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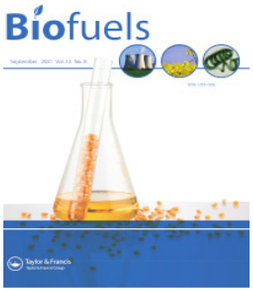

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Novel fusants of two and three clostridia for enhanced green production of biobutanol

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ABSTRACT

The objective of the present study is to improve biobutanol production by utilizing renewable resources of agriculture residues (i.e. wheat straws) in simultaneous saccharification and fermentation (SSF). Three strains of anaerobic gram-positive clostridia (*Clostridium beijerinckii*, *Clostridium thermocellum*, and *Thermoanaerobacterium saccharolyticum*) were fused through a protoplast fusion technique. Results show that protoplast fusion of thermophilic and mesophilic clostridia led to improved thermostability in a fermentation medium at 45 °C. This represents the optimum temperature for enzymatic hydrolysis. Results also show that the fused strain produced essential hydrolysis enzymes, which eliminated the need to add any enzymes during the hydrolysis step. Furthermore, results in the present study demonstrate that the fused culture of bacteria was able to tolerate the elevated concentration of acetone, butanol, and ethanol during production, which resulted in higher biobutanol production of 13.8 g/L. This study includes a comparison with the co-culture as a benchmark to account for the effects of protoplast fusion.

List of abbreviations: ABE: Acetone–butanol–ethanol; *Ca*: *Clostridium acetobutylicum*; *Cb*: *Clostridium beijernickii*; *Ct*: *Clostridium thermocellum*; *Ts*: *Thermoanaerobacterium saccharolyticum*; CBM: Clostridium basal medium; CBP: Consolidated bioprocessing; CFU: Colony-forming units; CGM: Clostridium growth medium; CMM: Cooked meat medium; DNS: Dinitrosalicylic acid; FPA: Filter paper assay; FPU: Filter paper unit; HPLC: High-performance liquid chromatography; MTC: Medium for thermophillic clostridia; NBRC: National Biological Resource Center medium; PCR: Polymerase chain reaction; PPM: Protoplasting medium; RM: Regeneration medium; RSD: Relative standard deviation; SSF: Simultaneous saccharification and fermentation; WS: Wheat straw

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Biobutanol; novel fusants; simultaneous saccharification and fermentation (SSF); co-cultures

Introduction

Over the last decade, biofuels such as bioethanol, biobutanol, and biodiesel have been promoted as a promising alternative to petroleum, and thus as an effective solution to mitigate climate change. Presently, biofuels provide approximately 3% of the total fuel necessary for road transportation worldwide, and higher rates are achieved in certain countries. The United States, Brazil, and the European Union (EU) are the world's three largest biofuel markets as they represented 85% of global production in 2010. North America is the world's leader in biofuel production, contributing 48% of biofuel production in the market. Biofuel production is projected to experience rapid growth in the foreseeable future, between 2017 and 2021, due to high oil prices, new feedstock availability, and advanced technology. Significant advances in the development of biofuel production, with a particular focus on more sustainable practices over the course of the past decade, have resulted in the development and classification of three generations of biofuels. The first generation of biofuels was made from edible sources such as wheat, corn, and sugars by the action of microorganisms. However, this generation was not successful, since it threatened traditional food supplies as well as biodiversity. In contrast, the second generation of biofuels, derived from lignocellulosic crops, has attracted much attention, because it is produced from residual non-food crops or agricultural residues that are sustainable resources. Finally, the third generation of biofuel, which is made from algae, has attracted much attention due to its ability to be cultured on lands that are unsuitable for agriculture [1].

Biobutanol is a biofuel that can be produced from renewable resources or biomass through different chemical and biological techniques. Biological butanol synthesis served as a great feedstock for industrial purposes until the 1950s. However, an increase in the cost of substrates and the availability of cheap raw oil for the petrochemical industries reduced the importance of the acetone–butanol–ethanol (ABE) fermentation process. The 1974 oil crisis, which resulted in a high cost of crude oil along with its environmental impacts, created a renewed interest in the biological production of butanol using microorganisms, which have the potential to produce cellulolytic enzymes [2].

Biobutanol, as a clean energy alternative, has some advantages over other biofuels, especially ethanol. Due to its physical properties, butanol can be mixed with gasoline; it also has greater potential to replace gasoline than ethanol, without any adjustments to automobile engines [3]. In contrast, ethanol can only be used as an additive to gasoline after engine modifications and with a maximum content of 85%. Moreover, the rate of evaporation of butanol is 6 times lower than that of ethanol, with its volatility being 13.5 times less than that of gasoline. Regarding

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ecological issues, butanol combustion releases no sulfur or nitrogen oxides into the environment. Moreover, biobutanol can be produced from the same renewable sources as ethanol. In the past, traditional substrates such as starch, glucose, molasses, and whey permeate have been used for butanol production. However, because of the high costs of the substrates affecting the price of butanol, the use of economically available substrates, including agricultural residues and wastes (wheat straw, corn fiber, rice straw, hardwood, waste paper, and annual and perennial crops), is being examined [4]. It should also be noted that pre-treatment of some agricultural residues such as corn fiber might produce fermentation inhibitors.

Biobutanol can be produced by anaerobic fermentation of sugar components using various species of Clostridia. Cellulolytic and solveontogenic Clostridia species such as *C. thermocellum*, *C. saccharobutylicum*, *C. cellulolyticum*, and *C. acetobutylicum* are among the best-studied biomass-metabolizing bacteria which have a significant potential to produce sustainable biofuel via consolidated bioprocessing (CBP). Among them, *Clostridium beijerinckii* and *Clostridium acetobuylicum* are the best-known strains for butanol fermentation, which have the ability to produce solvents from carbohydrates via two-stage fermentation. The advantage of using these strains is related to their ability to utilize both hexose and pentose sugars available in biomass, compared to traditional ethanol-producing yeast strains that are incapable of utilizing lignocellulosic hydrolysate sugars [5].

The main problem facing the enzymatic activity of *C. beijerinckii* and *C. acetobutylicum* during SSF is the low temperature of fermentation, which is around 35 °C, whereas the optimum temperature for enzymatic hydrolysis is 45 °C. This reduces the efficiency of enzymes, especially *cellulase*, thus leading to lower productivity of butanol and higher cost of fermentation [6].

The main objective of the present study is to enhance butanol production from non-edible agricultural biomass by improving the thermal stability (thermostability) of biobutanol-producing mesophilic bacterial strains such as *C. Beijerinckii* (*Cb*), and to enhance metabolism to break down longer sugar chains. In the present study, improvement of the bacterial strains through protoplast fusion with a more thermally stable thermophilic clostridial species, *C. Thermocellum* (*Ct*), was achieved. Another protoplast fusion was formed among the three strains *Cb*, *Ct* and *Thermoanaerobacterium saccharolyticum* (*Ts*) as the latter is known to metabolize longer sugars during hydrolysis. Biobutanol production was then examined in SSF using formed protoplast clostridial fusants and their corresponding co-cultures. Two different fusants of *CbCt* and *CbCtTs* were prepared and examined for stability in terms of biobutanol production using several growth cycles at 45 °C. The results of fermentation experiments conducted with *CbCt* and *CbCtTs* fusants were compared with results from co-cultures of the corresponding individual species. In the last part of the present study, the genetic stability of the fused strains was examined over several growth cycles.

Materials and methods

Chemicals and supplies

Clostridium beijerinckii (*Cb*; ATCC BA101), *C. thermocellum* (*Ct*; ATCC 27405), and *T. saccharolyticum* (*Ts*; ATCC 31907) were purchased from American Type Culture Collections. Wheat straw (WS) used in the current study was collected from Springridge Farm located in Milton, Ontario, and stored at room temperature. Before using it as a fermentation substrate, WS was ground into fine particles using a 1-mm sieve screen in a hammer mill. The moisture content of WS was reduced through heating in a conventional oven at 105 °C for 10 h until a constant weight was obtained. All the chemicals were purchased from Sigma-Aldrich Canada and were used without any further purification.

Media preparation

Clostridium basal medium (CBM) and Clostridium growth medium (CGM) were used for growing wild and fused clostridia strains. They were prepared according to the procedure published ealier [7–9]. National Biological Resource Center medium (NBRC) 979, containing 1.3g/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 or avicel cellulose, was used for growing *C. thermocellum* during the co-culture process [10]. The medium for thermophilic clostridia (MTC) was used for the growth of *T. Saccharolyticum* as well. It contained 5 g urea, 2 g citric acid tripotassium salt, 1.0 g sodium sulfate, 1.0 g potassium phosphate, 1.25 g citric acid monohydrate, 1.0 g magnesium chloride hexahydrate, 2.5 g sodium bicarbonate, 0.2 calcium chloride dihydrate, 1.0 g L-cysteine hydrochloride monohydrate, 0.1 g ferrous chloride, and 0.002 g resazurin per liter [11]. Regeneration Medium (RM) is a medium for the regeneration of bacterial cell walls and was prepared by the addition of stock solutions to a basal mixture. Then the liquid medium was poured into Petri dishes and left until it solidified to allow the growth of bacteria on its surface. Additional details on RM medium preparation are shown in [7,12]. Finally, cooked meat medium (CMM), which is supplemented with 0.1% glucose and composed of 100 g beef heart solids, 20 g casein/meat peptone, 2 g dextrose, and 5 g sodium chloride, was used to maintain the wild strains of bacteria [7,8].

Culture conditions

All Clostridia strains including wild and fused strains of *Cb*, *Ct*, and *Ts* were kept as a cell suspension in 30% v/v sterile glycerol and CMM at −82 °C in Eppendorf tubes inside the freezer. Before inoculation of the strains, their spores were heat shocked at 80 °C for 5 min and then the heat-shocked spore suspensions were grown overnight in CBM and CGM broth under severe anaerobic conditions [5]. All manipulations involving cells and protoplasts were carried out in an anaerobic glove box at a mean temperature of 25 ± 2 °C. To create an anaerobic environment inside the glove box, a vacuum pump was used for 10 min to evacuate the air from the chamber. Then during the inoculation, N_2 gas was purged through the box until all the serum bottles were properly sealed using a crimper. The glove box was cleaned routinely by wiping the work surface with 70% ethanol before and after the work. Also, the work surface

was exposed to the ultraviolet light to be sterilized for 1–2 h before inoculation of the bacteria.

Protoplast fusion and cell wall regeneration process

Three wild strains were used in the protoplast fusion formation: *C. beijerinckii* (*Cb*), *C. thermocellum* (*Ct*), and *T. saccharolyticum* (*Ts*). Fusants of *CbCt* and *CbCtTs* formed the final parent-fused strains. Genetic stability of the parent strains was then examined in 10 growth cycles for biobutanol producing capability. All experiments were carried out under blanket nitrogen in an anaerobic environment inside the glove box, while incubation under controlled temperature was done using an anaerobic chamber inside a shaking incubator. The process of fusion involves three major steps: protoplast formation, fusion, and cell wall regeneration [7,8]. During the first step of protoplast formation, overnight cultures of the wild strains were diluted 1:4 with fresh, sterile CBM containing 0.4% or 0.8% glycine. Protoplasts were formed after 60 min of incubation at 35 °C for *Cb*, and at 45 °C for *Ct* and *Ts*. To extract the protoplasts, the cell suspensions in PPM were centrifuged at 3300 rpm for 5 min and the cell pellets were resuspended in fresh PPM without lysozyme. During the second step or protoplast fusion, protoplast suspensions of the two and three Clostridia were mixed and centrifuged at 1500 rpm for 10 min at 20 °C. In the third step or protoplast regeneration, dilutions of the fused protoplasts were plated onto RM using a sterile inoculating loop and a spreader. The regenerated colonies were extracted and suspended in CBM medium with 30% (v/v) glycerol, and stored at −82 °C in labeled Eppendorf tubes [8].

Regeneration frequency of the protoplasts was calculated using Equation (1), where *a* is the number of colony-forming units (CFU) of cells before protoplasting per mL (units/mL), which was determined by counting viable cells after 24 h on agar plates; *b* is the CFU per mL of regenerated protoplasts after 48 h on RM plates in units/mL; and *c* is the CFU per mL of the non-protoplasted units (units/mL), which was calculated by viable counting of the protoplast suspension after dilution in CBM to bring about osmolysis of protoplasts [7].

$$\text{Regeneration Frequency} = 100(\text{b} - \text{c})/\text{a} \quad (1)$$

Clostridial co-culture process

Biobutanol production was also examined in SSF using clostridial co-culture strains. Fermentation experiments applied in this current study were done according to the co-culture technique developed by the Department of Fermentation Science and Technology at Tokyo University, Japan. During fermentation, 8% of *Ct* was cultured anaerobically at 60 °C in 100 mL of National Biological Resource Center (NBRC) medium 979 [10]. *Cb* was inoculated anaerobically within a CGM medium, and the culture was incubated at 35 °C. *Ts* was also cultured within an MTC medium in the strict anaerobic environment at 60 °C [11]. The co-culture experiments were conducted in 250-mL serum bottles. The butanol-producing *Cb* strain that grew exponentially was collected after 24 h by centrifugation, washed, and re-suspended in CGM containing 4% glucose. Tehn, 2 mL of this cell suspension was added to serum bottles containing both *Ct* and *Ts*, after the incubation temperature was decreased to 30–35 °C [10]. The same procedure was also applied by adding 1 mL of *Cb* cell suspension to the serum bottles containing *Ct* to obtain the *Cb-Ct* co-culture. The co-culture samples were then incubated at 30–35 °C for 3 days. Samples were then collected in Eppendorf tubes containing 30% (v/v) sterile glycerol and stored at −82 °C for further fermentation processes.

WS pre-treatment

During acidic pre-treatment, 4.5 g of dried WS were suspended in 50 mL of 1% dilute sulfuric acid (H_2SO_4) in 250-mL Wheaton serum bottles. The solution of WS–acid was then autoclaved at 121 °C for 60 min. After autoclaving, the lost water was added to maintain a constant volume. After adding water, the serum bottles were allowed to cool down to room temperature to make the WS ready for the SSF process [6].

SSF using fused and co-culture clostridia

In the present study, ABE were produced from pretreated WS using clostridia-fused and co-cultured bacteria in batch SSF. Experiments were conducted in 250-mL sealed Wheaton serum bottles. ABE concentrations, sugar consumption, pH changes and cell growth profiles were examined completely for all SSF experiments. The incubation temperature during fermentation was adjusted to 45 °C for fused strains and 35 °C and 45 °C for co-culture strains. After WS pretreatment, 40 mL of the growth medium (i.e. CBM) was added to the serum bottles to provide nutrients for the strain growth. Before inoculation, the pH of the solution was adjusted to 6.5 with 10 M sodium hydroxide. The inoculation part was carried out inside the anaerobic chamber, and all the manipulations were performed under a constant supply of N_2 gas. The serum bottles containing the culture medium and pretreated WS were inoculated with 7–8 mL of actively fused or co-culture strains. After the inoculation, the fermentation bottles were bubbled with N_2 gas for around 5–10 min. The serum bottles were sealed with blue neoprene rubber stoppers and a metallic cap using a vial crimper. The serum bottles were then transferred to the incubator set at the right fermentation temperature. The bottles containing co-culture strains were maintained at 35 °C and 45 °C, while those containing the fused strains were kept at 45 °C. The SSF procedure was applied for the first growth cycles of both fused and co-culture strains to check the stability of the bacteria and compare ABE production using fused and co-culture strains. SSF experiments were conducted in triplicate (i.e. repeated 3 times) for both fused and co-culture strains. Results reported were the averages of data collected from the corresponding experiments.

Analytical techniques and methods

Enzyme assay

The enzyme activity in the current study was quantified by the Filter Paper Assay (FPA) [13]. Cellulase enzyme activity

Table 1. Primer sequences and product size for biomarker genes.

Gene	Forward (5′–3′)	Reverse (5′–3′)	Product (bp)
bglA	ATCTGGACTCGGAGGTGTT	TTGTGCCATACCAACCAG	538
ald	ATGTTGCATGCGACCACTTC	TCGGATGCGGGATAATGT	463
xylB	ATACAGGTACGCCAAGAGGA	AGTAGTCAGCACCACCGCAT	620

was calculated in filter paper units (FPU) per milliliter of undiluted enzyme solution. During this assay, 2 mg of glucose was released from 50 mg of filter paper (4% conversion) in 60 min and designated as the intercept for calculating FPU for cellulose. The DNS assay method was used for measuring the reducing sugar concentration [14]. The assay procedure involved finding a dilution of the original enzyme stock such that a 0.5-mL aliquot of the dilution would catalyze 4% conversion in 60 min. The DNS reagent was prepared by mixing 10.6 g of 3,5 dinitrosalicylic acid and 19.8 g sodium hydroxide in 1416 mL distilled water. More details on the procedure for the FPA are published elsewhere [8]. The FPU was calculated using Equation (2), where *FPU* is filter paper activity (units/mL), and [*E*] is the concentration of enzymes releasing 2 mg glucose.

$$FPU = \frac{0.37}{[E]} \tag{2}$$

Genetic stability of the fused strains

Polymerase chain reaction (PCR) was used to test the stability of 10 generations of both fused strains by amplifying small amounts of DNA [15]. Genetic stability of *CbCt* and *CbCtTs* fused strains was determined by extracting the genomic DNA for several growth cycles using the MO BIO UltraClean Microbial DNA Isolation Kit. For the *CbCt* fusant, two biomarkers, β-glucosidase A gene (*bglA*) from *Ct* and aldehyde dehydrogenase gene (*ald*) from *Cb*, were selected for PCR. The *ald* gene chosen for *Cb* is predominantly involved in the oxidation of aldehydes and, finally, butanol production, while the *bglA* gene selected for *Ct* is predominantly involved in glucose metabolism. Primers for *bglA* and *ald* were designed using DNAMAN software to reduce primer dimers and ensure ~50% GC (guanine and cytosine) content in the primer as a percentage of total bases. They were designed within the *bglA* and *ald* gene targets to amplify ~538 bp and ~436 bp products, respectively. Table 1 shows the biomarker genes selected for PCR, as well as the number of base pairs (bp) in the product.

To determine the genetic stability of *CbCtTs* fused strains, the same process was applied for genomic DNA extraction for three generations and controls, including single cultures of *Cb*, *Ct*, and *Ts*. Three biomarkers, β-glucosidase A gene (*bgl*A), β-xylosidase B (*xylB*), and aldehyde dehydrogenase gene (*ald*) from *Ct*, *Ts*, and *Cb*, respectively, were selected for PCR analysis, and corresponding primer sequences can be found in Table 1. The PCR products were then detected and viewed on a 1% agarose gel to confirm size, quantity and purity. Samples from PCR analysis were loaded into the agarose gel wells within the electrophoresis chamber. The DNA samples that are negatively charged due to the phosphate molecule were loaded to the negative end of the chamber. After applying the electric current through the chamber, the negatively charged samples moved toward the positive end. DNA fragments of different sizes will travel different distances based on the pores in the agarose gel.

Hemocytometry

Cell count was determined using a hemocytometer (Qiujing XB-K-25) with 1/400 mm^2 unit area and 0.1 mm height under an optical microscope (Zeiss Axio Observer A1). Furthermore, cell count was obtained using the traditional viable cell counting. Samples were diluted at a rate of 10^7 and 0.1 mL of the final dilution was plated onto the agar plates. Plates were incubated overnight at the appropriate temperature for colonies to grow. Each growth colony represents one cell in the original sample. After counting the total number of colonies, it was multiplied by the dilution rate and reported as cells/mL.

UV/Vis spectrophotometry

Enzyme activity was measured using a pre-calibrated UV/Vis scanning spectrophotometer at 540 nm. Enzyme samples were analyzed by measuring absorbance against a reagent blank at the same wavelength. Before analysis, all tubes including the sample assay, blanks, standards, and controls were diluted in 0.2 mL of color-developed reaction mixture along with 2.5 mL of distilled water in the spectrophotometer cuvette, and analyzed.

High-performance liquid chromatography

Sugars and product concentrations were determined using high-performance liquid chromatography (HPLC – Perkin Elmer), which is equipped with an automatic sample injector and a refractive index detector. The mobile phase was 5 mM H_2SO_4. Three HPLC columns used throughout this study as follows: Shodex KC811 for measuring sugars, Shodex SP0810 for measuring inhibitors, and Aminex HPX-87H for measuring ABE solvents and acid concentrations. The flow rate was maintained at 0.6 mL/min for 1 h, whereas the temperature of the HPLC column was increased from 20 °C to 60 °C. Data were processed with the computer software Tubochrom Navigator. During the SSF process, a sample size of 1 mL was taken periodically, centrifuged at 15,000 *g* for 15 min and double filtered through 0.2-μm Polytetrafluoroethylene (PTFE) membrane filters prior to being analyzed.

Results and discussion

Table 2 displays the regeneration after 24–48 h at 35 °C for the mesophilic strains, and 45 °C for the thermophilic bacteria (i.e. *Cb*, *Ct* and *Ts* on agar plates).

From Table 2, it can be seen that the highest percentage of regenerated protoplasts after lysozyme treatment was observed with *Cb*, while the lowest was observed with *Ct*. Previous studies showed that the regular regeneration frequencies were in the range of 8–25%, which is in agreement with the regeneration frequencies obtained in the current study. Wood and Bhat (1988) demonstrated that Ca^{2+} and Mg^{2+} in the PPM could affect subsequent protoplast regeneration during protoplast formation. The required amount of both Ca^{2+} and Mg^{2+} for maximum

Table 2. Regeneration of *C. beijerinckii*, *C. thermocellum* and *T. saccharolyticum* (CFU/mL).

Bacterial strains	Cellsa (CFU/mL)	Regenerated protoplasts[b] (CFU/mL)	Non-protoplasted unitsc (CFU/mL)	Percent regeneration
Cb	7.5×10^6	2.3×10^6	1.2×10^6	14.6
Ct	6.4×10^6	1×10^6	6.5×10^5	5.4
Ts	8.1×10^6	2×10^6	1.1×10^6	11.1

[a] Cells were grown in CBM prior to protoplast formation. This value was determined from a viable cell count on agar plates after 48 h at 35 °C and 45 °C.
[b] Protoplasts were formed in PPM by lysozyme treatment (2.5 mg/mL) for 60 min, centrifuged, suspended in PPM and plated on RM. This value was determined after 48 h at 35 °C and 45 °C.
[c] Non-protoplasted units were determined from a viable cell count of the protoplast suspension after dilution in CBM to bring about osmotic lysis of protoplasts.

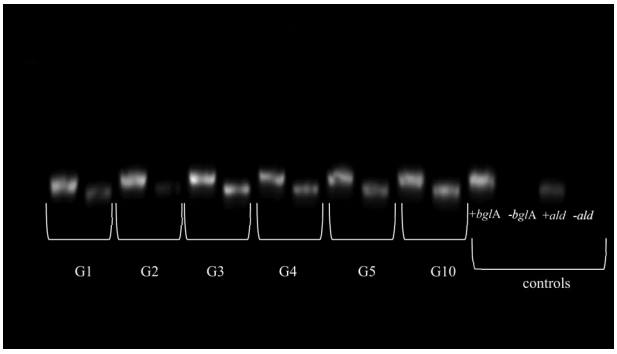

Figure 1. Agarose gel electrophoresis of PCR products for biomarkers. From left: generations 1 to 5 and 10 (G1–G5 and G10); first band (solid line) and second band (dotted line) are *bglA* and *ald* gene, respectively, representing *Ct* and *Cb*, positive and negative controls as designated.

regeneration seems to be concentrations over 25 mM. They concluded that concentrations of Ca^{2+} at the range of 10 mM or below in the PPM would have resulted in little or no regeneration of the protoplasts [16]. Therefore, $CaCl_2$ and $MgCl_2$ were added to PPM medium in the present study at concentrations of 50 mM to obtain the highest regeneration frequencies.

The electrophoresis results for all growth cycles of *CbCt* fused strains are shown in Figure 1. In this figure, G1 represents the first growth cycle or parent fusion, and G2, G3, G4 and G5 represent the second, third, fourth, and fifth growth cycles, respectively. To ensure long-term stability of the fused strain, the tenth growth cycle was analyzed as G10. As shown in Table 1, the biomarker control genes that were chosen for all growth cycles of *CbCt* fused strains were β-glucosidase A gene (*bgl*A) and aldehyde dehydrogenase gene (*ald*), from the wild strains of *Ct* and *Cb*, respectively. As shown in Figure 1 for all growth cycles of *CbCt* fused strains, the DNA fragments from the PCR are transferred to the negative end of the electrophoresis chamber to obtain the DNA profile. Since they have different molecular weights, they move down the gel at different speeds. Usually, the shortest DNA molecule moves the farthest. For instance, in the current study, the *ald* gene traveled farther than the *bglA* gene (Figure 1). It should be noted that the presence of the biomarker control genes in all growth cycles confirms the presence of genes from both wild strains in the fused strains. It also tells us the quantity of genetic information transferred to the fused strains through the protoplast fusion technique. For instance, as can be seen in Figure 1, in the second growth cycle (G2) the *ald* gene is less visible, which can lead to lower ABE productivity. It should also be mentioned that the strong expression of both biomarker genes in G10 confirms that the protoplast fusion was both successful and stable over the long term. Therefore, in the present study, the tenth generation of the *CbCt* fused strain was picked to examine the production of solvents through the SSF process.

A similar analysis was performed on *CbCtTs* fusant strains. Figure 2A shows both biomarker gene products for *bglA* and *xylB* of *Ct* and *Ts*, respectively. Results show that the fusant *CbCtTs* did not appear to be genetically stable. The *ald* gene biomarker product was the only product detected within the first two generations of growth, indicating the detectable presence of *Cb*. Controls showing the presence or absence of biomarkers in corresponding bacterial species can be seen in Figure 2B. This instability in *CbCtTs* fused strains can be attributed to several factors. First, the fusion represents a homologous crossover between the genomes of each species involved as long as each protoplast was successfully established. It does not ensure that large or whole portions of the genome will cross over because the process is random in the sense that it depends on what portions of the genome are homologous and where. One should also consider the variability in the species and the species metabolism; these differences can cause less homology in areas perhaps where the biomarkers were chosen. The biomarker genes are designed specifically for each strain; thus, they were chosen regarding genes involved in the specific metabolism of each strain, resulting in lower genetic detection if there is not enough homology.

Table 3 shows final concentrations of ABE, as well as the acid production that resulted from SSF for both *Cb* and *Ct* fused and co-culture strains. As shown in Table 3, fused *CbCt* were able to produce 23 g/L of total ABE and 13.82 g/L of biobutanol at 45 °C. However, their corresponding co-culture strains produced only 9.52 g/L of total ABE and 5.79 g/L. The same *Cb* strain (BA101) produced equivalent amounts of ABE solvents (i.e. 24 g/L) at the pilot plant scale using a corn steep water medium [17]. In the current study, a total solvent concentration of 23 g/L was obtained from the *CbCt* fusant at a laboratory scale. This demonstrates a high potential to enhance production of biofuel by utilizing the fused strains at an industrial scale.

Previous studies reported equivalent butanol productions of 13 g/L and 15.8 g/L that were obtained with glucose and corn as the main substrates [18,19]. WS, which was used in the present study as the substrate, is an agricultural waste rather than a food source, which highlights the importance of the proposed work. Figure 3 shows the ABE concentration profile during fermentation using *CbCt* fused strains.

Figure 3 shows that after 120 h of SSF, the *CbCt* fused strain produced 23 g/L of total ABE by consuming 36.01 g/L of sugars. Figure 3 also shows that solvent production of 6.75, 13.42, and 2.2 g/L of acetone, butanol, and ethanol,

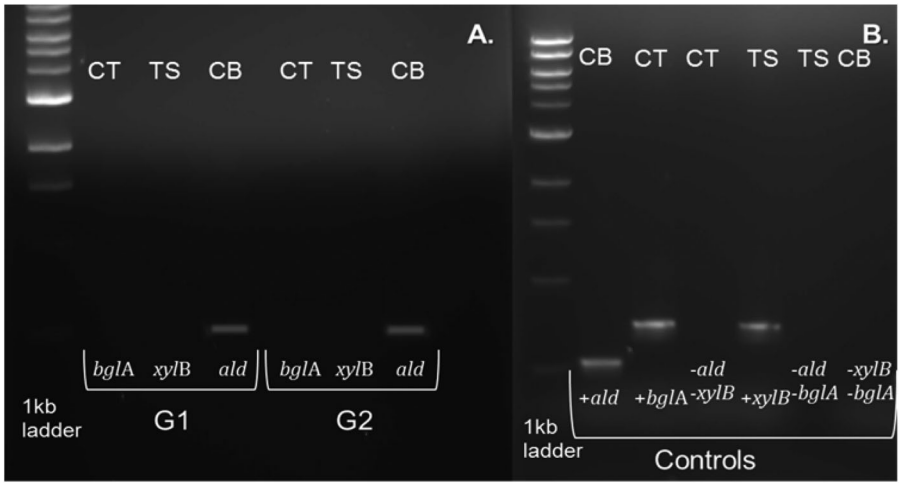

Figure 2. (A) Agarose gel electrophoresis of PCR products for biomarkers. From left: Generations 1 and 2 (G1, G2); third band in each generation: *ald*, respectively, representing *C. beijerinckii*. (B) Positive (band present) and negative controls (no bands) for the presence/absence of biomarkers in each corresponding strain as labeled.

Table 3. Fermentation results obtained with fused strains at 45 °C and co-cultured strains at 35 °C during 120 h of SSF.

		CbCt		*CbCtTs*	
Fermentation parameters		Fused	Co-culture	Fused	Co-culture
ABE (g/L)	Acetone	6.89	2.77	6.44	3.84
	Butanol	13.82	5.79	12.8	6.25
	Ethanol	2.29	0.96	4.69	1.95
Acids (g/L)	Acetic acid	1.75	2.21	1.82	2.46
	Butyric acid	0.87	1.17	0.95	1.3
Total sugars consumed (g/L)		36.01	25.26	38.3	27.24
Average cell proliferation rate (10^5 cells/mLh)		3.85	3.66	4.02	3.36
ABE yield ($Y_{ABE/S}$)[a]		0.48	0.24	0.49	0.29
Acetone yield ($Y_{A/S}$)[b]		0.14	0.07	0.13	0.09
Butanol yield ($Y_{B/S}$)[c]		0.28	0.14	0.26	0.15
Ethanol yield ($Y_{E/S}$)[d]		0.04	0.02	0.09	0.04

[a]$Y_{ABE/S}$ was calculated by dividing final ABE concentration by total sugars consumed.
[b]$Y_{A/S}$ was calculated by dividing final acetone concentration by total sugars consumed.
[c]$Y_{B/S}$ was calculated by dividing final butanol concentration by total sugars consumed.
[d]$Y_{E/S}$ was calculated by dividing final ethanol concentration by total sugars consumed.

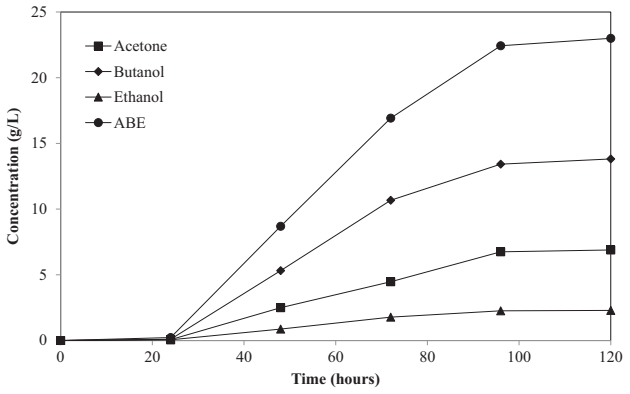

Figure 3. ABE concentration profile during SSF using *CbCt* fused strains at 45 °C (average RSD: 0.6%).

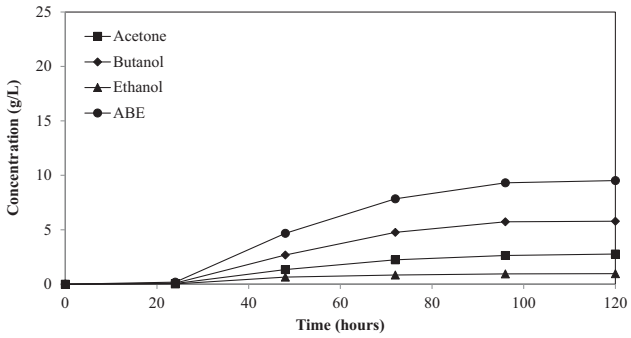

Figure 4. ABE concentration profile during SSF using *CbCt* co-culture strains at 35 °C (average RSD: 0.9%).

respectively, occurred between 24 and 96 h. During the next 24 h of fermentation, the total increase in solvent concentration was about 4%, from 22.02 g/L to 23.00 g/L at the end of fermentation. As shown in Table 3, the concentrations of acetone, butanol, and ethanol at the end of fermentation were 6.89, 13.82 and 2.2 g/L, respectively. Considering the concentration of butanol as shown in Figure 3, it can be observed that between 24 h and 96 h, it almost reached its maximum concentration of about 13.42 g/L. After this time, the total increase in butanol concentration was about 2.75%, from 13.42 to 13.82 g/L at the end of fermentation. Apparently, the fused strain showed a strong tolerance to butanol toxicity. This confirms that the protoplast fusion technique created strains with a higher level of tolerance to butanol toxicity, which led to an enhanced butanol production of around 14 g/L. It should also be mentioned that this novel strain experienced butanol toxicity after 96 h, as shown in Figure 3. Table 3 displays the total sugars consumed by both co-culture and fused strains during SSF. It also shows the average bacterial cell proliferation rate for all strains.

The co-culture strains corresponding to the fused ones were examined for ABE production at 35 °C and 45 °C. Figure 4 shows the ABE concentration profile during SSF

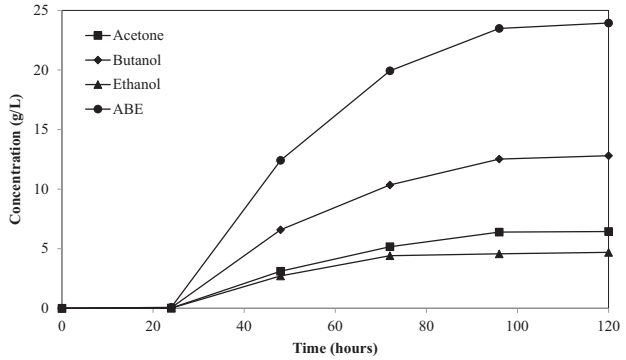

Figure 5. ABE concentration profile during SSF using *CbCtTs* fused strains at 45 °C (average RSD: 0.29%).

using *CbCt* co-culture strains at 35 °C. As can be observed from this figure, *CbCt* co-culture strains produced 2.77 g/L of acetone, 5.8 g/L of butanol, and 0.96 g/L of ethanol by the end of fermentation. Previous studies done on this method reported about 2 g/L of butanol using crystalline cellulose as the main substrate [10]. However, in the current study, a concentration of 5.8 g/L was obtained from WS substrate using the *CbCt* co-culture strain. It should also be noted that none of the solvents produced in the co-culture experiment had toxic effects on bacterial cells since they were far below the toxic range of the cell cultures.

Comparing the results of solvents produced using co-culture strains to those produced by the fused strain suggests that *CbCt* fused strains were able to produce around 139% more butanol than *CbCt* co-culture strains by the end of fermentation. Table 3 summarizes results for the ABE concentration profile during SSF, which was determined for both *CbCtTs* fused and *CbCtTs* co-culture strains. Table 3 shows that the final butanol concentration achieved using the *CbCtTs* fused strain is comparable to results of previous studies done on butanol production using *Cb* as the butanol-producing strain. One study [4] reached a butanol production level of around 13.00 g/L from glucose during SSF using *C. beijerinckii*, whereas in the current study 12.80 g/L of butanol was obtained with WS as the substrate [20]. Another study that applied SSF and gas stripping for butanol removal from the system showed butanol production of up to 12.70 g/L [4]. However, the fused strains in the present study produced almost the same butanol concentrations without employing gas stripping or other butanol removal methods. The total ABE produced in the current study was about 23.94 g/L for *CbCtTs* fused strains, which is relatively high compared to previous studies. The concentration of furfural as an inhibitor during the SSF was also measured in this study. It was in the range of 0.01 to 0.03 g/L for all strains, which is considerably less than the inhibitory amount of 1 g/L [7]. Therefore, in this study, the negative effects of furfural on the fermentation process were neglected. Table 3also shows that *CbCtTs* co-culture strains produced about 12.04 g/L of total ABE and 6.25 g/L of butanol at 35 °C, which is lower than the butanol produced by fused strains at 45 °C. This demonstrates that the optimum temperature for enzyme activity necessary for saccharification and fermentation is 45 °C as indicated earlier. Figure 5 displays the concentration of ABE during SSF using *CbCtTs* fused strains.

Figure 5 shows that the *CbCtTs* fused strains produced 23.94 g/L of total ABE after 120 h of SSF. It can also be observed that the production of solvents was most predominant between 24 h and 96 h with the production of 6.39 g/L acetone, 12.52 g/L butanol, and 4.57 g/L ethanol during that time period. The final concentrations of acetone, butanol, and ethanol were ∼ 6.44, 12.80 and 4.69g/L, respectively. It can be observed that during the 96 h period the solvent reached a concentration of 23.48 g/L. However, during the following 24 h, there was a small increase of about 2% in total ABE concentration from 23.48 g/L to 23.94 g/L [21,22]. However, as the figure shows, the genetic improvement of bacterial strains through protoplast fusion resulted in novel strains with a higher butanol tolerance level followed by high butanol production at the range of 12.80 g/L, in the case of *CbCtTs* fused strains. It should also be mentioned that acetone and ethanol concentrations in the current study did not contribute to solvent toxicity, in which the toxic level of acetone and ethanol on cell growth was 70 g/L and 50–60 g/L, respectively [23,24]. As shown in Table 4, the *CbCtTs* fused strains produced 23.94 g/L of total ABE after 120 h of SSF by consuming 38.3 g/L of total sugars. Figure 6 displays the ABE concentration profile during SSF using *CbCtTs* co-culture strains at 35 °C.

As shown in Figure 6, during the first 24 h of fermentation, there was not a significant increase in the solvent concentration due to bacterial growth and enzymes produced for saccharification of polysaccharides into monomeric sugars. This was followed by a considerable increase in solvent production until the end of fermentation when the concentrations of acetone, butanol and ethanol reached 3.84, 6.25, and 1.95 g/L, respectively. Since the butanol concentration was less than 7 g/L after 120 h, butanol toxicity does not seem to be a major concern. Acetone and ethanol concentrations were also not considered toxic to the co-culture strains since their levels were far below the toxic range. Figure 6 demonstrates that the *CbCtTs* co-culture strains were able to produce lower amounts of solvents compared to their corresponding fused strains. Comparing the biobutanol concentrations, it can be observed that the *CbCtTs* fused strains were able to produce 104% more butanol than were the *CbCtTs* co-culture strains. Results show that *Cb*, which is the butanol-producing bacteria, was not able to produce any biobutanol at 45 °C. However, *Ct* and *Ts*, which are the two thermophilic strains, were able to produce some amounts of ethanol at thehigher temperature of 45 °C [11].

Butyric acid and acetic acid were the two acids generated during SSF using the fused and co-culture strains in the present study [24]. As previously described, these acids are produced during the acidogenic phase of the ABE metabolic pathway. Table 3 shows that the highest concentrations of acids were produced using the *CbCtTs* co-culture strain. This strain produced about 2.46 g/L of acetic acid and 1.30 g/L of butyric acid. It was followed by the *CbCt* co-culture strain that produced 2.21 g/L of acetic acid and 1.17 g/L of butyric acid. Therefore, co-culture strains produced greater amounts of acids compared to fused strains.

Changes in pH were also measured during the experiments. Figure 7 shows the change in pH during SSF for all bacterial strains. As shown in this figure, the lowest pH of

Table 4. Concentrations of released glucose from samples and enzyme activity for *CbCt* and *CbCtTs* fused strains.

	CbCt (fused)		*CbCtTs* (fused)	
Dilution #	Abs 540 nm	Glucose (mg/0.5 mL)	Abs 540 nm	Glucose (mg/0.5 mL)
1	0.587	2.561	0.635	2.764
2	0.434	1.889	0.485	2.111
3	0.282	1.228	0.327	1.424
Enzyme activity (FPU/mL)	68.51		75.51	

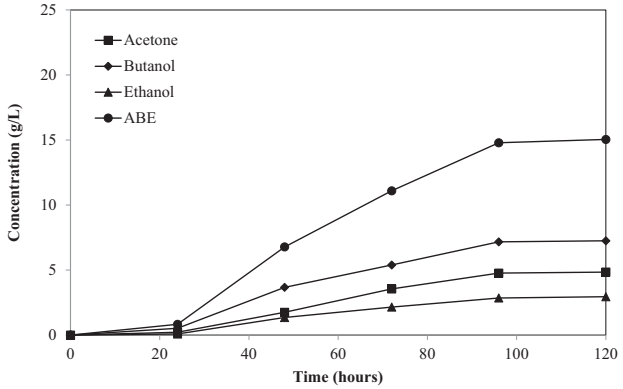

Figure 6. ABE concentration profile during SSF using *CbCtTs* co-culture strains at 35 °C (average RSD: 0.24%).

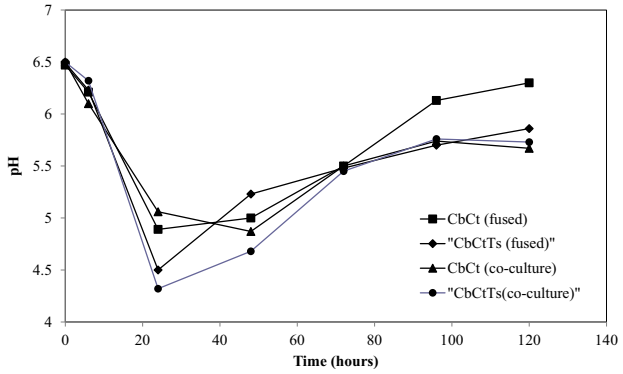

Figure 7. Changes in pH during SSF for all fused and co-culture bacterial strains (average RSD: 3.4%).

about 4.32 was obtained for *CbCtTs* co-culture strains after 24 h of fermentation. The pH for *CbCtTs* fused strains, which produced around 1.82 g/L of acetic acid and 0.95 g/L of butyric acid, dropped from 6.49 to around 4.50 in the first 24 h before increasing to 5.23 at 48 h. In the case of *CbCt* fused strains, which produced about 1.75 g/L of acetic acid and 0.87 g/L of butyric acid, the pH dropped to 4.89 after 24 h. However, for *CbCt* co-culture strains the pH dropped to around 4.9 after 48 h of fermentation. From Figure 7, it can be concluded that the reduction in pH at the beginning of fermentation is due to the production of acetic acid and butyric acid, while the subsequent increase in pH can be attributed to the production of acetone, butanol and ethanol as solvents during the solventogenesis stage [25].

Table 3 shows that the total sugar amount consumed with *CbCt* fusant was 36.01g/L, while it was 25.26g/L for *CbCt* co-culture strains. A similar comparison can be made for *CbCtTs* fused and co-culture strains. According to these results, the two fused strains (*CbCt* and *CbCtTs*) consumed more sugars compared to their corresponding co-culture strains. Generally, fused strains consumed higher sugars than the amount of sugars consumed in previous studies

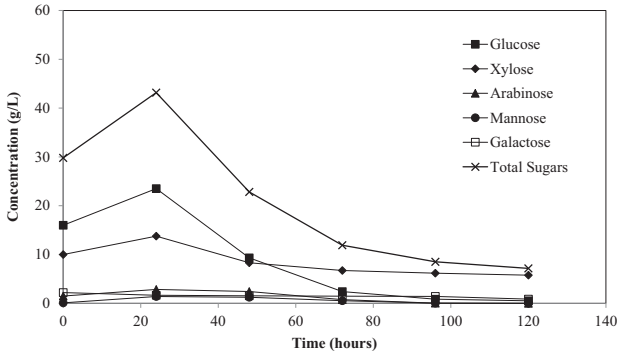

Figure 8. Individual sugar concentrations with respect to total sugars during SSF for *CbCt* fused strains (average RSD: 1.65%).

using only *Cb* as the fermentative bacteria [8]. This shows that the fused strains have the potential to consume more sugars compared to their wild strains. This eventually led to higher solvent production from fused strains compared to co-culture strains. Figure 8 displays the amount of remaining sugars, including glucose, xylose, galactose, arabinose, and mannose, at the end of the fermentation process in the system using *CbCt* fused strains.

This figure shows that total sugar concentrations increased in the first 24 h of fermentation. The increase in the total sugar is the result of saccharification of polysaccharides into monomers such as glucose, xylose, mannose, galactose and arabinose by the action of the enzymes released from the fused strains. This can obviously be observed for glucose, xylose, and arabinose, while the increase in the concentrations of galactose and mannose was relatively minor. As shown in Figure 8, arabinose and mannose were completely consumed by the end of fermentation. It should also be mentioned that during the first 24 h of fermentation, the concentration of sugars increased by around 44% in the system. This is attributable to the hydrolysis enzymes released from the bacteria, which lead to breaking the polysaccharides into monomeric sugars in the system. Compared to *Ct*, *Cb* is more capable of breaking down pentose sugars such as xylose. Therefore, there was a steady decline in the xylose concentration, from 13.76 to 5.76 g/L at the end of fermentation; however, the glucose concentration declined more rapidly (from 23.5 g/L at 24 h to 0.52 g/L at 120 h) due to its consumption by the two bacteria (*Cb* and *Ct*), which are able to consume both pentose and hexose sugars. It can also be concluded that pentose sugars such as xylose were not completely consumed at the end of fermentation. However, six-carbon sugars such as glucose were mostly consumed, and only very small amounts (0.52 g/L) remained in the system. This suggests that the *CbCt* fused strains preferred to consume glucose compared to other monomeric sugars. Other hemicellulose-derived sugars such as arabinose and mannose were totally consumed.

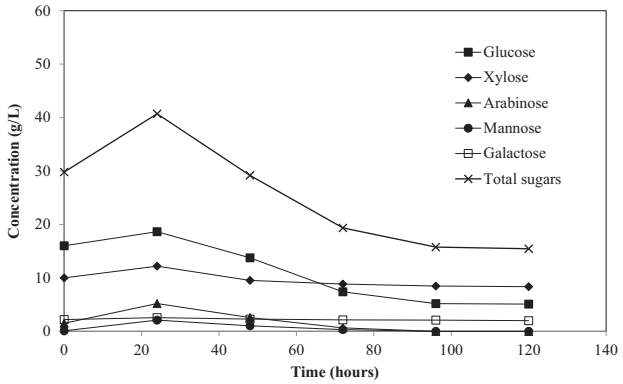

Figure 9. Individual sugar concentrations with respect to total sugars during SSF for *CbCt* co-culture strains (average RSD: 1.3%).

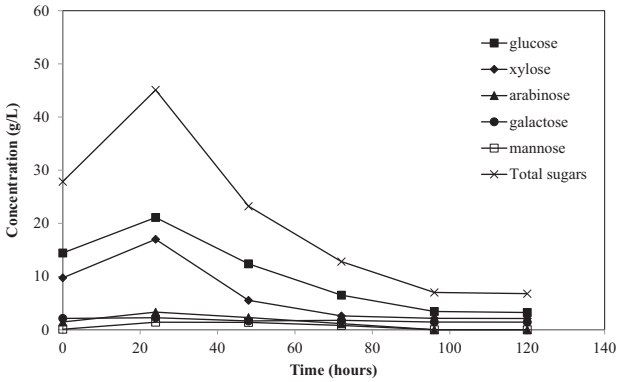

Figure 10. Individual sugar concentrations with respect to total sugars during SSF for *CbCtTs* fused strains (average RSD: 0.8%).

According to Figure 9, the amount of total sugar that remained in the system at the end of fermentation using *CbCt* co-culture strains was double of that of the fused strains.

Arabinose and mannose were completely consumed by the end of SSF using *CbCt* co-culture strains. It can also be seen that *CbCt* co-culture strains preferred to consume glucose than hemicellulose-derived sugars (i.e. xylose). Therefore, a considerable decrease in the glucose concentration can be seen at the end of the fermentation process (from 18.65 g/L at 24 h to 5.1 g/L at 120h). However, lesser amounts of pentose sugars such as xylose were consumed during the fermentation process. Figure 10 shows changes in the total and individual sugar concentrations during SSF with *CbCtTs*.

As shown in Figure 10, sugar concentrations increased during the first 24 h of fermentation. This is simply due to the saccharification of cellulose and hemicellulose into monomers, such as glucose, xylose, mannose, arabinose, and galactose, by the action of the enzymes released from the fused strains. A higher increase in sugar concentration was mainly obtained by glucose and xylose compared to all other individual sugars. The increases in arabinose and mannose concentrations, by contrast, were considerably lower. The increase in sugar concentrations was followed by a sharp decrease during the next few days of fermentation due to the consumption of sugars and the production of solvents. Figure 10 shows that during 24 h of saccharification, a 57% increase in sugar concentration was observed for the *CbCtTs* fused strains without the addition of any enzymes.

Most consumption of both glucose and xylose can be seen between 24 h and 48 h, where the glucose and xylose remaining in the system dropped from 21.1 to 12.38 g/L and from 17 to 5.52 g/L, respectively. At the end of fermentation, only 3.24 g/L of glucose and 2.11 g/L of xylose remained. It can also be seen that compared to xylose, greater amounts of glucose were consumed during the 120 h using *CbCtTs* strains. Thus, higher percentages of glucose during the first few hours of fermentation inhibited consumption of xylose during the following hours. *Ts* is a bacterium that is able to utilize five-carbon sugars such as xylose. Therefore, compared to the previous case described in this study (*CbCt* fused), *CbCtTs* fused strains were capable of using greater amounts of xylose during the 120 h of fermentation. It should be noted, however, that xylose was not consumed completely and about 2.11 g/L

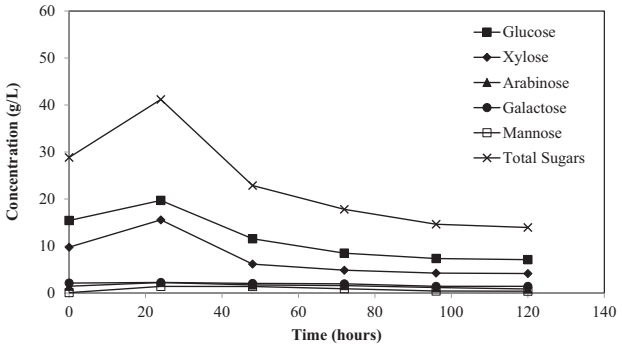

Figure 11. Individual sugar concentrations with respect to total sugars during SSF for *CbCtTs* co-culture strains (average RSD: 0.36%).

remained in the system at the end of fermentation. Considering other sugars, it can clearly be seen that no traces of arabinose and mannose remained at the end of SSF, and they were completely consumed; however, only a small amount of galactose remained at the end of fermentation. Figure 11 displays the changes in the concentrations of individual sugars with respect to the initial concentration of total sugars at the beginning of SSF using *CbCtTs* co-culture strains.

Figure 11 shows that after 120 h of fermentation, around 13.94 g/L of total sugars remained in the system. As stated for *CbCtTs* fused strains, in the case of *CbCtTs* co-culture strains, glucose was consumed more rapidly compared to xylose, which shows the preferential consumption of glucose over xylose by the three co-culture strains. As a result, at the end of fermentation, the remaining glucose dropped from 15.52 to 7.1 g/L, whereas the xylose concentration decreased from 9.75 to 4.15 g/L. This shows that compared to glucose, a lower percentage of xylose was consumed by *CbCtTs* co-culture strains. Figure 11 shows that arabinose and mannose were almost completely consumed at the end of fermentation. However, about 7.1 g/L glucose, 4.1 g/L xylose, 1.43 g/L galactose, and 13.94 g/L of total sugars remained in the system. The amount of total sugars consumed for *CbCtTs* co-culture strains was 27.24 g/L, which shows a decline of about 40% in sugar consumption compared to their corresponding fused strains.

The average cell proliferation rate for all bacterial strains with respect to sugar consumption is shown in Table 3. From this table, it can be concluded that more bacterial cells tend to metabolize a greater amount of total sugars and produce more solvents. Figures 12 and 13 display the changes in cell concentration for both fused and co-culture

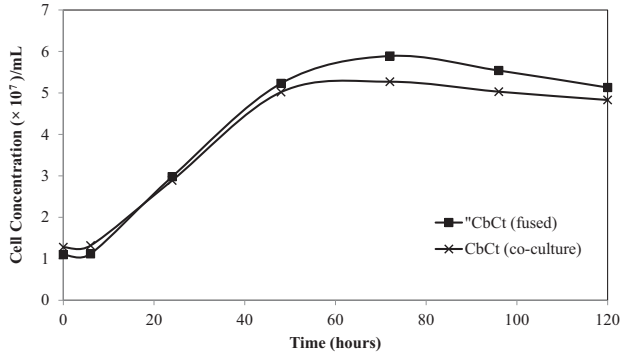

Figure 12. Changes in cell concentration for *CbCt* fused and *CbCt* co-culture strains during SSF (average RSD: 3.4%).

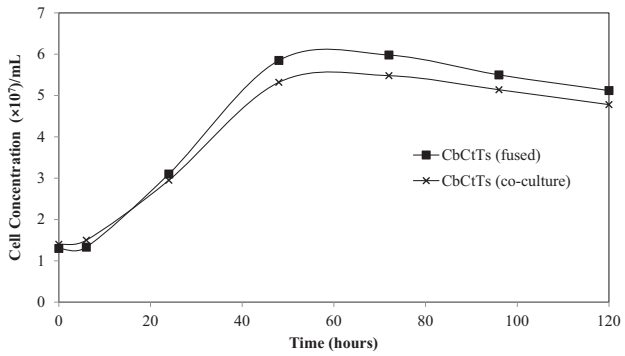

Figure 13. Changes in cell concentration for *CbCtTs* fused and *CbCtTs* co-culture strains during SSF (average RSD: 4.1%).

strains. During the first 6 h, all bacteria experienced a lag phase. In this phase, the cell concentration remained almost constant because the strains were adjusting themselves to the medium, temperature, and pH levels. After this phase, a sharp increase in cell concentration can be seen, where the cells grew exponentially by feeding on the nutrients present in the culture medium and entering the exponential phase. The exponential phase ended after 72–84 h, when the cell concentration decreased slowly upon entering the decay phase. This phase corresponds to the solventogenic phase in the SSF process, where butanol toxicity inhibits the cell growth.

As shown in Figure 12, an increase in cell concentration can be seen for *CbCt* fused strains between 12 h and 60 h, when the strains grew exponentially. It was followed by the stationary phase during the next 36 h when the cell concentration remained almost constant. As described before, *CbCt* fused strains are capable of tolerating higher butanol concentrations, of around 12.5 g/L. This can clearly be seen until 84 h, when there was no decline in cell concentration. Moreover, this strain exhibited a wider stationary phase until 96 h, in which the butanol concentration was less than 13.42 g/L. This was followed by a decay phase at 96 h, when the cell concentration decreased slowly due to butanol toxicity in the range of 13.42 g/L. The corresponding *CbCt* co-culture strains showed a growth phase between 12 h and 72 h, when the butanol concentration reached around 4.76 g/L. Since the co-culture strains produced lower butanol levels compared to their fused strains, the stationary phase was longer for the co-culture strains, and they did not experience a considerable decay phase.

Figure 13 shows the changes in cell growth of *CbCtTs* fused and co-culture strains during SSF. A similar analysis of *CbCtTs* fused and co-culture strains showed that these strains also exhibited a lag phase during the first 6 h of fermentation. This was followed by an exponential phase, when the cells grew rapidly, between 12 and 72 h. During the following 24 h, both fused and co-culture strains experienced a stationary phase until 96 h when the fused cells entered the decay phase, due to butanol toxicity at concentrations of more than 12.5 g/L. However, the cell concentration for the corresponding co-culture strains remained almost constant, without any significant changes for the rest of SSF.

Total ABE yield was calculated for total ABE production with respect to total sugar consumption during 120 h of fermentation. Results listed in Table 3 show that yields obtained for the fused strain were generally higher than for the corresponding co-culture. The total sugar produced in the hydrolysis of WS was around 55 g/L. This was almost equivalent to the total sugar concentration reported in previous studies, which was in the range of 55–65 g/L in hydrolysate [4]. According to Table 3, the total ABE yield for *CbCt* fused strains was around 0.48, with an acetone yield of 0.14, a butanol yield of 0.28, and an ethanol yield of about 0.04. Furthermore, the total ABE yield obtained from *CbCt* fused strains was 0.40 of the total ABE and 0.26 of biobutanol. These results are comparable to other results, in which a total ABE yield of 0.41 was achieved [4]. However, it should be mentioned that in those studies, a gas stripping method and a bioreactor were used to increase the solvent production. On the other hand, the total ABE yield of the *CbCtTs* fused strains was 0.49, which was almost the same as that obtained from *CbCt* fused strains. In this case, the acetone yield slightly decreased from 0.14 to 0.13, and the butanol yield dropped from 0.28 to 0.26. Moreover, the ethanol yield increased from 0.04, in the case of *CbCt* fused strains, to 0.09 in the case of *CbCtTs* fused strains, which shows the capability of the three fusants for ethanol production compared to the two-fusants process. It should be mentioned that in this case, total ABE yield is still a little higher than that reported in the previous study [4], in which *Cb* was the only bacterium to produce biobutanol. Besides, the total ABE yield obtained from *CbCt* co-culture strains was 0.24 which was the smallest yield; however, *CbCtTs* co-culture strains reported an ABE yield of 0.29, which is higher than the previous case. Overall, it can be concluded that the two and three fused strains were able to produce higher yields of total ABE and biobutanol compared to their corresponding co-culture strains.

Table 4 displays the glucose concentrations released from the filter paper for each sample, and the corresponding enzyme activity in filter paper units per mL (FPU/mL). In the current study enzymes were produced by both thermophilic and mesophilic bacterial strains. According to the literature, both thermophilic and mesophilic bacteria are able to produce enzymes that hydrolyze cellulose and hemicellulose into monomers. Then the strain metabolizes them to acids and ABE solvents [24]. *Ct* is proven to produce both cellulose-degrading enzymes and cellulosome cellulases; hence, it has been considered as a significant strain for decades [26]. In the current study, the fused strains were able to produce enzymes required for cellulose

hydrolysis, such as endoglucanase, exoglucanase and β-glucosidase, during the SSF process.

As shown in Table 4, *CbCt* fused strains produced 68.51 FPU/mL cellulolytic enzymes, and *CbCtTs* fused generated 75.51 FPU/mL enzymes. Compared with the activity of commercial enzymes, such as accellerase 1,500 with an activity of 43.21 FPU/mL [27], the enzyme activity of the fused strains applied in the current study revealed a new technique for enzymatic hydrolysis associated with the conversion of lignocellulosic feedstock to biofuels during SSF.

Conclusions

Results showed that fused strains showed superiority in terms of butanol production and yield compared to co-culture strains (production of 13.82 g/L by *CbCt* fused strains at 45 °C compared to 5.79 g/L by their corresponding co-culture at 35 °C). Similarly, the *CbCtTs* fused strains produced 12.80 g/L of butanol, compared to 6.25 g/L of butanol produced by their co-culture counterparts. These results demonstrate that genetic improvements by the protoplast fusions resulted in higher levels of tolerance to produced butanol toxicity. Furthermore, both fused strains showed the potential to produce enzymes required for saccharification and fermentation. While *Ct* was able to produce enzymes for degradation of cellulose and cellobiose to monomeric sugars, *Ts* and *Cb* had the potential to hydrolyze hemicellulose to pentose sugars and ferment them to biobutanol. The genetic stability results showed that *CbCt* fused strains displayed genetic stability during 10 growth cycles. *CbCtTs* fused strains did not demonstrate genetic stability although they demonstrated clear stability in ABE production over 10 cycles.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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