

Article

Butyric Acid Production from Wheat Straw Biomass by Simultaneous Saccharification and Fermentation and High-Productivity Reactors [†]

Nasib Qureshi ^{1,*}, Thaddeus C. Ezeji ², Haibo Huang ³ and Ronald E. Hector ¹

¹ BioEnergy Research Unit, National Center for Agricultural Utilization Research (NCAUR), Agricultural Research Service (ARS), United States Department of Agriculture (USDA), 1815 N University Street, Peoria, IL 61604, USA

² College of Food, Agricultural and Environmental Sciences, Departments of Animal Sciences and Food, Agricultural and Biological Engineering, The Ohio State University, 1680 Madison Avenue, Wooster, OH 44691, USA

³ Department of Food Science and Technology, Virginia Tech, 1230 Washington St. SW, Blacksburg, VA 24061, USA

* Correspondence: nasib.qureshi@usda.gov

[†] Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity provider and employer.

[‡] These authors contributed equally to this work.

Abstract

Sustainable production of biofuels and chemicals by green fermentation routes is becoming attractive. In this paper, the production of butyric acid by fermentation of wheat straw is studied. This conversion process requires biomass milling, pretreatment, hydrolysis, fermentation, and recovery. Of these five processing steps, pretreatment, hydrolysis, fermentation, and recovery are particularly cost-intensive. In the current study we combined simultaneous saccharification and fermentation in one unit operation for the substrate of wheat straw. In the integrated process, 90 g L⁻¹ of wheat straw was simultaneously saccharified and fermented to 20.8 g L⁻¹ of total acetic and butyric acids, of which butyric acid is the main product. As a result of the integration of saccharification with fermentation into a single streamlined workflow, the economics of the process significantly improved. In the fermentation process in which water was recycled, 39.9 g L⁻¹ total acid (acetic acid + butyric acids) was produced. This is comparable to 40.8 g L⁻¹ total acid produced in the glucose-fed control fermentation. Using cell-recycling technology, acid productivity was increased by 8–10 fold (800–1000%) from wheat straw hydrolysate as a feed. In this article, product yield, productivity, and selectivity were deemed to have high potential.

Keywords: butyric acid; wheat straw; high productivity; yield; water recycle; selectivity



Academic Editor: Diomi Mamma

Received: 26 August 2026

Revised: 2 October 2026

Accepted: 5 October 2026

Published: 9 October 2026

Copyright: © 2026 by the authors.

Licensee MDPI, Basel, Switzerland.

This article is an open access article distributed under the terms and

conditions of the [Creative Commons Attribution \(CC BY\)](https://creativecommons.org/licenses/by/4.0/) license.

1. Introduction

Butyric acid is an important chemical that is used for making chemicals (additives to fibers, polymer monomer, flame retardant, and bioenergy), and pharmaceuticals (electrolyte balance, acidulant and extractant). Esters of this acid can be used to make perfumes, and in food for increasing fruit fragrance and aromatic additives [1,2]. It is well known as a treatment for cancer and gastrointestinal diseases [3–7]. It is also used in medicines such as antianxiety medication to enhance oxidative phosphorylation and suppress inflammation. Furthermore, it has applications in animal feed (in which it may provide energy, promote

water and sodium absorption) [1]. Currently, it is produced from petrochemicals, reaching quantities of 140,000–150,000 MT year⁻¹ (metric tons per year), and its selling price is \$1.39–2.21 kg⁻¹. It can be produced from low-cost feedstocks such as agricultural biomass which are produced in large quantities in the Midwestern region of the United States with costs as low as \$50–100 MT⁻¹ (metric tons). This biomass is available for bioconversion to biofuels and biochemicals such as butyric acid (according to the 2023 billion ton report). In addition to agricultural biomass, it can also be produced via fermentation of food waste [8] and household and municipal organic wastes such as sludge [9]. Using these resources it is possible to reduce environmental pollution and produce butyric acid at a lower cost, as these wastes are available at low and economical prices.

The microbial strains that can produce butyric acid include *Clostridium butyricum* ZJUCB [10], *C. tyrobutyricum* NRRL 67062 [11], *C. tyrobutyricum* ATCC 25755 (also known as *C. thermobutyricum* ATCC 25755) [12] and *Escherichia coli* [13]. It is pointed out that *C. tyrobutyricum* ATCC 25755 can also utilize cellulosic sugars (hexose and pentose) simultaneously. This strain can accumulate up to 45 g L⁻¹ total acetic and butyric acids in the reactor. Other strains that are known to produce butyric acid include *Bacteroides melaninogenicus*, *Treponema phagedenis* and *Peptococcus, asacelarolyticus* [14]. It is hypothesized that achieving economically viable butyric acid production will require the use of low-cost biomass, implementation of novel pretreatment methods, integration of process steps, recycling of water or fermentation broth and energy-efficient product recovery. Additionally, developing high-productivity reactors will be necessary to achieve the desired economic feasibility.

In order to produce butyric acid from agricultural biomass, these residues have to be pretreated using dilute H₂SO₄ or NaOH at a high temperature (usually 160 °C). The use of pretreatment chemicals such as acid or alkali at high temperature generates biomass inhibitors including furfural, hydroxymethyl furfural (HMF), syringaldehyde, glucuronic acid, coumaric acid, ferulic acid, and phenolic acid [15]. These inhibitors are sugar degradation products and arrest/inhibit cell growth and fermentation. Additionally, the application of alkali or acid for biomass pretreatment at high temperature is not environmentally friendly. For that reason, we developed a pretreatment technique for wheat straw, sweet sorghum bagasse or corn stover, in which biomass is subjected to hot water at 190 °C and no chemicals are used. As soon as the temperature reaches 190 °C, cooling of the reactor is initiated. This method is called “hot water pretreatment” and it does not generate inhibitors at concentrations that arrest cell growth or fermentation. This technique has already been tested and is routinely used in our reactors. Use of hot water has a number of additional advantages, including the following: (i) it is more economic than using alkali or acid; (ii) hot water is not corrosive to the reactors and process equipment; (iii) it has reduced health hazards; and (iv) it is environmentally friendly. Pretreatment of biomass is essential because it disintegrates biomass cell wall structure and makes it accessible to hydrolytic enzymes. In addition to the use of low-cost biomass feedstocks, pretreatment, saccharification, and fermentation, numerous studies have explored alternate methods for recovering butyric acid [12,16–20] from fermentation broth. These recovery processes also make the economics of butyric acid production attractive.

There are two process integration methods that can be used to produce butyric acid from biomass: (i) separate hydrolysis and fermentation (SHF); and (ii) simultaneous saccharification and fermentation (SSF). In the first method, there are two separate unit operations called “hydrolysis” and “fermentation,” while in the second method there is only one unit operation called “simultaneous saccharification and fermentation”. In the latter operation, hydrolysis or saccharification will be conducted in the fermentation conditions. In fact, in the SSF process, the need for a hydrolysis reactor is eliminated. Elimination of the hydrolysis reactor reduces capital and operational costs, thus economizing the production

of butyric acid. It is also noteworthy that recycling water is essential, as these biological conversion processes require large amounts of water. The use of large amounts of water increases processing costs and adds to the cost of wastewater treatment after the recovery of butyric acid.

The traditional method of fermentation is a batch process which results in low productivity due to low cell concentration and hence cell concentration should be increased. Cell-recycling techniques include centrifugation to separate cells from the fermentation broth, membrane filtration, and settling the cells in the conical reactor. Recycling cells via centrifugation and membrane filtration is superior to settling the cells as cell-settling methods require an increased amount of time, thus decreasing the overall productivity [21]. After the separation of cells from the fermentation broth, they are returned to the bioreactor.

The objectives of this study include developing a simultaneous saccharification and fermentation process for butyric acid production using wheat straw as a feedstock (low-cost agricultural biomass), recycling water, and developing a cell-recycling process to improve reactor productivity. The cell recycling would be performed by employing centrifuge and membrane filtration and the wheat straw would be pretreated using hot water at 190 °C. The cell recycling productivities achieved in this work are the highest reported for butyric acid production using wheat straw hydrolysate. Simultaneous saccharification and fermentation of cellulosic biomass has been documented for butanol production and reported elsewhere [see Supplementary Material Paper I and II].

2. Materials and Methods

2.1. Micro-Organism, Propagation and Stock Solutions

Spores of lyophilized *Clostridium tyrobutyricum* ATCC 25755 were obtained from the Agricultural Research Service Culture Collection (NRRL, Peoria, IL, USA) and were propagated as described elsewhere [12]. Reinforced Clostridial Medium (RCM; Difco™, Becton Dickinson and Company, Sparks, MD, USA) was also prepared as described in the above article and stored at 4 °C. Glucose (Fisher Scientific, Fair Lawn, NJ, USA) and xylose (Sigma Chemicals St. Louis, MO, USA) solutions were prepared by dissolving 400 g each of the sugars in 1.0 L distilled water separately and filter-sterilized by passing through a 0.20 µm filter (Whatman International Ltd., Maidstone, UK) and the solutions were stored at 4 °C. When needed, known volumes of these solutions were aseptically transferred to inoculum preparation tubes or screw-capped bottles in a Biological Safety Cabinet (NUAIRE, Plymouth, MN, USA).

For inoculum preparation, 5–7 mL RCM was transferred to a plastic tube with a cap (Medical Caplugs, EVERGREEN Labware Products, Test Tube 17 × 100 mm 2-Pos Cap; Rancho Dominguez, CA, USA) and to this medium 250 µL of stock glucose solution was added, resulting in a glucose concentration of 14–20 g L⁻¹. This was followed by creating an anaerobic environment in the medium by sparging oxygen-free nitrogen gas at a flow rate of 30 mL min⁻¹ for 10 min. To prepare inoculum, 0.30 mL (300 µL) of spore suspension was transferred to a 1.5 mL sterile plastic microcentrifuge tube with a lid (Bio Plastic Inc., San Rafael, CA, USA) and the suspension was heat-shocked at 75 °C for 2 min on a heating block (Cole-Parmer, Chilling Heating block, Vernon Hills, IL, USA). Then, 100 µL of heat shocked suspension was added to the above tube, followed by incubation at 35–37 °C for approximately 36 h or until the cell growth was visible. The incubation was carried out in an anaerobic jar (BBL GasPak™, Sparks, MD, USA). When the strain was actively growing and producing fermentation gases, this indicated that the culture/strain was ready for inoculation in larger-volume medium (30 mL). The inoculum developed here was called stage I inoculum.

For stage II inoculum, 27.75 mL of RCM and 2.25 mL of glucose or xylose stock solutions (volume; 30 mL) were transferred to a sterile 50 mL Fisherbrand™ plastic tube and anaerobic conditions were created as described above. To generate anaerobic conditions, oxygen-free nitrogen gas flow rate was increased 4–5 fold compared to that listed above. This tube was inoculated with 3 mL of stage I inoculum and incubated at 35 °C for 16–18 h. For incubation, the tube was placed in an anaerobic jar (BBL, GasPak™). To prepare butyric acid production medium, specified concentrations of sugar solution and RCM (usually 2 fold) were autoclaved separately at 121 °C for 15 min and mixed when cooled to room temperature. For butyric acid production, anaerobic conditions were created by sparging oxygen-free nitrogen gas.

2.2. Control Fermentation

The control fermentation was performed in a 2.5 L reactor (BioFlo 2000 Fermenter, New Brunswick, NJ, USA) with 1.0 L working volume. To prepare the reactor, 100 g glucose was dissolved in 500 mL distilled water and transferred to the fermenter. Also, 38 g of RCM was dissolved in 500 mL distilled water and transferred to a 1.0 L Pyrex™ bottle, (Charlottesville, PA, USA). Both the fermenter containing glucose and the bottle containing RCM were autoclaved separately at 121 °C for 20 min and the two solutions were mixed when cooled to approximately 50 °C. The total volume in the reactor was 1.0 L with 100 g L⁻¹ glucose and 38 g L⁻¹ RCM. Anaerobic conditions were created by sparging oxygen-free nitrogen gas at a flow rate of 1.0 vvm (volume per unit volume per min) for 15 min. Upon cooling the reactor to 35 °C, 100 mL of stage II inoculum was added and agitation, pH, and temperature were controlled at 100 rpm, 6.5, and 35 °C, respectively. Samples (0.5–1.0 mL) were taken at regular intervals to measure sugar and acetic and butyric acid concentrations. A simple schematic diagram of this process is shown in Figure 1A.

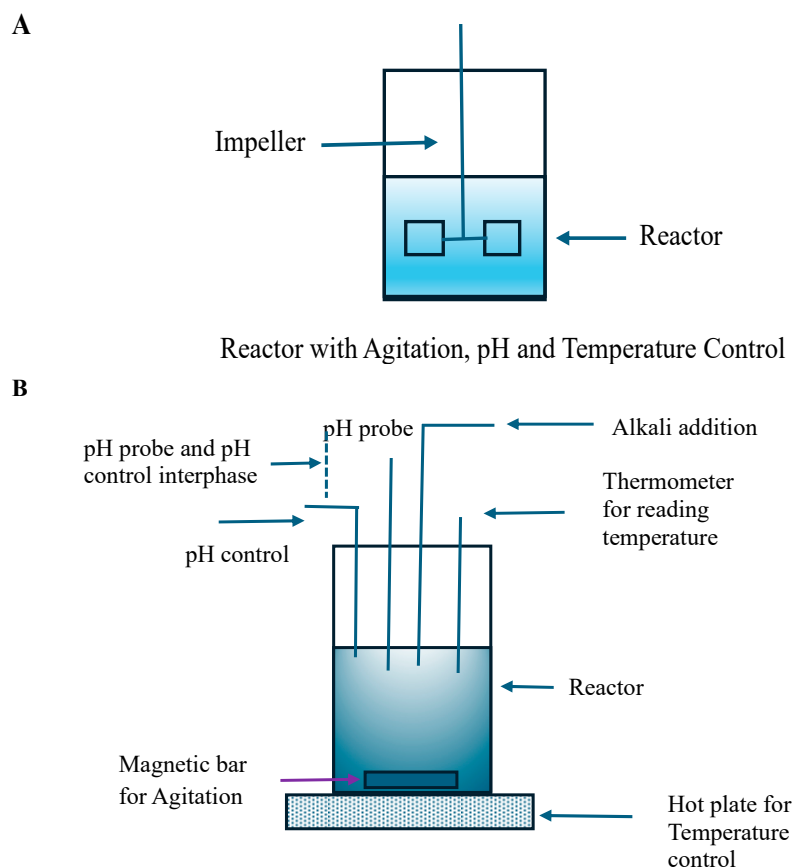


Figure 1. Cont.

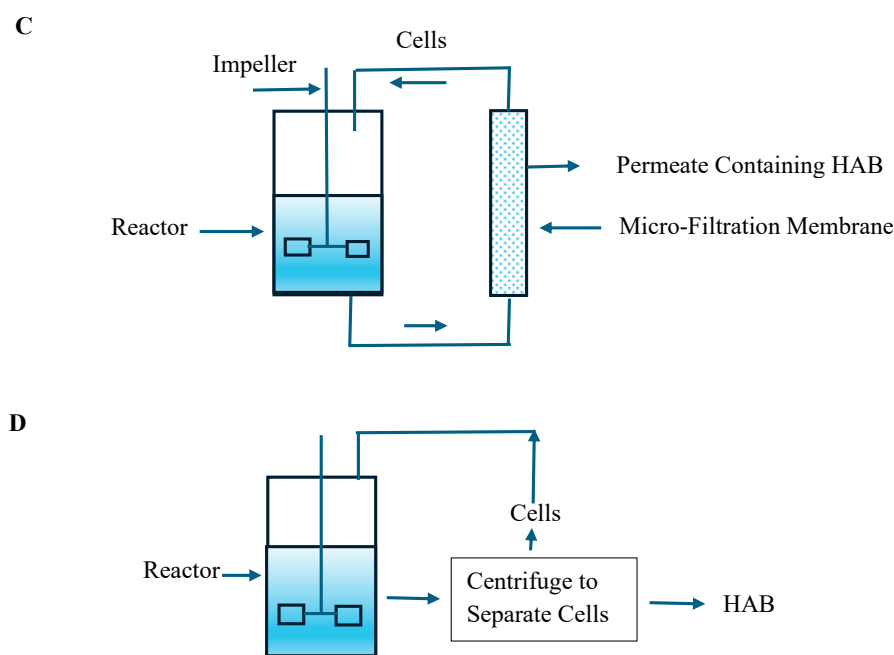


Figure 1. Schematic diagrams of production of butyric acid from glucose, wheat straw and wheat straw hydrolysate. (A) Reactor used to control fermentation (provided with agitation, pH and temperature control); (B) Reactor used for simultaneous saccharification and fermentation (SSF; included agitation, pH and temperature control); (C) Membrane cell-recycling fermentation (membrane reactor with agitation, pH and temperature control); (D) Cell recycling using centrifugation (provided with pH and temperature control; vigorous gas release caused adequate agitation).

2.3. Wheat Straw Pretreatment

Wheat straw was obtained from a local farmer (Manito, IL, USA); its details were provided in a previous article [12]. The straw's cultivars were FS 634 and FS 645, where FS stands for Farm Service. Prior to pretreatment, the straw was milled and sieved through a 1.27 mm screen. This was followed by making a suspension in distilled water by mixing 9 g of wheat straw with 91 mL of distilled water, which was then transferred to 200 mL stainless-steel reactor (Labomat BFA-12, Mathis USA Inc., Concord, NC, USA) followed by transferring them to the oven for heat pretreatment. The oven was programmed to heat up to 190 °C at a rate of 2.6 °C min⁻¹ followed by cooling to 35 °C at a rate of 6 °C min⁻¹. The holding time at 190 °C was zero min. This protocol was adapted because at these conditions, threshold concentrations of biomass inhibitors such as furfural, hydroxymethyl furfural (HMF), syringaldehyde, glucuronic acid, coumaric acid, ferulic acid, and phenolic acid [15] were not generated.

2.4. Wheat Straw Hydrolysis

When needed, 100 mL of pretreated wheat straw was transferred to a 250 mL screw-capped PyrexTM glass bottle (Charleroi, PA, USA) and the biomass hydrolytic enzymes were added followed by transferring the bottles to a rotating shaker at 150 rpm, 45 °C temperature, and 6.0 pH for 72 h. In these conditions the biomass was liquified and an adequate amount of sugars were released. For liquification or biomass hydrolysis, the following three enzymes were added at concentrations of 12 mL L⁻¹ biomass suspension and their activities were as follows: [(cellulase, Celluclast 1.5 L, enzyme activity 751 ± 35 U mL⁻¹; Sigma Chemicals St. Louis, MO, USA), (β-glucosidase, activity 380 ± 19 U mL⁻¹; Novozyme 188, Sigma Chemicals), (xylanase, activity 9837 ± 190 U mL⁻¹; Viscostar, Dyadic Corporation, Jupiter, FL, USA)].

2.5. Simultaneous Saccharification and Fermentation

For simultaneous saccharification and fermentation experiments, 9 g of wheat straw and 91 mL of distilled water were transferred to the Mathis reactor (Concord, NC, USA) and mixed well followed by pretreatment at the conditions described under the subheading “Wheat Straw Pretreatment”. After the reactor was cooled to 35 °C, the pH was adjusted to 6.0 and hydrolytic enzymes were added at the level mentioned above (12 mL L⁻¹; see the Section titled Wheat Straw Hydrolysis for details) and the contents were transferred to a 250 mL screw-capped PyrexTM glass bottle. Following this, 3.8 g RCM was added and the bottle was incubated at 35 °C (on a rotary shaker) at approximately 100 rpm for 12 h. After 12 h, the bottle was inoculated with 7–10% (*v/v*) of stage II inoculum and transferred to a hot plate to control agitation at approximately 100 rpm using a magnetic stirrer bar. Both pH and temperature were controlled at 6.0 (using 5M NaOH) and 35 °C, respectively. Fermentation was continued until glucose and xylose were completely utilized. From this bottle/reactor, 0.5–1.0 mL samples were taken, centrifuged at 12,000 rpm for 5 min (Eppendorf 5417C, Cole Parmer, Vernon Hills, IL, USA) and stored at −18 °C until they were needed for measuring sugars and acids. Figure 1B shows a schematic diagram of this set-up.

2.6. Water Recycling

Water recycling was performed by using the final fermentation broth from the SSF experiment. To recycle water, the liquid containing acids was separated from the fibrous particles by filtering it through a cheese cloth. Additionally, suspended particles were removed using a centrifuge (Sorvall LYNX 6000 Centrifuge, Thermo Scientific, Waltham, MA, USA) at 4000 rpm for 20 min. From the clear solution, acids were removed using XAD-4 resin. To prepare for the removal of acids, 60 g of XAD-4 was washed twice with 60 mL (each time) of 50% (*v/v*) methanol. This was followed by washing XAD-4 twice with 60 mL of milli-Q water each time. To remove acids, 50 mL of fermentation broth was treated with 50 g of XAD-4. After the removal of acids, the liquid was supplemented with sugars (total sugars approximately 100 g L⁻¹; glucose and xylose) and RCM. The individual sugars were glucose 58.59 g L⁻¹ and xylose 40.85 g L⁻¹. The RCM concentration in the separated liquid was 38 g L⁻¹. The solution containing broth, sugars and RCM was filter-sterilized using a 0.20 µm filter (Whatman). Fermentation was initiated by inoculating 3.5–5.0 mL (~ 7 to 10%, *v/v*) of stage II inoculum. The pH, agitation and incubation temperature were controlled at 6.5, 100 rpm and 35 °C, respectively. These studies were performed in a 100 mL PyrexTM glass bottle (Charlrooi, PA, USA) and the total volume in the reactor was approximately 65.0 mL.

2.7. Cell Recycling

For cell recycling, MidGee Hoop-type ultrafiltration membrane (hollow fiber cartridge; Cytiva.com) was supplied by Cytiva (Global Life Sciences Solutions, USA, LLC, Cytiva, Marlborough, MA, USA). The membrane made of polysulfone with 2 fibers and 16 cm² filtration area was used for this study. The membrane was sterilized by circulating 80–100 °C hot water for 2 h. The water flux at different inlet pressures is presented in Table 1. In order to concentrate cells in the reactor, cell mass was grown in a 1.0 L PyrexTM bottle anaerobically with 500 mL medium. After cell growth, fermentation medium was circulated through the membrane using a Masterflex peristaltic pump (Cole Parmer, Vernon Hills, IL, USA) and size 14 norprene tubing until a final volume of 40 mL was reached. The cells were returned to the reactor and permeate was discarded. A schematic diagram of this process is shown in Figure 1C.

Table 1. Rate of water permeation (flux) through the MidGee Hoop membrane at 25 °C. Water was circulated through the membrane at a flow rate of 2.09 L h^{−1}.

Inlet Pressure, [psi]	Outlet Pressure, [psi]	Permeate Side Pressure [psi]	Water Flux [mL 15 s ^{−1}]	Water Flux [L m ^{−2} h ^{−1}]
0.00	0.00	0.00	1.40	210
3.70	3.40	0.00	6.00	900
6.50	6.00	0.00	8.30	1245

For the same purpose (cell separation by centrifugation), a cell mass was grown in a 1.0 L PyrexTM glass bottle containing 500 mL medium. The cell mass was centrifuged (Sorvall LYNX 6000 centrifuge) aseptically in 200 mL bottles. The clear liquid was discarded and the cell mass was transferred to a 50 mL capacity sterile screw-capped plastic tube (Fisherbrand, Pittsburgh, PA, USA). To the cell mass, 20 mL wheat straw hydrolysate was transferred and the pellet was suspended in it. The total volume of the cell mass plus wheat straw hydrolysate was 26.15 mL (calculated volume). Within 10 min of adding hydrolysate, the fermentation became highly vigorous and significant amounts of gases started coming out. A schematic diagram of this process is presented in Figure 1D.

2.8. Use of a Low Concentration of RCM

In the above study, 38 g L^{−1} (3.8%) RCM was used. In order to economize further, an experiment was run with second fermentation without adding RCM. An initial concentration of glucose was 25.58 g L^{−1}. Fermentation was run for 50 h and during this time all the glucose was utilized, suggesting that a low concentration of RCM can be used.

2.9. Analyses

Samples were analyzed for sugar using a Thermo Ultimate HPLC with a BioRad 87P column. Details of the sugar, and cell concentration measurements were provided in our previous article [12]. For acid measurement, samples were analyzed using a Shimadzu HPLC, Bio-Rad 87H column, along with a Bio-Rad Carbo-H cartridge [12]. The productivity in batch systems was calculated as the acid concentration in g L^{−1} divided by the fermentation time and is presented as g L^{−1} h^{−1}. Fermentation time was the difference between inoculation and when fermentation ceased. The productivity in continuous fermentation conditions was calculated as the acid produced in g L^{−1} multiplied by dilution rate and is presented as g L^{−1} h^{−1}. Dilution rate is defined as feed flow rate in mL h^{−1} per unit reactor volume and is presented as h^{−1}. Yield was calculated as the total acids produced in g L^{−1} divided by sugar utilized in g L^{−1} and is presented as g g^{−1}. Specific productivity was calculated as productivity in g L^{−1} h^{−1} divided by dry weight cell concentration and is presented as h^{−1}. Dry cell weight was measured at 100 °C. This was performed by measuring the weight of a sample of cell suspension. To calculate cell concentration, a correlation was plotted between optical density (x) at λ₅₄₀ nm and g dry weight cell concentration (y) and the correlation is presented as: $y = 0.214x^3 - 0.159x^2 + 0.2588x - 0.0009$. Prior to measuring the optical cell density, the sample was diluted 10–20 fold. After obtaining the dry weight cell concentration of the diluted sample it was multiplied by the dilution factor to determine the actual cell concentration in the broth [12].

SAS 9.4 was used to perform an Analysis of Variance (ANOVA) to compare the various treatments in the cell-recycling fermentations at a significance level of $p \leq 0.05$. The two replicates of total acid production, in all the reactors, were compared to the glucose control using Duncan's multiple range test when wheat straw hydrolysate served as the feedstock. The acid produced using wheat straw hydrolysate was also compared with the glucose control using Duncan's mean separation test. The data presented in figures are an average of two replicates with standard deviation.

3. Results and Discussion

3.1. Control Fermentation

In order to compare the results of simultaneous saccharification and fermentation, a control experiment was run with approximately $100\text{--}105\text{ g L}^{-1}$ glucose in the feed. Over the course of fermentation, 8.45 g L^{-1} acetic acid and 32.3 g L^{-1} butyric acid were produced, thus totaling 40.75 g L^{-1} total acids (Figure 2A). The microbial strain resulted in a cell mass concentration of 2.88 g L^{-1} . These results are presented in Figure 2B. During this period of fermentation, a productivity and yield of $0.34\text{ g L}^{-1}\text{ h}^{-1}$ and $0.41\text{ g acids g}^{-1}\text{ sugar}$ were obtained, respectively (Table 2). Sugar in excess of approximately 105 g L^{-1} could not be used because over this concentration the strain experiences substrate inhibition. Also, the maximum acid tolerance of this strain is approximately $40\text{ to }45\text{ g L}^{-1}$ [12]. The specific productivity of the strain is a measure of efficiency of the microorganism. Using glucose as a substrate, this efficiency or specific productivity (for total acids) was 0.10 h^{-1} . In the current experiment a butyric acid selectivity of 0.79 was obtained while in the previous experiment [12] it was 0.82 . These selectivity values are comparable. The productivity and yield obtained in the control experiment, performed previously, were $0.23\text{ g L}^{-1}\text{ h}^{-1}$ and 0.41 g g^{-1} , respectively. Though the acid yields in the two experiments were the same, the productivities were different. The possible reason for the reduced productivity in the previously reported paper [12] may have been due to inhibition caused by the increased level of glucose (126.5 g L^{-1}). It should be noted that in the xylose-fed (117.1 g L^{-1}) reactor, a productivity of $0.23\text{ g L}^{-1}\text{ h}^{-1}$ was also obtained [12]. The fermentation parameters obtained when using glucose as a control are compared with SSF process when using wheat straw as a substrate (Table 2). In the SSF process a productivity of $0.25\text{ g L}^{-1}\text{ h}^{-1}$ and yield of 0.40 g g^{-1} were obtained. In the wheat straw SHF process, the butyric acid concentration, productivity, and yield were 22.15 g L^{-1} , $0.27\text{ g L}^{-1}\text{ h}^{-1}$, and 0.43 g g^{-1} , respectively [12].

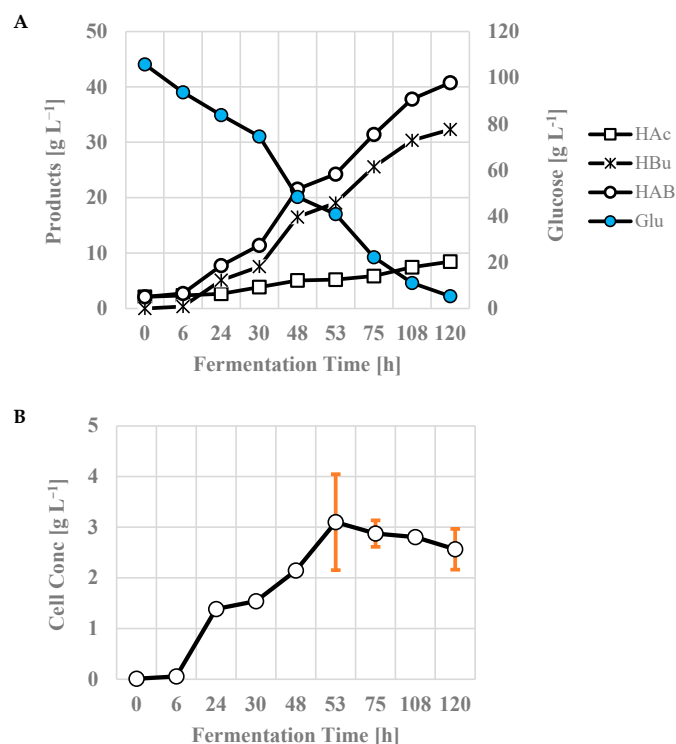


Figure 2. Production of butyric acid from glucose in a control batch reactor using *C. tyrobutyricum* ATCC 25755. (A) Fermentation time vs. glucose and products. (B) Fermentation time vs. cell concentration in the reactor. HAc—acetic acid, HBu—butyric acid, HAB—total acetic and butyric acids, Glu—glucose.

Table 2. Fermentation parameters of butyric acid production from sugars (glucose and glucose + xylose), and wheat straw biomass. Data on cell-recycling fermentations are also presented.

Fermentation Type and Feedstock	Total Acid Produced [g L ⁻¹]	Productivity [g L ⁻¹ h ⁻¹]	Acid Yield [g g ⁻¹]	Max Cell Conc [g L ⁻¹]	Specific Productivity [h ⁻¹]
^e Control, glu	40.8 ^a	0.34 ^c	0.41 ^a	2.9 ^c	0.10 ^b
WS biomass, SSF	20.8 ^b	0.25 ^c	0.40 ^a	-	-
^f Recycled ferm, glu + xyl	39.9 ^a	0.35 ^c	0.40 ^a	2.1 ^c	0.18 ^a
* Cell recycle membrane, WSH	9.8 ^c	2.93 ^a	0.42 ^a	37.5 ^b	0.08 ^c
Cell recycle centrifuge, WSH	18.0 ^b	2.58 ^b	0.45 ^a	46.7 ^a	0.05 ^d

Values sharing the same letter within a column are not significantly different ($p \leq 0.05$). - Could not be measured due to WS biomass suspended solids in fermentation broth. ^e glu—glucose. ^f glu and xyl—glucose and xylose. * Continuous fermentation at a dilution rate of 0.30 h⁻¹. WS—wheat straw; WSH—wheat straw hydrolysate.

The reader is advised that the initial substrate concentration in the control, and water-recycling experiments were about 100–105 g L⁻¹. This is because the strain cannot produce more than 40–45 g L⁻¹ acid from approximately 100–105 g L⁻¹ glucose. Use of greater than this amount of sugar would result in the presence of residual sugar and slow fermentation due to glucose inhibition. In our SSF process, a wheat straw concentration of 90 g L⁻¹ was used, which contained 54.1 g L⁻¹ sugars. Use of greater than 90 g L⁻¹ wheat straw may have caused inhibition. To avoid this inhibition, it is suggested that a fed-batch reactor be used for future studies. It is also suggested that the two systems (control and SSF) should not be compared in terms of their final acid concentration.

3.2. Pretreatment of Wheat Straw

The ultimate purpose of this study is to produce butyric acid from lignocellulosic biomass and for that reason wheat straw was used as a substrate. It is essential to pretreat and hydrolyze the biomass as this microbial strain is not capable of enzymatic hydrolysis. Usually, pretreatment is performed using chemicals such as ammonia, alkali and acids. It is noted that the use of these chemicals is not environmentally friendly. The use of ammonia, alkali such as NaOH and KOH and acids like HCl and H₂SO₄ results in the generation of fermentation inhibitors that severely inhibit fermentation. For these reasons we developed our pretreatment method that uses hot water. This pretreatment method heats up the wheat straw and water to 190 °C followed by cooling them to room temperature [12]. It should be noted that the hydrolysis method requires different pH and temperature conditions than the fermentation. Hydrolysis requires a pH of 5.0 and temperature of 45 °C while butyric acid fermentation needs to be operated at pH of 6.5 and temperature of 35 °C. In order to check that the strain was not deficient in sugars, it was essential to perform wheat straw hydrolysis at an approximate pH of 6.0 and temperature of 35 °C. The results of this hydrolysis are presented in Figure 3. The hydrolysis was performed for 72 h and at the end of this period a sugar concentration of 27.56 g L⁻¹ had accumulated in the hydrolysis reactor. It is noteworthy that within 4 h of hydrolysis, the culture accumulated 11.21 g L⁻¹ total sugars. At 12 h of hydrolysis the concentration of total sugars in the reactor was 18.63 g L⁻¹. This experiment suggested that the average rate of sugar generation was 1.55 g L⁻¹ h⁻¹, while average rate of sugar utilization in the fermenter was 0.83 g L⁻¹ h⁻¹. The rate of sugar generation was approximately two fold of sugar utilization. Since the rate of sugar generation was faster than its utilization, it was ascertained that the strain was not deficient in sugars.

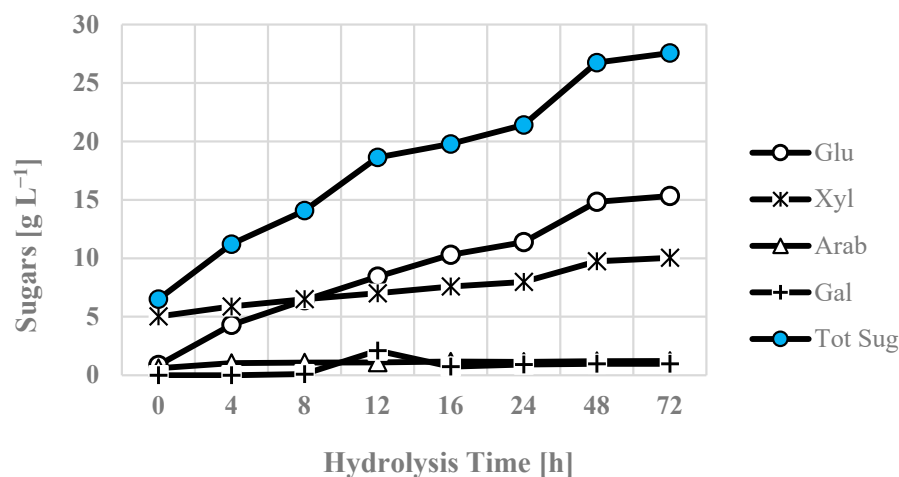


Figure 3. Hydrolysis of WS using enzymes in a batch process under fermentation conditions (pH 6.0, and temperature 35 °C). Glu—glucose, Xyl—xylose, Arab—arabinose, Gal—galactose, Tot Sug—total sugar.

3.3. Simultaneous Saccharification and Fermentation

To run fermentation with simultaneous saccharification, the enzymatic hydrolysis/saccharification was performed for 12 h and at that time the culture was inoculated with the microbial strain or inoculum. At the initiation of the fermentation, the sugar concentration in the reactor was 20.85 g L⁻¹. The butyric acid fermentation profile in the reactor is shown in Figure 4A. At 17 h of fermentation the strain produced 4.92 g L⁻¹ total acids. The fermentation was operated for 84 h, as evidenced by the release of fermentation gases, and at that time fermentation ceased. At the end of fermentation, 20.80 g L⁻¹ total acids had accumulated in the reactor. As reported in Figure 4B, the sugar profile in the reactor suggests that the culture was not deficient in sugars until 72 h when the total sugar was 3.92 g L⁻¹. At the end of fermentation (84 h) there was 2.10 g L⁻¹ unused sugar. This experiment suggested that simultaneous saccharification and fermentation was successful. During this fermentation process, 90 g L⁻¹ wheat straw was used. Based on mass balance 90 g L⁻¹ wheat straw had 54.05 g L⁻¹ total sugars of which 2.10 g L⁻¹ were unused and the yield was 0.40 g g⁻¹. This yield is comparable to the control experiment's yield of 0.41. The yield based on the biomass was 0.23 g g⁻¹ WS. From the sugar profile in this experiment, the strain did not use arabinose and galactose (Figure 4B). There are three options to utilize unused sugar: (i) dispose of final fermentation broth in the water streams; (ii) perform anaerobic digestion of the waste stream to produce methane gas; and (iii) mutate the butyric acid-producing strain so that it can use all the sugars. Disposal of residual sugar containing broth is not environmentally friendly and would cause environmental pollution. For this reason, disposal via streams is not favorable. Anaerobic digestion is also disregarded as it may require large anaerobic tanks and may not be economical. Hence, it is suggested that the microbial strain should be adapted or engineered to use these sugars. Use of a butyric acid-producing engineered strain to ferment all the sugars would be more economically viable as it would result in a higher butyric acid concentration and higher acid yield. In this SSF experiment a productivity of 0.25 g L⁻¹ h⁻¹ was achieved (Table 2). This productivity is 26% lower than the control experiment. The main reasons for the lower productivity include low wheat straw sugar concentration and possibly inhibition due to biomass. We were not able to measure the cell concentration due to the presence of fibrous biomass in the reactor and the samples.

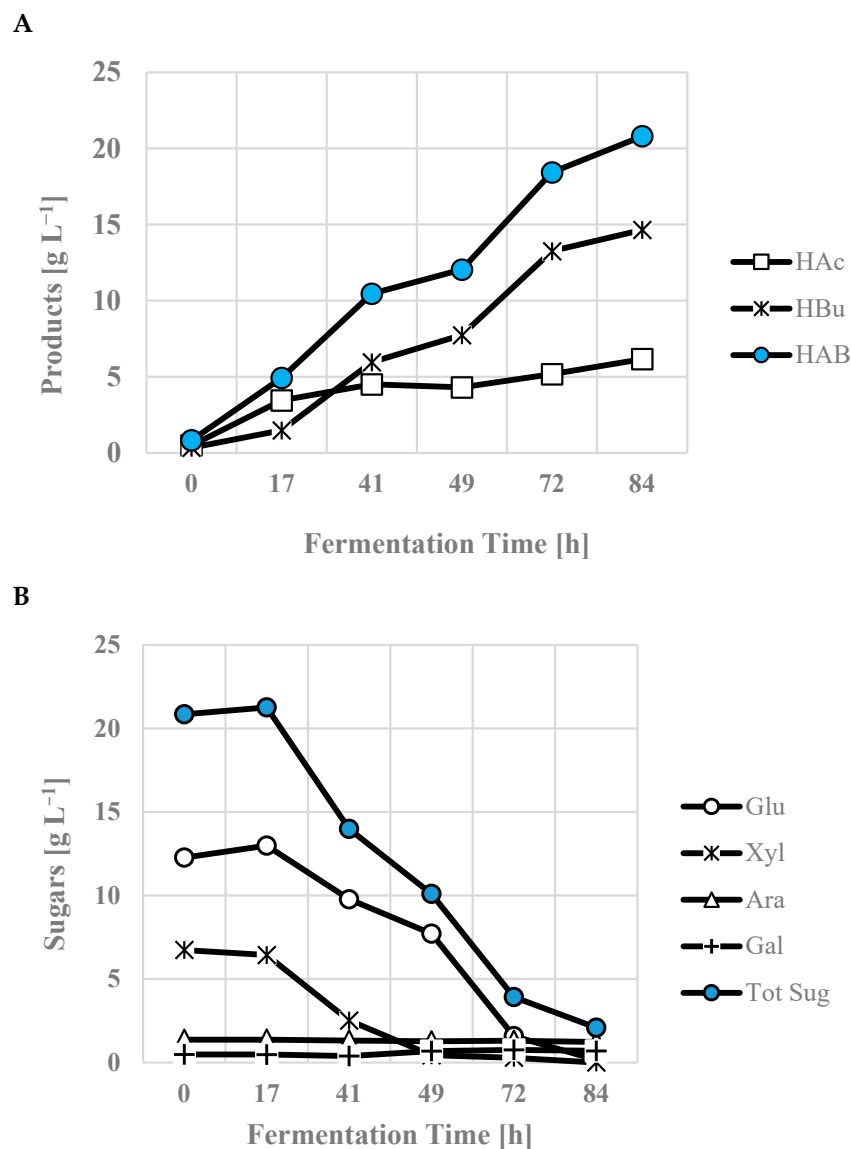


Figure 4. Simultaneous saccharification, and fermentation of wheat straw in a batch reactor employing *C. tyrobutyricum* ATCC 25755 to produce butyric acid. (A) Fermentation time vs. acids. (B) Fermentation time vs. sugars.

There were several suggested reasons as to why fermentation may have ceased before all the sugars were used, including: (i) inhibition due to product, (ii) nutrient limitation, (iii) loss of microbial activity, (iv) the availability of insufficient sugars, (v) and any other reason that could be attributed to the presence of toxic chemicals in the wheat straw or wheat straw hydrolysate. Inhibition due to the final product (acid) was disregarded as the strain can tolerate up to 45 g L⁻¹ butyric acid. In the current experiment, 20.80 g L⁻¹ acids were present, which is less than 50% of the amount this strain can tolerate. Nutrient limitation was also disregarded as the fermentation was started with a high RCM concentration of 38 g L⁻¹. Loss of microbial activity, availability of insufficient sugars, and presence of toxic chemicals in the biomass hydrolysate are valid reasons and will be investigated in future, as stated in the Conclusions Section.

In some clostridial fermentations such as *C. saccharobutylicum* P262, there are four types of cells, namely (i) growing cells; (ii) solvent- or product-forming cells; (iii) cells that neither form product nor grow but still require maintenance energy; and, (iv) dead cells [22]. The exact ratio of these types of cells varies from strain to strain. It is likely that in

C. tyrobutyricum ATCC 25755 fermentation, similar phenomena may occur. It appears that nearly all the cells are actively participating in product formation or fermentation elicitors may be present. For that reason a high specific productivity of 0.18 h^{-1} was obtained. In the control fermentation a specific productivity of 0.10 h^{-1} was obtained.

In the SSF process, the dynamics of generation of sugars and their utilization has been changing. In this system, the sugars are generated and simultaneously used to produce acids. The profiles of sugars during the hydrolysis are presented in Figure 4B. As can be seen in this figure, the strain was never deficient in sugars except when fermentation ended. During the SSF process, estimation of cell mass by optical density (OD) measurement was not possible, as wheat straw residues interfered with OD measurement. An alternative to this is enumerating the cell numbers or colony-forming units on agar plates. However, this is an approximation, as growing or colony-forming units may differ from acid-producing cells.

3.4. Water Recycling

Next, an experiment was performed to find out whether water or fermentation broth can be recycled. This could not be done until acids were removed from the fermentation broth because the maximum tolerance of the strain was approximately $40.0\text{--}45.0 \text{ g L}^{-1}$. The sediments were removed from the fermentation broth by centrifugation followed by the removal of acids by XAD-4 adsorbent resin. In the beginning of the experiment, the fermentation broth contained 6.16 g L^{-1} acetic acid, 14.65 g L^{-1} butyric acid (total acids 20.81 g L^{-1} ; Table 2). After recovery of acids from the broth, the residual acid concentrations were 0.98 g L^{-1} acetic acid and 0.48 g L^{-1} butyric acid (1.46 g L^{-1} total acids; Table 3). Then cellulosic sugar solution was added to the reactor. Figure 5A presents the fermentation profile of the recycled broth. Fermentation was run until the strain ceased acid production, as evidenced by the slow release of fermentation gases. The culture produced 39.88 g L^{-1} total acids of which 4.06 g L^{-1} was acetic acid and 35.82 g L^{-1} butyric acid (butyric acid selectivity 0.90). The maximum cell concentration was 2.05 g L^{-1} at 66 h, which is 29% lower than the control experiment. After XAD-4 treatment, two phenomena were observed: (i) reduced cell mass growth and (ii) increased fermentation activity. It is likely that XAD-4 treatment leached out some unknown chemicals that inhibited cell growth and enhanced specific fermentation activity. Fermentation took 114 h to complete with a productivity of $0.35 \text{ g L}^{-1} \text{ h}^{-1}$ (Table 2). This productivity is comparable to the control run's productivity of $0.34 \text{ g L}^{-1} \text{ h}^{-1}$. The sugar concentrations at the beginning and end of fermentation were 99.44 and 0.60 g L^{-1} , respectively (Figure 5B). Our previous study reported that *C. tyrobutyricum* ATCC 25755 [12] utilizes glucose and xylose simultaneously (Figure 5B) which is further confirmed by the current study.

Table 3. Concentrations of acids and sugars before and after removal of acids from fermentation broth by XAD-4.

Process	HAc	HBu	HAB	Glu	Xyl	Total Sugar
Before XAD-4 treatment, g L^{-1}	6.16	14.65	20.81	0.16	0.00	0.16
After XAD-4 treatment, g L^{-1}	0.98	0.48	1.46	0.00	0.00	0.00

HAc—acetic acid; HBu—butyric acid; HAB—total acetic and butyric acid; Glu—glucose; Xyl—xylose.

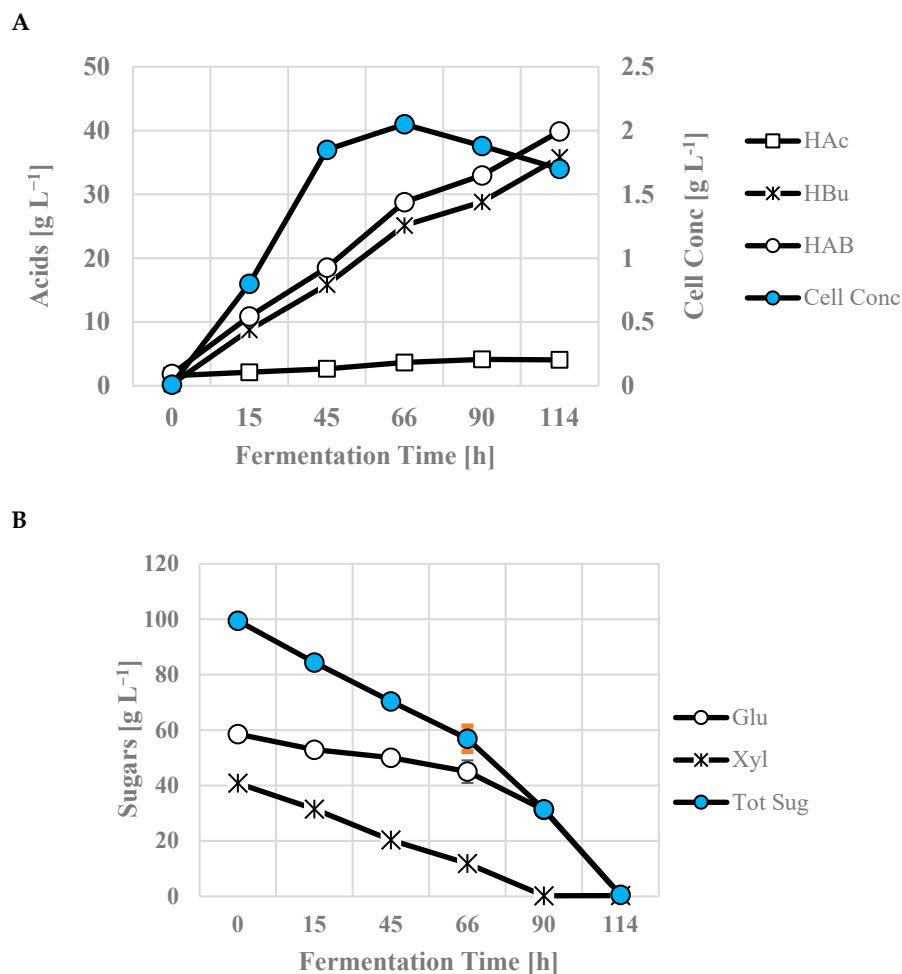


Figure 5. Production of butyric acid from cellulosic sugars and recycled water/fermentation broth. (A) Fermentation time vs. acids and cell concentration. (B) Fermentation time vs. sugars.

3.5. Cell Recycling

In the batch reactor, the total acid productivities were 0.25 to 0.35 g L⁻¹ h⁻¹. These productivities are low for commercial fermentation and hence require improvement. To improve productivity, cell immobilization and membrane cell-recycling techniques are the two important methods. The immobilized cell techniques include adsorption/adhesion, entrapment, encapsulation, and covalent bond formation [23]. Adsorption can be performed inside the reactor on solid supports such as bone-char, glass beads and glass wool. This process (adsorption) does not require any chemicals and studies have been conducted on the use of immobilized cell reactors such as a fibrous bed reactor for butyric acid production [24,25]. Entrapment can be performed by cell inclusion in gel such as calcium alginate. Covalent bond formation requires the use of chemicals that form a bond with the cell; cell support and the bonding process may inactivate cells. Another immobilization method used to increase the cell concentration in the reactor is self-aggregation or the use of a flocculent microbial strain [26]. Cell immobilization can lead to substrate, nutrient, and product diffusion barriers and hence has some limitations. Membrane or cell recycling is a unique technique and can be used in numerous fields [27]. Cell recycling in fermentation [21,28,29] is a method in which a cell concentration in the range of 20–50 g L⁻¹ can be achieved. This technique has not been applied to produce butyric acid from agricultural biomass hydrolysates.

There are three methods of cell recycling, including: (i) growing cells in a reactor followed by concentrating them by centrifugation and transferring to a smaller size reactor,

(ii) using a membrane to concentrate cells, and (iii) settling in a conical tank and returning to the reactor. In a non-cell-recycling batch reactor, a cell concentration of the order of $2.5\text{--}3.0\text{ g L}^{-1}$ is achieved, which can be increased >10 fold by cell recycling. For this study, the first method of cell concentration by centrifugation was applied because it is simple and does not require a membrane, which can increase the process cost. A cell concentration of 46.7 g L^{-1} was achieved which resulted in a reactor productivity of $2.58\text{ g L}^{-1}\text{ h}^{-1}$. The reactor was fed with wheat straw hydrolysate and acid productivity increased 8–10 fold. Cell recycling was performed to improve butyric acid productivity by increasing the cell concentration in the reactor. It should be noted that the fermentation time was reduced from 84 h to only 7 h. Figure 6A shows the fermentation profile of butyric acid production from wheat straw hydrolysate and Figure 6B shows sugar utilization by the centrifuged cells. At the end of fermentation, all sugars except arabinose were utilized by the strains. In these cell-recycling experiments, acid yields were improved because the sugars were not utilized for cell growth. Rather, sugars were directed to product formation and this was the reason for the improved yields.

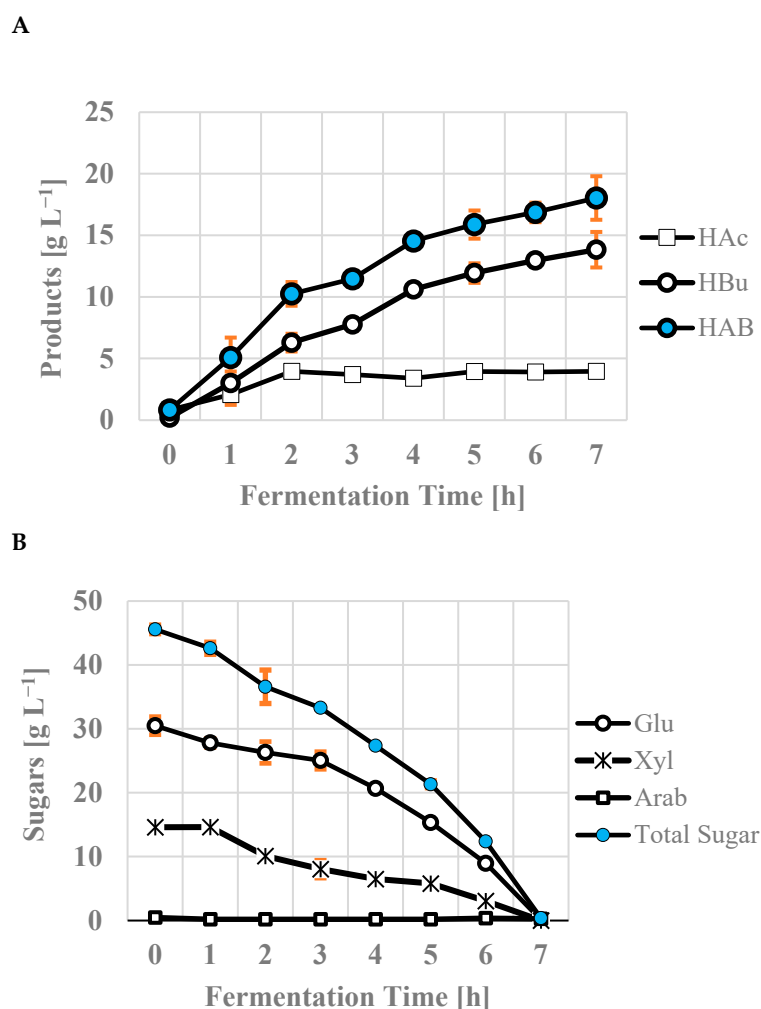


Figure 6. Production of butyric acid from wheat straw hydrolysate in cell recycle reactors. (A) Fermentation time vs. products. (B) Fermentation time vs. sugars.

The membrane cell recycle reactor was operated at a single dilution rate of 0.30 h^{-1} , which resulted in a productivity of $2.93\text{ g L}^{-1}\text{ h}^{-1}$. This dilution rate was chosen because in our *C. acetobutylicum* P262 (since renamed as *C. saccharobutylicum* P262) continuous fermentations, this was the lowest and adequate dilution rate [30]. *C. saccharobutylicum* and

C. tyrobutyricum are similar strains and hence we chose a dilution rate of 0.30 h^{-1} . In the current reactor a cell concentration of 37.5 g L^{-1} was obtained. It should be noted that in the centrifuged cell fermentation, a lower productivity was reported than that achieved during membrane cell recycling even though the cell concentration in the centrifuged cell fermentation was higher. The reason behind this discrepancy was that in centrifuged cell fermentations, some cells formed flocks or self-aggregates which created sugar and nutrient diffusional problems, thus resulting in lower productivity. Also in actual practice, the centrifuged cell batch fermentation would have lag and down time which would cause reduced productivity. Concentrating cells by settling is beyond the scope of this strain because under active stages of fermentation, cells do not settle; rather, they float and do not suspend in the fermentation broth.

In the above study RCM was used at a concentration of 38 g L^{-1} , which is high. Hence, an attempt was made to check if a second fermentation process could be performed without RCM supplementation. The concentration of glucose was adjusted to 25.6 g L^{-1} and fermentation was run (Figure S1; see the Supplementary Material). The culture initiated vigorous fermentation and in 50 h, all the glucose was used. In this fermentation, 11.0 g L^{-1} of total acids were produced with a productivity of $0.22\text{ g L}^{-1}\text{ h}^{-1}$ and acid yield of 0.43 g g^{-1} . This productivity is comparable to wheat straw's SSF productivity. The low initial glucose was adjusted because a high concentration of glucose exerts osmotic pressure on the culture and, hence, leads to cell growth inhibition. This study was successful because we were able to produce butyric acid from wheat straw using simultaneous saccharification alongside a fermentation technique. Also, the water used was recycled, and increased acid productivity (8–10 fold or 800–1000%; Table 2) was achieved.

During the recovery of acids using XAD-4 resin, it was noticed that the amount of glucose reported before recovery was 0.16 g L^{-1} . After recovery it was 0.0 g L^{-1} , suggesting that the resin adsorbed glucose. To avoid glucose adsorption, fermentation should be run until all the glucose is used by the culture. In our previous studies, lactose was used as a substrate [31] and lactose adsorption did not occur. In the study conducted by Ennis et al. [31], a molecular sieve and adsorbent resin were used to remove fermentation products (acetone, butanol and ethanol) of *C. acetobutylicum* P262. Alternately, it is suggested that XAD-7 and XAD-16 should be tested in our butyric acid recovery fermentations. Zeolites and XAD resins are important adsorbents and have the potential to be used at industrial scale [32]. Though using cellulosic biomass offers challenges, recent advances in pretreatment, saccharification and fermentation have shown potential [33]. Recent studies on the use of corn and cellulosic biomass [34,35] demonstrate that using these feedstocks in combination with science and technology's cutting edge can meet the economic challenges. New data on the economics of producing butyric acid using these feedstocks and fermentation route suggest that butyric acid can be produced at a price competitive to petroleum produced butyric acid [34,35]. Although no economic analysis is presented in the current article, a new article that discusses this has been submitted to the Fermentation journal [35]. This newly submitted paper involves economic analysis of wheat straw to butyric acid conversion. In our batch fermentations, cell concentrations of the order of $2.1\text{--}2.9\text{ g L}^{-1}$ were obtained; these concentrations are low. Hence, this strain should be adapted to increase cell growth, butyric acid tolerance, and production as the other strains were improved using a similar approach [36].

The specific productivities obtained in various fermentations are compared (Table 2) with the control run. In the control fermentation, in which glucose was used as a substrate, a specific productivity of 0.10 h^{-1} was obtained. In the fermentation process in which water was recycled, a specific productivity of 0.18 h^{-1} was achieved. However, in the case of cell-recycling fermentation, in which a membrane was used, a specific productivity of

0.08 h⁻¹ was obtained. This suggested that recycling affected the cells negatively. At this stage, it is not clear why the cells were affected negatively.

One of the possibilities could be the use of the peristaltic pump and tubing that was used to circulate the fermentation broth. Peristaltic pump rotors press against the tubing carrying cell broth and this may have inactivated some cells under pressure. Table 4 compares butyric acid production from various cellulosic feedstocks, including corn stover, wheat straw, paper mill sludge, recycled paper hydrolysate and wheat straw hydrolysate. Our SSF process results on butyric acid concentration and yield are comparable to the results obtained by other authors (Table 4). The productivities obtained in our wheat straw hydrolysate experiments are superior to those of other authors.

Table 4. A comparison of production of butyric acid from cellulosic biomass.

Microorganism	Substrate	Reactor Configuration	Butyric Acid Conc. [g L ⁻¹]	Yield [g g ⁻¹]	Productivity [g L ⁻¹ h ⁻¹]	Ref.
<i>C. tyrobutyricum</i> RPT-4213	Corn stover, wheat straw	Batch	7.15–10.40	0.39–0.48	0.16–0.22	[37]
<i>C. tyrobutyricum</i> RPT-4213	Paper mill sludge	Batch	0.7–8.00	0.36	0.19	[38]
<i>C. tyrobutyricum</i> DSMZ 2637	Wheat straw hydrolysate	Batch	19.00–20.00	0.30–0.45	-	[39]
<i>C. tyrobutyricum</i> ZJU 8235	Recycled paper hydrolysate	Batch stirred tank	10.26 ± 0.43	0.36	0.29	[24]
<i>C. tyrobutyricum</i> ATCC 25755	Wheat straw, SSF	Batch integrated	20.80 ^A	0.40 ^A	0.25 ^A	This work
<i>C. tyrobutyricum</i> ATCC 25755	Wheat straw hydrolysate	Continuous cell recycle	9.80–18.00 ^A	0.42–0.45 ^A	2.58–2.93 ^A	This work

SSF—Simultaneous saccharification and fermentation. A—these parameters were taken from Table 2.

The objectives of this study were to develop a simultaneous saccharification and fermentation process for producing butyric acid from wheat straw, water or recycled fermentation broth, and to develop a cell-recycling technique to produce butyric acid with high productivity. These processes have been developed. The productivity was improved by 8–10 fold using a filtration membrane. At a dilution rate of 0.30 h⁻¹, the residence time was 3.33 h. The membrane was operated for 72 h (21 residence times) in continuous mode. At the end of the fermentation process, the permeate flux was found to be 232.5 L m⁻² h⁻¹ (inlet pressure 4 psi and outlet pressure 0.0 psi) (see Supplementary Table S1). At this inlet pressure the permeate flux was normal, however, at an inlet pressure of 5.0 psi it was fouled. After completion of the cell-recycling membrane study, the membrane was cleaned with water.

4. Conclusions

In this study, butyric acid was produced from wheat straw in an integrated fermentation that combined simultaneous saccharification and fermentation in a single unit operation. Use of wheat straw as a feedstock (under the integrated fermentation) produced 20.8 g L⁻¹ total acids from 90 g L⁻¹ wheat straw, with butyric acid as the predominant product. In this SSF system, a productivity and yield of 0.25 g L⁻¹ h⁻¹ and 0.40 g g⁻¹ were obtained, respectively. These results of developing a SSF process for the conversion of wheat straw to butyric acid are highly encouraging. In future, this concentration of acid would be increased by processing higher wheat straw concentration, perhaps in the range of 150–160 g L⁻¹. It is expected that such a high concentration of wheat straw would result in the production of approximately 40 g L⁻¹ acid, which is comparable to the amount of acid production in the control experiment. Fermentation parameters such as productivity

and yield would also be increased. Increased production of acids would result in economic recovery of the product. Also, improved fermentation parameters would benefit process economics. Furthermore, the acid concentration would be increased by using a fed-batch reactor. In the SSF experiment, 2.10 g L⁻¹ sugars (arabinose and galactose) remained unutilized. The possible reasons for the presence of these residual sugars include loss of microbial activity, insufficient sugar availability, and the presence of toxic chemicals in the biomass hydrolysate. Use of these residual sugars would increase the product concentration and yield and would benefit the process economics of producing butyric acid from agricultural residues. Moreover, we successfully recycled water or fermentation broth from the above SSF process. It was recycled after removing butyric acid from the above reactor by XAD-4 adsorbent resin. Fermentation of recycled water resulted in an acid concentration, productivity and yield of 39.88 g L⁻¹, 0.35 g L⁻¹ h⁻¹, and 0.40 g g⁻¹, respectively. The results from all of the above experiments can be compared with the control fermentation in which 40.8 g L⁻¹ acids were produced from 100–105 g L⁻¹ glucose with a productivity and yield of 0.34 g L⁻¹ h⁻¹ and 0.41 g g⁻¹, respectively. In separate experiments, productivity was improved 8–10 fold (2.58 to 2.93 g L⁻¹ h⁻¹) when cells were recycled by employing centrifuge or membrane with wheat straw hydrolysate as a feed medium. This is the highest productivity reported for butyric acid produced from this substrate.

Subheadings: Subheadings presented in Materials and Methods section are related to procedures and in the Results and Discussion section they are related to Results and Discussion.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/fermentation12100477/s1>, Figure S1. Second fermentation of recycled broth after removal of acids by XAD-4 resin. For the second fermentation, no RCM was added to the fermentation medium. In 50 h of fermentation all the sugar was used. Glu—glucose. Table S1. Water flux study using Cytiva membrane at 25 °C after using the membrane for cell recycle experiment. Rate of circulation of water was 2.09 L·h⁻¹. The flux at inlet pressure of 5 psi suggested that the membrane was fouled by the fermentation broth.

Author Contributions: N.Q.—conception, running experiments and preparing the manuscript; T.C.E.—writing and reviewing manuscript; H.H.—writing and reviewing the manuscript; and R.E.H.—supervision of the project and reviewing the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: Financial support was provided by the United States Department of Agriculture, Agricultural Research Service (CRIS Number 5010-30600-008-00D).

Institutional Review Board Statement: This study did not involve animals or humans.

Data Availability Statement: All data generated in this study are presented in this paper.

Acknowledgments: N.Q. would like to thank Stephanie Thompson and Sarah Frazer for providing excellent technical assistance. N.Q. would like to thank Mukti Singh (USDA, ARS, Peoria, IL, USA) for performing the statistical analysis.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Jiang, L.; Fu, H.; Yang, H.K.; Xu, W.; Wang, J.; Yang, S.T. Butyric acid: Applications and recent advances in its bioproduction. *Biotechnol. Adv.* **2018**, *36*, 2101–2117. [[CrossRef](#)] [[PubMed](#)]
2. Dwidar, M.; Park, J.-Y.; Mitchell, R.J.; Sang, B.-I. The future of butyric acid in industry. *Sci. World J.* **2012**, 471417. [[CrossRef](#)] [[PubMed](#)]
3. Watson, R.; Wright, C.J.; McBurney, T.; Taylor, A.J.; Linforth, R.S.T. Influence of harvest date and light integral on the development of strawberry flavour compounds. *J. Exp. Bot.* **2002**, *53*, 2121–2129. [[CrossRef](#)] [[PubMed](#)]

4. Chen, Z.-X.; Breitman, T.R. Tributyrin: A prodrug of butyric acid for potential clinical application in differentiation therapy. *Cancer Res.* **1994**, *54*, 3494–3499. [[PubMed](#)]
5. Williams, E.A.; Coxhead, J.M.; Mathers, J.C. Anti-cancer effects of butyrate: Use of microarray technology to investigate mechanisms. *Proc. Nutr. Soc.* **2003**, *62*, 107–115. [[CrossRef](#)] [[PubMed](#)]
6. Rephaeli, A.; Zhuk, R.; Nudelman, A. Prodrugs of butyric acid from bench to bedside: Synthetic design, mechanisms of action, and clinical applications. *Drug Dev. Res.* **2000**, *50*, 379–391. [[CrossRef](#)]
7. Pouillart, P.R. Role of butyric acid and its derivatives in the treatment of colorectal cancer and hemoglobinopathies. *Life Sci.* **1998**, *63*, 1739–1760. [[CrossRef](#)] [[PubMed](#)]
8. Stein, U.H.; Wimmer, B.; Ortner, M.; Fuchs, W.; Bochmann, G. Maximizing the production of butyric acid from food waste as a precursor for ABE-fermentation. *Sci. Total Environ.* **2017**, *598*, 993–1000. [[CrossRef](#)] [[PubMed](#)]
9. Rozsenberszki, T.; Kalauz-Simon, V.; Fejes, R.; Bakonyi, P.; Kook, L.; Kurdi, R.; Nemestothy, N.; Belafi-Bako, K. Carboxylic acid production from kitchen waste and sewage sludge digestate inoculation via acidogenic fermentation at high organic load. *Waste Biomass Valoriz.* **2025**, *16*, 3733–3743. [[CrossRef](#)]
10. He, G.-Q.; Kong, Q.; Chen, Q.-H.; Ruan, H. Batch and fed-batch production of butyric acid by *Clostridium butyricum* ZJUCB. *J. Zhejiang Univ. Sci.* **2005**, *6*, 1076–1080. [[CrossRef](#)] [[PubMed](#)]
11. Qureshi, N.; Liu, S.; Saha, B.C. Butyric acid production by fermentation: Employing potential of the novel *Clostridium tyrobutyricum* strain NRRL 67062. *Fermentation* **2022**, *8*, 491. [[CrossRef](#)]
12. Qureshi, N.; Ashby, R.D.; Nichols, N.N.; Hector, R. Novel technologies for butyric acid fermentation: Use of cellulosic biomass, rapid bioreactor, and efficient product recovery. *Fermentation* **2024**, *10*, 142. [[CrossRef](#)]
13. Wang, L.; Chauliac, D.; Moritz, B.E.; Zhang, G.; Ingram, L.O.; Shanmugam, K.T. Metabolic engineering of *Escherichia coli* for the production of butyric acid at high titer and productivity. *Biotechnol. Biofuels* **2019**, *12*, 62. [[CrossRef](#)] [[PubMed](#)]
14. Jiang, L.; Wang, J.; Liang, S.; Wang, X.; Cen, P.; Xu, Z. Production of butyric acid from glucose and xylose with immobilized cells of *Clostridium tyrobutyricum* in a fibrous-bed bioreactor. *Appl. Biochem. Biotechnol.* **2010**, *160*, 350–359. [[CrossRef](#)] [[PubMed](#)]
15. Ezeji, T.C.; Qureshi, N.; Blaschek, H.P. Butanol production from agricultural residues: Impact of degradation products on *Clostridium beijerinckii* growth and butanol fermentation. *Biotechnol. Bioeng.* **2007**, *97*, 1460–1469. [[CrossRef](#)] [[PubMed](#)]
16. Oh, H.W.; Lee, S.C.; Woo, H.C.; Kim, Y.H. Energy-efficient recovery of fermented butyric acid using octyl acetate extraction. *Biotechnol. Biofuels Bioprod.* **2022**, *15*, 46. [[CrossRef](#)] [[PubMed](#)]
17. Fu, H.; Wang, X.; Sun, Y.; Yan, L.; Shen, J.; Wang, J.; Yang, S.-T.; Xiu, Z. Effects of salting-out and salting-out extraction on the separation of butyric acid. *Sep. Purif. Technol.* **2017**, *180*, 44–50. [[CrossRef](#)]
18. Zaman, N.K.; Law, J.Y.; Chai, P.V.; Rohani, R.; Mohammad, A.W. Recovery of organic acids from fermentation broth using nanofiltration technologies: A review. *J. Phys. Sci.* **2017**, *28*, 85–109. [[CrossRef](#)]
19. Chun, J.; Choi, O.; Sang, B.-I. Enhanced extraction of butyric acid under high-pressure CO₂ conditions to integrate chemical catalysis for value-added chemicals and biofuels. *Biotechnol. Biofuels* **2018**, *11*, 119. [[CrossRef](#)] [[PubMed](#)]
20. Wu, Z.; Yang, S.-T. Extractive fermentation for butyric acid production from glucose by *Clostridium tyrobutyricum*. *Biotechnol. Bioeng.* **2003**, *82*, 93–102. [[CrossRef](#)] [[PubMed](#)]
21. Du, J.; McGraw, A.; Lorenz, N.; Beitle, R.R.; Clausen, E.C.; Hestekin, J.A. Continuous fermentation of *Clostridium tyrobutyricum* with partial cell recycle as a long-term strategy for butyric acid production. *Energies* **2012**, *5*, 2835–2848. [[CrossRef](#)]
22. Qureshi, N.; Paterson, A.H.J.; Maddox, I.S. Model for continuous production of solvents from whey permeate in a packed bed reactor using cells of *Clostridium acetobutylicum* immobilized by adsorption onto bonechar. *Appl. Microbiol. Biotechnol.* **1988**, *29*, 323–328. [[CrossRef](#)]
23. Laponi, M.J.; Mendez, M.B.; Trelles, J.A.; Rivero, C.W. Cell immobilization strategies for biotransformations. *Curr. Opin. Green Sustain. Chem.* **2022**, *33*, 100565. [[CrossRef](#)]
24. Huang, J.; Dai, H.; Yan, R.; Wang, P. Butyric acid production from recycled waste paper by immobilized *Clostridium tyrobutyricum* in a fibrous-bed bioreactor. *J. Chem. Technol. Biotechnol.* **2016**, *91*, 1048–1054. [[CrossRef](#)]
25. Jiang, L.; Wang, J.; Liang, S.; Cai, J.; Xu, Z.; Cen, P.; Yang, S.; Li, S. Enhanced butyric acid tolerance and bioproduction by *Clostridium tyrobutyricum* immobilized in a fibrous bed bioreactor. *Biotechnol. Bioeng.* **2011**, *108*, 31–40. [[CrossRef](#)] [[PubMed](#)]
26. Santos, L.D.; Sousa, M.D.B.; Guidini, C.Z.; de Resende, M.M.; Cardoso, V.L.; Ribeiro, E.J. Continuous ethanol fermentation in tower reactors with cell recycling using flocculent *Saccharomyces cerevisiae*. *Process Biochem.* **2015**, *50*, 1725–1729. [[CrossRef](#)]
27. Li, J.; Lu, C.; Sun, J.; Peng, H.; Li, Q.; Hosseini, S.S.; Zhu, Y.; Sun, M.; Ma, B. Membrane recycling and resource utilization: Latest progress and prospects. *J. Environ. Sci.* **2025**, *156*, 346–359. [[CrossRef](#)] [[PubMed](#)]
28. Caverio-Olguin, V.H.; Dishisha, T.; Hatti-Kaul, R. Membrane-based continuous fermentation with cell recycling for propionic acid production from glycerol by *Acidipropionibacterium acidipropionici*. *Microb. Cell Factories* **2023**, *22*, 43. [[CrossRef](#)] [[PubMed](#)]
29. Mehaia, M.A.; Cheryan, M. Lactic acid from acid whey permeate in a membrane recycle bioreactor. *Enzyme Microb. Technol.* **1986**, *8*, 289–292. [[CrossRef](#)]

30. Qureshi, N.; Maddox, I.S. Continuous solvent production from whey permeate using cells of *Clostridium acetobutylicum* immobilized by adsorption onto bonechar. *Enzyme Microb. Technol.* **1987**, *9*, 668–671. [[CrossRef](#)]
31. Ennis, B.M.; Qureshi, N.; Maddox, I.S. Inline toxic product removal during solvent production by continuous fermentation using immobilized *Clostridium acetobutylicum*. *Enzyme Microb. Technol.* **1987**, *9*, 672–675. [[CrossRef](#)]
32. Faisal, A.; Zarebska, A.; Saremi, P.; Korelskiy, D.; Ohlin, L.; Rova, U.; Hedlund, J.; Grahn, M. MFI zeolite as adsorbent for selective recovery of hydrocarbons from ABE fermentation broths. *Adsorption* **2014**, *20*, 465–470. [[CrossRef](#)]
33. Fu, H.; Yang, S.-T.; Wang, M.; Wang, J.; Tang, I.-C. Butyric acid production from lignocellulosic biomass hydrolysates by engineered *Clostridium tyrobutyricum* overexpressing xylose catabolism genes for glucose and xylose co-utilization. *Bioresour. Technol.* **2017**, *234*, 389–396. [[CrossRef](#)] [[PubMed](#)]
34. Qureshi, N.; Mishra, S.; Liu, Z.; Singh, M.; Hector, R.E.; Ezeji, T.C.; Ashby, R.D.; Jackson, M.A.; Singh, V. Butyric acid production from corn: Economics of fermentative production of a novel biobased chemical. *J. Ind. Microbiol. Biotechnol.* **2016**, *53*, kuag009. [[CrossRef](#)] [[PubMed](#)]
35. Qureshi, N.; Mishra, S.; Liu, Z.; Singh, M.; Hector, R.E.; Ezeji, T.C.; Ashby, R.D.; Jackson, M.A.; Singh, V. A novel process of butyric acid production by fermentation of biomass: Toward a sustainable economic evaluation. *Fermentation*, 2026; submitted.
36. Hughes, S.R.; Gibbons, W.R.; Bang, S.S.; Pinkelman, R.; Bischoff, K.M.; Slininger, P.J.; Qureshi, N.; Kurtzman, C.P.; Liu, S.; Saha, B.C.; et al. Random UV-C mutagenesis of *Scheffersomyces* (formerly *Pichia*) *stipitis* NRRL Y-7124 to improve anaerobic growth on lignocellulosic sugars. *J. Ind. Microbiol. Biotechnol.* **2012**, *39*, 163–173. [[CrossRef](#)] [[PubMed](#)]
37. Liu, S.; Bischoff, K.M.; Leathers, T.D.; Qureshi, N.; Rich, J.O.; Hughes, S.R. Butyric acid from anaerobic fermentation of lignocellulosic biomass hydrolysates by *Clostridium tyrobutyricum* strain RPT-4213. *Bioresour. Technol.* **2013**, *143*, 322–329. [[CrossRef](#)] [[PubMed](#)]
38. Liu, S.; Duncan, S.; Qureshi, N.; Rich, J.O. Fermentative production of butyric acid from paper mill sludge hydrolysates using *Clostridium tyrobutyricum* NRRL B-67062/RPT 4213. *Biocatal. Agric. Biotechnol.* **2018**, *14*, 48–51. [[CrossRef](#)]
39. Baroi, G.N.; Baumann, I.; Westermann, P.; Gavala, H.N. Butyric acid fermentation from pretreated and hydrolysed wheat straw by an adapted *Clostridium tyrobutyricum* strain. *Microb. Biotechnol.* **2015**, *8*, 874–882. [[CrossRef](#)] [[PubMed](#)]

Disclaimer/Publisher’s Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.