sugars during SSF (35 °C) for *CbCt* (co-culture) strains (average RSD = 1.7 %)

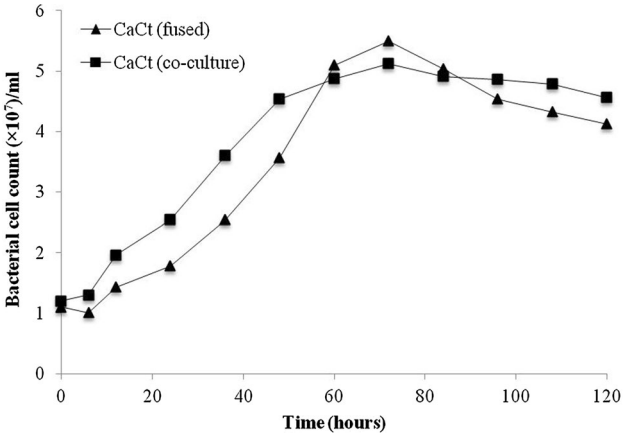


**Fig. 10** Change in cell concentration for *CaCt* (fused) and *CaCt* (co-culture) strains during SSF (average RSD = 3.4 and 2.85 %, respectively)

while small trace of glucose (~0.56 g/L) was observed. This suggests a near-complete consumption of glucose unlike *CaCt* (fused) strain that still had 9 % (2.61 g/L) of glucose remaining in the system. Other hemicellulotic sugars like arabinose and mannose were completely consumed. Figure 9 shows sugars remaining in the system with respect to the initial concentration of total sugars using *CbCt* (co-culture) strains. Total sugar consumption for this strain was around 65 % (28.76 g/L, Table 1) and is the highest among the two co-culture strains examined.

Total sugars remained in the system was double what was obtained with *CbCt* (fused) strain. During the saccharification phase during 24 h, an additional 14 g/L of sugars were released into the system, while 52 % of total sugars (i.e., 28 % xylose, 17 % glucose, 6.7 % galactose) remained in the system at the end of SSF. Arabinose and mannose were completely consumed.

## Cell growth of fused and co-culture strains

Figures 10 and 11 show the changes in cell concentration observed for both fused strains and corresponding co-culture strains. All strains exhibit a lag phase where the cell concentration was almost constant. During this phase, the strains acclimatize to culture medium, pH levels and surrounding temperature. The lag phase for *CaCt* (fused) and *CbCt* (fused) strains was up to 6 h. Co-culture strains exhibited similar lag phases. After this phase, the cells grew exponentially and entered the solventogenic phase. The exponential phase ended at 72–84 h into the SSF. Butanol concentration at 84 h was 10.45 g/L with *CaCt* (fused) strain (Fig. 1), which corresponded to the concentration range in which cell inhibition was initiated due to butanol toxicity. The concentration of cells decreases after 84 h and enters the decay phase.

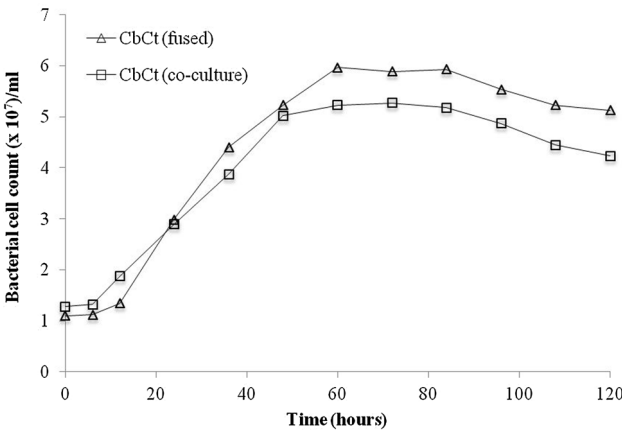


**Fig. 11** Change in cell concentration for *CbCt* (fused) and *CbCt* (co-culture) strains during SSF (average RSD = 4.52 and 2.85 %, respectively)

A similar analysis of *CbCt* (fused) strains show that the concentration of cells increased exponentially between 12 and 60 h after which the cells reach a stationary stage for the next 36 h. Due to relatively higher butanol tolerance of this strain, even concentrations as high as 12.5 g/L observed at 84 h did not initiate a cell decline. This strain showed a wider stationary phase from 60 to 96 h during which the butanol concentration varies from 7.8 to 13.42 g/L. The strain entered the decay phase at 96 h when butanol concentration reached close to 13.42 g/L.

Co-culture strains produce relatively lower butanol (not in the range of toxic levels) than their corresponding protoplast fused strains. Growth phase was recorded for *CbCt* (co-culture) between 12 and 72 h with a butanol concentration going up to 4.76 g/L. The stationary phase was observed for the rest of the SSF time without a distinct change into the decay phase.

**Table 3** Concentration of glucose released from samples including calculated enzyme activity for *CbCt* (fused) and *CaCt* (fused) strains

| Dilution # | *CbCt* (fused) | | *CaCt* (fused) | |
|---|---|---|---|---|
| | Abs 540 nm | Glucose (mg/0.5 mL) | Abs 540 nm | Glucose (mg/0.5 mL) |
| 1 | 0.586 | 2.551 | 0.563 | 2.451 |
| 2 | 0.456 | 1.985 | 0.403 | 1.754 |
| 3 | 0.282 | 1.228 | 0.247 | 1.075 |
| Enzyme activity (FPU/ml) | 68.52 | | 58.73 | |

**Table 4** ABE and individual solvent yield for all growth cycles of protoplast fused and co-culture strains

| Strains growth cycles | ABE yield ($Y_{ABE/S}$)[a] | Acetone yield ($Y_{A/S}$)[b] | Butanol yield ($Y_{B/S}$)[c] | Ethanol yield ($Y_{E/S}$)[d] |
|---|---|---|---|---|
| *CbCt* (fused) | 0.57 | 0.17 | 0.34 | 0.06 |
| *CaCt* (fused) | 0.57 | 0.17 | 0.34 | 0.05 |
| *CbCt* (co-culture) | 0.33 | 0.10 | 0.20 | 0.03 |
| *CaCt* (co-culture) | 0.28 | 0.08 | 0.17 | 0.03 |

[a] $Y_{ABE/S}$ was calculated by dividing final ABE concentration with total sugars consumed during SSF

[b] $Y_{A/S}$ was calculated by dividing final acetone concentration with total sugars consumed during SSF

[c] $Y_{B/S}$ was calculated by dividing final butanol concentration with total sugars consumed during SSF

[d] $Y_{E/S}$ was calculated by dividing final ethanol concentration with total sugars consumed during SSF

### Enzyme assay analysis

Table 3 summarizes the amount of glucose released from the filter paper for each sample and the corresponding enzyme activity (i.e., endoglucanase and exoglucanase, and β-glucosidase) in filter paper units per ml (FPU/ml). *CbCt* (fused) strain has demonstrated a capability to produce higher biobutanol. Cellulosome producing *Ct* and its ability to ferment sugars offers a promising future in biofuels. Distinct from the fungal cellulases, the *Ct cellulase* has a very high enzyme activity on cellulose [33].

Previous studies have demonstrated higher biobutanol productivity for *CbCt* (fused) strains without addition of any external enzymes [8]. Thermophilic and mesophilic enzymes were able to digest cellulose and hemicellulose into sugars and finally into solvents and acids. *Ct*, an anaerobic thermophile, cellulose degrading enzymes and cellulosomes cellulases, had been a subject of study for decades [34]. Therefore, the fused strains should have been able to produce all the enzymes associated with cellulose hydrolysis during the SSF experiments. From Table 3, *CbCt* (fused) strains produced cellulolytic enzymes in the amount of 68.52 FPU/ml and *CaCt* (fused) produced enzymes in the amount of 58.73 FPU/ml. These values can be compared with the activity of many cellulase enzymes derived from other organisms such as *Trichoderma reesei* (ATCC 26921) whose activity reported as 85.1 FPU/ml [35] and accellerase (1500) with an activity of 43.21 FPU/ml [36]. The enhanced enzyme activity of *CbCt* (fused) strains and its relative similarity to cellulase activity from commercial sources represents an improvement in the enzymatic hydrolysis associated with SSF of lignocellulose.

### Yield calculations

Table 4 compares overall ABE yield with respect to total sugars consumptions in Table 1. Genetic modification of butanol-producing strains (*Cb* and *Ca*) has allowed for lower solvent inhibition and enhanced productivity and yield. Total ABE yield for *CbCt* (fused) strain was 0.57, with acetone yield of 0.17, butanol yield in the range of 0.34 and ethanol yield of 0.06. The ABE yield obtained using the fused strains are significantly higher than that reported earlier for the parenting strains of 0.4 and 0.42 [4]. In the case of *CaCt* (fused) strain was 0.57, with acetone yield of 0.17, butanol yield of 0.34 and ethanol yield of 0.05. *CbCt* (co-culture) strains recorded an ABE yield of 0.33 and *CaCt* (co-culture) strains with an ABE yield of 0.28. According to these results, *CaCt* (fused) and *CbCt* (fused) strains produced higher yields of biobutanol as compared to their co-culture counterparts.

## Conclusions

Novel clostridial strains were developed and tested for enhanced ABE production. SSF was performed using the batch approach using renewable green substrates of WS and by utilizing novel clostridia protoplast fused strains. Results in the present study demonstrated that this novel development eliminated the need to add external enzymes

during the SSF process, allowed for SSF to be conducted at an elevated temperature of 45 °C, enhanced the activity of internally produced enzymes and tolerated butanol toxicity to new limits. These characteristics will be of prime importance while commercializing this technology as lower enzyme costs leads to more profitability.

Protoplast fused strains in general produced higher concentration of biobutanol and other solvents when compared to co-cultures. This confirms the superiority of the process as opposed to a co-culture. The *CbCt* (fused) strains produced 13.81 g/L of butanol (yield of 0.34) as compared to their co-culture counterpart which only produced about 5.79 g/L (yield of 0.2). A similar conclusion can be derived from *CaCt* (fused) strains with butanol concentration of 12 g/L (yield of 0.34) as compared to 4.53 g/L (yield of 0.17) achieved when using the co-culture strains.

**Acknowledgments** 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 at Ryerson University in Toronto, Canada.

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