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Two-Stage Bioconversion of Cellulose to Single-Cell Protein and Oil via a Cellulolytic Consortium

Eric Charles Peterson [1,2,*], Christian Hermansen [1,*], Ashriel Yong [1], Rowanne Siao [1], Gi Gi Chua [1], Sherilyn Ho [1], Coleen Toledo Busran [1], Megan Teo [1], Aaron Thong [1], Melanie Weingarten [1] and Nic Lindley [1]

[1] Singapore Institute of Food and Biotechnology Innovation (SIFBI), Agency for Science, Technology, and Research (A*STAR), 31 Biopolis Way, Nanos, Singapore 138669, Singapore
[2] Institut National de la Recherche Scientifique—Eau Terre Environnement (INRS-ETE), 490 Rue de la Couronne, Quebec City, QC G1K 9A9, Canada
* Correspondence: eric.peterson@inrs.ca (E.C.P.); christian_hermansen@sifbi.a-star.edu.sg (C.H.)

Abstract: A novel approach for converting non-edible plant biomass into single-cell protein and oil (SCPO) via consolidated bioprocessing has been established, leveraging aerotolerant thermophilic cellulolytic consortia consisting mainly of *Thermoanaerobacterium thermosaccharolyticum*, *Sporolactobacillus* spp. and *Clostridium sensu stricto* to achieve the rapid and complete conversion of crystalline cellulose into a consistent cocktail of lactate, acetate and ethanol. This cocktail is an excellent substrate for cultivating organisms for SCPO production and food and feed applications, including *Cyberlindnera jadinii*, *Yarrowia lipolytica* and *Corynebacterium glutamicum*. Cultivation on this cocktail resulted in yields ($Y_{X/S}$) of up to 0.43 ± 0.012 g/g, indicating a yield from cellulose ($Y_{X/Cellulose}$) of up to 0.27 ± 0.007 g/g (dwb). The resulting SCPO was rich in protein (42.5% to 57.9%), essential amino acids (27.8% to 43.2%) and lipids (7.9% to 8.4%), with unsaturated fatty acid fractions of up to 89%. Unlike fermentation feedstocks derived from easily digested feedstocks (i.e., food waste), this approach has been applied to cellulosic biomass, and this mixed-culture bioconversion can be carried out without adding expensive enzymes. This two-stage cellulosic bioconversion can unlock non-edible plant biomass as an untapped feedstock for food and feed production, with the potential to strengthen resiliency and circularity in food systems.

Keywords: fermentation; cellulose; lignocellulosic biomass; single-cell protein; single-cell oil; amino acids; food and feed

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1. Introduction

Lignocellulosic biomass represents an attractive feedstock for generating sustainable alternative food and feed ingredients, especially with respect to the growing aquaculture industry. Researchers have shown that the fermentation of softwoods into monomeric sugars can be used to generate yeast biomass [1,2] and that this whole-cell microbial biomass is an excellent feed ingredient for salmon aquaculture [3]. In general, there is a growing movement for the use of single-cell protein and oil as a food and feed ingredient as part of the rapidly growing alternative protein sector [4,5].

While there is interest in using lignocellulosic residues as a feedstock for single-cell protein, bottlenecks in conventional fermentation, such as enzyme addition and sterile conditions, add substantial costs. Direct bioconversion by cellulolytic organisms (i.e., consolidated bioprocessing [6]), in combination with approaches leveraging niche microbial communities, may be able to overcome these cost limitations. Specifically, while much

attention has been paid to the hydrolysis of lignocellulosic biomass for the recovery of fermentable sugars, there is growing interest in the production of volatile fatty acids and other fermentation end-products as a substrate for microbial growth. This approach comprises a two-stage fermentation process, where residues are first fermented anaerobically to end-products and then fermented aerobically to single-cell protein.

While efforts have succeeded in demonstrating this approach using less recalcitrant food waste residues [7], to date, this approach has not been applied exclusively to cellulosic materials as a starting feedstock. Using microbial cellulolytic consortia, the application of these approaches to cellulosic materials could represent a major improvement in substrate availability, potentially unlocking cellulosic residues as a new source of protein and oils for food and feed use. Considering the ample availability of lignocellulosic residues from global agrifood and forestry sectors, this approach could lead to increases in both food security and sustainability through the enhanced circularization of food systems, which is being similarly pursued for the production of biofuels, biocomposites and bioplastics [8].

Anaerobic cellulose degradation is a well-characterized process, with *Acetivibrio thermocellus* (formerly *Clostridium thermocellum*) used as a model cellulolytic microorganism [9]. This microbe was substantially investigated for bioethanol production but also shows promise for the bioconversion of lignocellulose to mixtures of soluble products that also include a high fraction of organic acids alongside ethanol. These end-products are an ideal substrate for other microorganisms and should be considered an intermediate building block. Importantly, these cellulolytic microorganisms are capable of rapid cellulose utilization [9]. This high rate of hydrolysis is due to the production of cellulosomes, which are enzymatic complexes with several subunits (cellulases, hemicellulases, chitinases, etc.) that docks the cell to lignocellulose [10,11]. Through attachment, cellulosomes permit simultaneous hydrolysis and uptake by the microorganism in such a way that feedback inhibition in the boundary layer is mitigated, allowing microbe–enzyme synergy [12], which is in part achieved by biofilm formation [13]. Furthermore, additional advances in cellulosic bioconversion may be yielded through studies of mixed cellulolytic consortia, which are effective cellulose utilizers capable of adapting to improve conversion performance [14–16], and these communities should be explored further.

Recent work has highlighted the ability of microbial consortia to hydrolyze complex biomass. For instance, consortia composed predominantly of the class *Clostridiales* have been studied to convert sugar cane bagasse to hydrogen [17]. Other studies have shown that consortia are effective in the delignification of rice straw, demonstrating the potential industrial relevance of consortia in pulping processes [18]. Co-culture systems with *Clostridia* have been shown to be capable of utilizing a wider range of substrates, including lignocellulosic biomass, food waste and industrial waste, while having improved substrate utilization, enhanced product yield, good stability and scalability [19]. *Clostridium* co-culture systems are commonly utilized to produce biofuels, including hydrogen and methane, as well as solvents and organic acids [20]. Two-stage bioprocesses for the production of single-cell protein from wastewater and food waste are an emerging technique of interest, wherein hydrolytic organisms anaerobically acidify the soluble organic load from wastewater, followed by a second aerobic fermentation stage where these end-products can be utilized as a substrate for the production of microbial biomass [7]. However, the potential production of single-cell protein and oil in a two-stage bioconversion of solid cellulose has hitherto been unexamined. Here, the use of aerotolerant thermophilic cellulolytic consortia to convert cellulose to a cocktail of acetic acid, butyric acid, lactic acid and ethanol has been evaluated, followed by the characterization of a second aerobic stage for primary fermentation utilization for three microorganisms that are well established for use

as food and feed ingredients, namely *Cyberlindnera jadinii* [21], *Yarrowia lipolytica* [22] and *Corynebacterium glutamicum* [23].

The overall objective of this article is to establish a proof of concept for a novel consolidated bioprocess approach for the direct conversion of cellulose to single-cell protein and oil via a naturally enriched cellulolytic consortium, which overcomes the need for enzymatic or chemical saccharification. To achieve this, a cellulolytic consortium was enriched from the soil, and the cellulose-derived fermentation products were evaluated for the production of single-cell protein in a two-stage bioprocess. Unlike conventional biomass bioconversion techniques based on axenic conditions and saccharification to monomeric sugars, this study presents a fundamentally different strategy for converting cellulosic residues into SCPO as a new food or feed source. By leveraging mixed cellulolytic cultures for the consolidated bioconversion of cellulose to a bioavailable cocktail of substrates, the two-stage cellulose-to-protein process demonstrated here holds the promise of a paradigm shift in bioprocessing to increase sustainability and strengthen both food security and resilience with circular food systems.

2. Materials and Methods

2.1. Strains, Enrichment and Reagents

Experiments were conducted using *Acetivibrio thermocellus* ATCC 27405 or a soil enrichment, wherein 1 g of Singaporean soil was added to 100 mL of sterile water sparged with nitrogen gas in a Hungate bottle. This soil solution was then used as inoculum (1% *v*/*v*) for anaerobic bottles containing 50 mL of a Reinforced Clostridial (RM) medium [24]; 5 g/L Avicel® microcrystalline cellulose was incubated at 60 °C and 200 rpm shaking for 48 h, with *A. thermocellus* cultivated under identical conditions. All Hungate bottles were prepared anaerobically via alternate N_2 sparging and vacuum cycles facilitated via a syringe manifold, followed by autoclaving at 121 °C and 115 psi for 20 min. Anaerobic bottle cultures were similarly prepared and used as seed cultures for 2 L bioreactors. Strains for the production of microbial biomass included *Yarrowia lipolytica* (CBS 2070), *Cyberlindnera jadinii* (ATCC 9950) and *Corynebacterium glutamicum* (DSM 20300), which were cultivated on yeast extract–peptone (YP) media containing the following reagents: yeast extract (10 g/L), peptone (20 g/L) and sodium acetate (20 g/L), followed by adjustment to pH 7.0 with sodium hydroxide (2 M). Seed cultures of 50 mL were incubated overnight for 24 h at 30 °C and 250 rpm in 250 mL shake flasks. All pure strains were maintained at −80 °C with 15% (m/m) glycerol. All reagents were purchased from Sigma-Aldrich, Burlington, MA, USA.

From Sigma-Aldrich, Burlington, MA, USA, 17 amino acid standard solution (analytical grade), L-tryptophan (Trp) (BioUltra grade), L-asparagine (Asn) (HPLC grade), L-glutamine (Gln) (BioUltra grade), L-cysteine (Cys) (BioUltra grade), norvaline (≥98%), HCl (37%) and phosphoric acid (H3PO4) (85%) were obtained. Derivatization agents of 9-fluorenylmethyl chloroformate (FMOC) (≥99%), O-phthaldialdehyde (OPA) (≥99%), 3-mercaptopropionic acid (≥99%) and 3,3′-dithiodipropionic acid (DTDPA) (99%) were also purchased from Sigma-Aldrich. Methanol (HPLC grade), acetonitrile (LCMS grade) and a 10 N sodium hydroxide solution (NaOH) were obtained from Fisher Chemical. Borate buffer and a 4 mL screw glass vial (p/n: 5067-0246) with a PTFE/silicone septa cap (p/n: 5183-4305) were obtained from Agilent Technologies. Deionized water (18.2 MΩ, 0.2 µm) (Sartorius Arium Pro VF Type 1 water system) was used to prepare standard solutions, buffers and reagents. The aqueous-based mobile phase was filtered through a 0.45 µm Claristep hydrophilic filter (Sartorius, Goettingen, Germany) for HPLC analysis.

2.2. Microbial Solubilization of Crystalline Cellulose

The conversion of crystalline cellulose to soluble end-products was carried out in 100 mL Hungate bottles using a 50 mL RM medium loaded with 5 g/L Avicel® with biological triplicates for each time-point, as well as in a 2 L Biostat® B bioreactor (Sartorius, Goettingen, Germany), using a 1 L RM medium loaded with 20 g/L Avicel. Hungate bottles were prepared anaerobically, autoclaved as described above and inoculated with 0.5 mL seed cultures. The bioreactor was sparged with 2 VVM N_2 for 5 min, followed by autoclaving at 121 °C and 115 PSI for 20 min. Subsequently, the medium was sparged with sterile N_2 passed through a 0.22 um filter while the reactor was cooled to 60 °C, with anaerobic conditions ensured via a dissolved oxygen probe and anaerobic indicator. The bioreactor was inoculated with a 5% (*v*/*v*) seed culture of either *A. thermocellus* or the soil consortium prepared in anaerobic bottles as described above. In bioreactors, pH was controlled at 7.0 via the automatic addition of 3 M KOH. Samples were taken at 12 h intervals for 48 h in anaerobic bottles and 60 h in bioreactors and quantified for acetic acid, lactic acid, butyric acid and ethanol by HPLC. The suspended solids were recovered by centrifugation, washed and assayed for residual cellulose and cell protein by a Bradford assay as a growth indicator since standard optical or gravimetric methods for assessing microbial growth could not be used due to interference from residual insoluble microcrystalline cellulose.

2.3. Microbial Conversion of Soluble End-Products to Single-Cell Protein and Oil (SCPO)

Fermentation broth from cellulolytic consortium cultured on 20 g/L Avicel was directly used for the fermentation of food-grade microorganisms *Y. lipolytica*, *C. jadinii* and *C. glutamicum*, wherein 300 mL aliquots of fermentation broth were added to 500 mL bioreactors (MiniBio 500; Getinge Applikon; Delft, The Netherlands) and autoclaved as described above, followed by inoculation with respective seed cultures normalized to 0.1 optical density at 600 nm (OD600). Bioreactors were operated at 30 °C, with initial aeration and mixing of 0.5 VVM and 1000 rpm, and dynamic dissolved oxygen (DO) control was set, where the gassing and stirring speed increased when the DO level dropped below 10%. Samples were taken at regular intervals and measured for OD600 and substrate consumption via HPLC. After 24 h, the reactor contents were harvested, centrifuged, washed and quantified for cell dry weight and total nitrogen. Amino acid and lipid composition analyses were also performed in the case of *Y. lipolytica* and *C. jadinii* to assess their nutritional value.

2.4. OD600, Cell Dry Weight and Sample Handling

OD600 was measured for triplicate sample aliquots diluted 10 to 20 times with the fermentation media with absorbance measured at the wavelength of 600 nm and values accepted within the linear range of 0.2–0.8 absorbance units. Cell dry weight (CDW) was conducted in triplicate in 5 mL aliquots in pre-weighed centrifuge tubes, which were centrifuged for 5 min at 13,000 rpm, with supernatant decanted and the pellet dried in the oven at 60 °C for 48 h followed by gravimetric analysis. For total nitrogen and compositional analysis for both amino acid and lipids, 50 mL aliquots of reactor contents were centrifuged for 15 min at 3900 rpm and decanted, with pellets freeze-dried for 48 h (Freezone 2.5; Labconco, Kansas City, MO, USA) after freezing at −80 °C.

2.5. Cellulose Utilization

Residual cellulose in fermentation samples was measured using a modified Acid Detergent Fibre (ADF) method [25]. Briefly, fermentation samples were sealed in serum bottles with 2 equivalent volumes of ADF solution and autoclaved for 45 min. Subsequently, bottle contents were vacuum-filtered and washed with 3 equivalent volumes of boiling

water to remove any ADF residue, followed by gravimetric analysis. All samples were measured in triplicate.

2.6. Bradford Protein, Amino Acid and Total Nitrogen Analysis

Bradford protein was determined with a 2 mL sample of reactor contents centrifuged at 13,000 rpm for 5 min, with 0.5 mL of 1 M NaOH added to the decanted pellet, vortexed and placed in a 70 °C water bath for one hour, followed by neutralization via the addition of 0.5 L of 1 M HCl and centrifugation as given above. Samples were then quantified for total protein by Bradford protein assay (Bio-Rad Bradford Quick StartTM kit, Bio-Rad Laboratories, Hercules, CA, USA) and compared to a Bovine Serum Albumin (BSA) standard curve at 595 nm in triplicate.

Protein hydrolysis was performed according to the procedure reported by Teo et al. [26]. Briefly, the sample-to-hydrolysis reagent ratio was kept constant at 10:1. Two hydrolysis methods were used for the quantification of amino acids: (i) acid hydrolysis [27–29] in 6 N HCl with 3,3′-dithiodipropionic acid (DTDPA) 2% (w/v) in 0.2 N NaOH at 110 °C for 22 h and (ii) alkaline hydrolysis in 4.2 N NaOH at 110 °C for 4 h. Both hydrolysis reactions were carried out under blanketed nitrogen conditions.

The amino acids content was quantified based on protocol reported by Agilent [30] using Agilent Infinity II 1260 HPLC system equipped with Agilent 1260 Infinity quaternary eluent pump and diode array detector (DAD) (Agilent Technologies, Santa Clara, CA, USA). Mobile phase A consisted of 10 mM disodium hydrogen phosphate and 10 mM sodium tetraborate buffer, in pH 8.2 adjusted with concentrated HCl (37%); mobile phase B consisted of acetonitrile/methanol/deionized water (45/45/10, $v/v/v$), sonicated for 10 min. The flow rate was 1.5 mL/min with the column temperature maintained at 40 °C and injection volume of 1 µL. The amino acids were derivatized online with o-phthaldialdehyde (OPA) for the primary amino acid at a UV wavelength of 338 nm and 9-fluorenylmethylchloroformate (FMOC) for the secondary amino acid at 262 nm. The separation was achieved by the analytical column (Agilent AdvanceBio AAA, C18, 4.6 × 100 mm, 2.7 µm) attached with a guard column, Agilent AdvanceBio AAA (4.6 × 5 mm, 2.7 µm). The elution gradient system was performed with slight modifications such as 0–0.35 min, 2% B; 13.4–13.5 min, 57% B; 15.7 min, 100% B; 15.8–24.5 min; 2% B. A total of 21 amino acids standard mixture together with 3-[(2-carboxyethyl)disulfanyl]alanine (Cys-X) [27,28] in 0.1 N HCl at the concentration ranges of 0.005 mM to 0.5 mM were prepared for the standard calibration curves. Norvaline was added into working standards and samples as internal standard at a final concentration of 0.1 mM. Data analysis was performed in OpenLab CDS Chemstation edition (Rev.C.01 07 SR4) software (Agilent Technologies, Santa Clara, CA, USA) for the HPLC system operation and analysis. Microsoft Excel (Version 2011) (Microsoft Corporation, Albuquerque, NM, USA) was used to perform all statistical analysis.

Total nitrogen was determined via the Dumas method using a DumaTherm (Gerhardt, Königswinter, Germany), with a protein conversion factor of 6.25 to calculate crude protein fraction.

2.7. Lipid Analysis

For lipid analysis, 1 mL of a prepared hydrochloric acid solution (8.3 M) was added to 200 mg samples in a screw-capped glass vial, and 10 mg of pyrogallic acid was added to minimize oxidative degradation during analysis. The mixture was heated at 70 °C with intermittent shaking for 40 min. Subsequently, the hydrolysates were vigorously agitated with 10 mL diethyl ether and petroleum ether (1:1), followed by the separation and drying of the solvent for the gravimetric analysis of total lipid content. For compositional lipid

analysis, dried fats were first methylated to fatty acid methyl esters (FAMEs) by combining samples with 2 mL BF3 (14% in methanol) and heating at 100 °C for 45 min, followed by extraction with 1 mL hexane for injection into the GC. FAMEs were analyzed by gas chromatography equipped with a flame ionization detector and a fused silica capillary column (Supelco SP2560; 100 m × 0.25 mm × 0.20 µm; Supelco, Bellefonte, PA, USA), with helium for a carrier gas (1.0 mL/min). A 1 µL sample was injected and split at 25:1, with injector and detector temperatures at 220 °C and 250 °C, respectively. The oven programme was initially set at 100 °C for 4 min before ramping at 3 °C/min to 240 °C and finally held for another 17 min. Data were processed using OpenLAB CDS Chemstation software (C.01.10; Agilent Technologies, Santa Clara, CA, USA).

2.8. End-Product Quantification

Acetic acid, butyric acid, lactic acid and ethanol were quantified by high-performance liquid chromatography (Agilent Infinity II 1260 HPLC; Agilent Technologies, Santa Clara, CA, USA) using a diode array detector (G7115A DAD). Briefly, the fermentation broth was filtered through 0.45 µm hydrophilic filters (Sartorius, Göttingen, Germany), and 20 µL was injected into the HPLC for analysis. The analytes were separated on an ion-exchange column (Aminex HPX-87H; 300 × 7.8 mm, 35 °C; Bio-Rad, Hercules, CA, USA), utilizing a mobile phase of 10 mM H_2SO_4 at 0.6 mL/min and detected using a refractive index detector (30 °C, positive polarity). Quantification was carried out by comparing against standard calibration curves using the OpenLab CDS Chemstation edition (C.01.07, Agilent Technologies, Santa Clara, CA, USA). Fermentable sugars and other organic acids were readily analyzed using this method but were not detected unless otherwise reported.

2.9. Titre, Rate, Yield Calculation and Statistical Analysis

Titre, rate and yield coefficients were calculated from the results of cellulose, end-products, crude protein by total nitrogen, lipid content and CDW measured in triplicate on dry weight basis at the bioreactor fermentation end-point of 60 hrs using the methodology described by Konzock and Nielsen [31]. All statistical analyses were performed using Microsoft® Excel® for Microsoft 365 MSO (v2412 Build 16.0.18324.20092) 64-bit. Data analysis and visualization was aided by Daniel's XL Toolbox add-in for Excel, version 7.3.2 [32]. One-way analysis of variance (ANOVA) was used to identify statistically significant differences ($p < 0.05$) among the means of biomass, protein and lipid yield coefficients from cellulose for *C. jadinii*, *Y. lipolytica* and *C. glutamicum*. The Bonferroni–Holm post hoc test was then used to identify significant differences ($\alpha = 0.05$) between the various combinations of means.

2.10. Molecular Analysis

For total nucleic acid extraction, 2 mL of fermentation broth was centrifuged at 3900 rpm for 10 min. The supernatant was removed, and the pellet was re-suspended in solution CD1 from DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany) and lysed using TissueLyser II (Qiagen, Hilden, Germany) at 30 Hz for 2 min. The samples were subjected to DNA extraction according to the manufacturer's instructions. DNA clean-up was performed using Mag-Bind® TotalPure NGS (Omega Bio-Tek, Norcross, GA, USA). DNA yield and purity were determined using NanoDrop™ 2000 Spectrophotometer (Thermo Scientific, Leipzig, Germany). For amplicon metagenomics sequencing, 16S rRNA gene amplicon library construction and sequencing were carried out on the Illumina Novaseq PE250 platform with focus on V3–V4 hypervariable region using primers 341F and 806R (NovogeneAIT Genomics, Singapore).

3. Results and Discussion

3.1. Cellulose Bioconversion in Hungate Bottles

Anaerobic cellulolytic consortia established via enrichment from soil have been shown to be efficacious in cellulose utilization [16], and the ease of generating a replicable consortium from widely available environmental sources such as soil enables simple access for industrially relevant microorganisms without the need for laborious isolation or restrictive licencing agreements. Thus, the cellulose hydrolysis efficiency of a cellulolytic consortium enriched from Singaporean soil was compared to *A. thermocellus* on 5 g/L crystalline cellulose in 100 mL Hungate bottles. As shown in Figure 1a,b, both *A. thermocellus* and the consortium show rapid hydrolysis rates, eliminating cellulose within 48 h with comparable growth as measured by cell protein content. For both treatments, cellulose hydrolysis is essentially complete by the end of the 48 h period in Hungate bottles, with 3.6 ± 1.4% cellulose remaining for the cellulolytic consortium and 6.1 ± 2.0% for *A. thermocellus*.

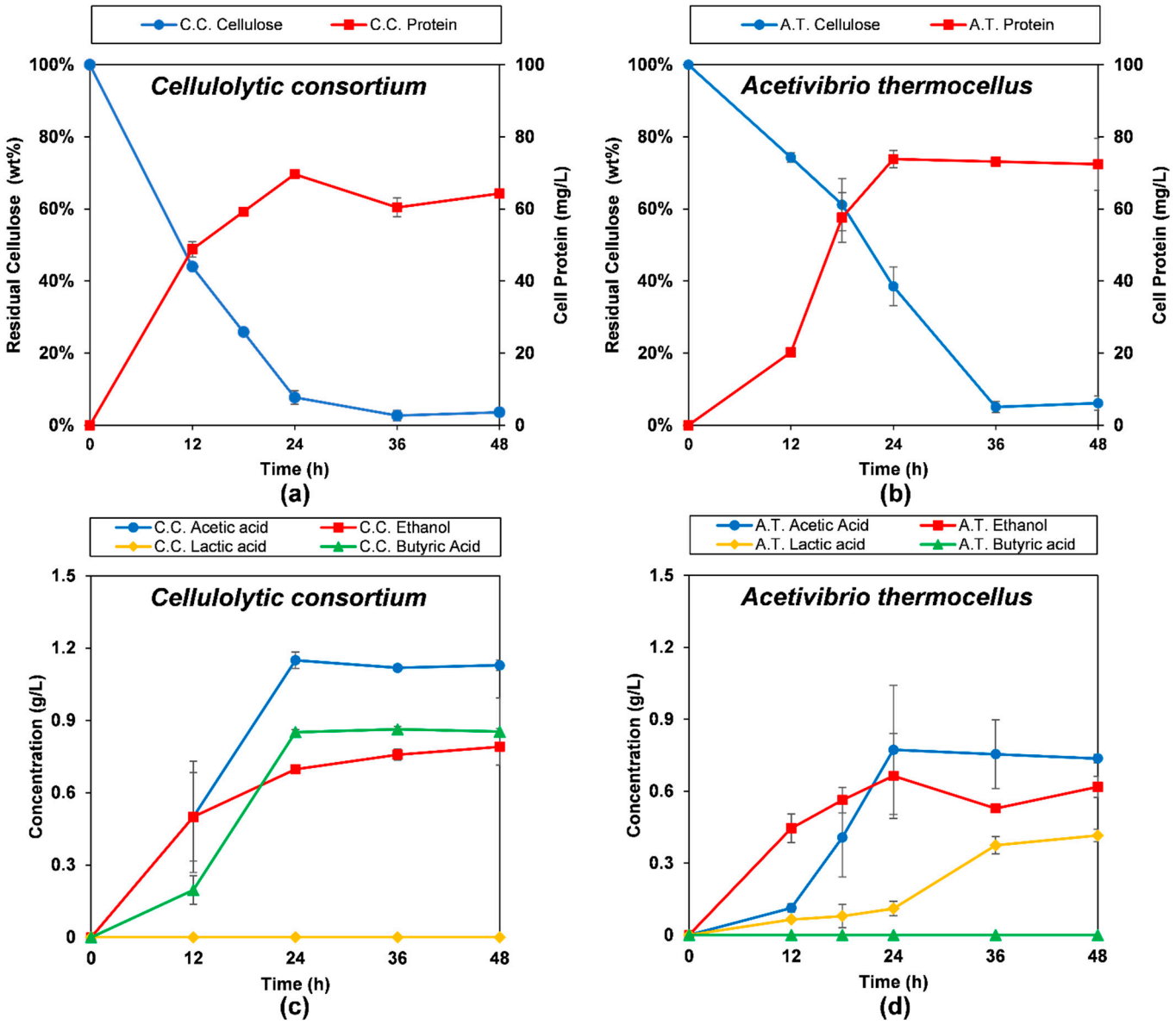


Figure 1. Cellulose bioconversion to end-products in Hungate bottles with 5 g/L cellulose by the cellulolytic consortium (C.C.) and *A. thermocellus* (A.T.): (**a**) cellulose utilization and Bradford protein of C.C. cultures; (**b**) cellulose utilization and Bradford protein of A.T. cultures; (**c**) end-products for C.C cultures.; (**d**) end-products for A.T. cultures.

It is important to note that reducing conditions were not created in Hungate bottles prior to cultivation, and no reducing agent was used. Despite this, the cellulolytic consortium showed immediate cellulolytic activity, growth and end-product accumulation, while *A. thermocellus* shows a noticeably slower rate of cellulose hydrolysis. For instance, after 24 h, 38.5 ± 5.4% residual cellulose was observed for the pure culture of *A. thermocellus*, compared to only 7.7 ± 0.2% for the cellulolytic consortium. This slower cellulose utilization with the pure culture may imply that the consortium has a higher tolerance to residual oxygen and generates reducing conditions more quickly.

The end-product profiles, as analyzed by HPLC, are presented in Figure 1c,d. In Hungate bottles, the cellulolytic consortium produces a consistent mixture of acetic acid, butyric acid and ethanol but no lactic acid (Figure 1c). In contrast, *A. thermocellus* in Hungate bottles produces acetic acid, lactic acid and ethanol but no butyric acid (Figure 1d).

A. thermocellus is known to produce a mixture of mainly ethanol and acetate, with a ratio of roughly 2:1 in batch fermentation and 1:1 in continuous fermentation [33]. In Hungate bottles, the expected end-product profile consisting mostly of acetic acid (1.06 ± 0.02 g/L) and ethanol (0.62 ± 0.04 g/L) is observed, with some lactic acid (0.42 ± 0.04 g/L). Interestingly, the cellulolytic consortium has a very different end-product profile from that of *A. thermocellus*, with acetic acid (1.13 ± 0.02 g/L) and ethanol (0.79 ± 0.08 g/L) as major products but also butyric acid (0.85 ± 0.10 g/L) present without lactic acid. This must be due to metabolic differences between this pure culture and the multiple members in the community structure of a mixed microbial culture, and thus, community structure analysis is merited.

3.2. Metagenomic Sequencing of Cellulolytic Consortium

To characterize the composition of the cellulolytic consortium directly enriched from cellulose, amplicon sequencing was employed to elucidate microbial community structure. The composition of the microbial consortium is visualized by a KRONA display [34] (Figure 2), showing that the community is overwhelmingly Clostridia dominated by *Thermoanaerobacterium* (species *T. thermosaccharolyticum*) at the genus level (81.2%), followed by 8.3% *Sporolactobacillus* spp. and 4.0% *Clostridium sensu stricto*, accounting for 93.5% of the identified operational taxonomic units (OTUs). Genera with below 1% relative prevalence include *Limosilactobacillus* (0.4% *L. fermentum*; 0.3% *L. reuteri*), Acetobacter (0.7% *A. pasteurianus*) and *Thermosinus* (0.9% *T. carboxydivorans*).

T. thermosaccharolyticum, formerly classified under the genus *Clostridium*, is a thermophilic obligate anaerobe investigated for butanol and hydrogen production from starch, corn stover and xylan [35]. *Sporolactobacillus* is a genus of facultative anaerobic lactic acid bacteria, and the species *S. inulinus* is an efficient lactic acid producer, capable of not only producing but also tolerating over 200 g/L lactic acid from hydrolyzed peanut meal [36,37]. *Clostridium sensu stricto* is considered the true *Clostridium* genus, including thermophilic and obligate anaerobic species with diverse acetic acid, butyric acid and ethanol production capabilities [38]. The type species is *C. butyricum*, but the genus also includes the thermophilic *C. thermobutyricum*, which produces butyric acid from glucose and acetate substrates [39,40]. Additionally, undefined mixed cultures have previously been explored for butyric acid production from rice straw, with butyric acid and acetic acid as the main products [41], and this supports the observed production of butyric acid from the cellulolytic consortium cultivated in Hungate bottles.

Figure 2. KRONA display at the genus level in the composition of the cellulolytic consortium enriched anaerobically on cellulose at 60 °C and characterized by 16S rRNA amplicon metagenomic sequencing.

These microorganisms are not reported to be independently efficient cellulose converters, but the consortium appears to be highly adapted to niche cellulose utilization in anaerobic thermophilic environments. *T. thermosaccharolyticum* has been used in co-culture with *A. thermocellus* for the consolidated bioprocessing of microcrystalline cellulose and corn straw for enhanced ethanol production on account of its ability to metabolize pentose sugars and produce higher concentrations of ethanol [42]. Similarly, their co-culture enhances cellobiose utilization and hydrogen production while changing the end-product profile towards butyric acid instead of lactic acid when cultured on corn by-products [43].

The presumed role of the identified microorganisms in the cellulolytic consortium is as follows. *Clostridium sensu stricto* is the de facto cellulose hydrolyzer, synthesizing powerful enzyme complexes known as cellulosomes that dock to cellulose and produce soluble glucose. This glucose is metabolized by *Clostridium sensu stricto* through a modified glycolysis pathway to pyruvate. However, non-cellulolytic microorganisms such as *T. thermosaccharolyticum* are capable of more efficient glucose uptake and benefit from incomplete cellulose utilization by the hydrolyzing *Clostridium sensu stricto* [44]. *T. thermosaccharolyticum* is also

reported to produce lactic acid and ethanol in a glucose-limited continuous culture, with the end-product profile depending on the pH and dilution rate [45]. The end-product profile of the cellulolytic consortium cultivated in Hungate bottles resembles that of *T. thermosaccharolyticum* cultivated continuously at a low pH of 5.4, which agrees well with an observed pH after 24 h of 5.09 ± 0.02 in the bottles. A population of homofermentative Sporolactobacillus is thought to persist on account of their resistance to lactic acid, ethanol and organic acids produced by themselves and other lactic acid bacteria and are likely to proliferate when end-products are accumulated in concentrations inhibitory of other microorganisms in the cellulolytic consortium.

3.3. Cellulose Bioconversion in Bioreactor Studies

To characterize the consortium's performance under more intensive conditions, stirred-tank bioreactor studies were conducted with higher substrate loadings (i.e., 20 g/L) and robust mixing and compared to *A. thermocellus*, as shown in Figure 3. At this higher substrate loading, the overall rate of cellulose was reduced compared to lower substrate loadings (i.e., 5 g/L cellulose). Specifically, for both treatments, residual cellulose hydrolysis after 48 h was 12.7% for the cellulolytic consortium and 33.3% for *A. thermocellus*, which is markedly higher than the values reported above for Hungate bottles. This can be attributed to the recalcitrance of cellulose when used as a substrate, which is highly resistant to hydrolysis and can represent a rate-limiting step in anaerobic cellulose utilization, and this would reduce overall rates for utilization as substrate loading increases. Even after 60 h, while 3.0% residual cellulose remained for the cellulolytic culture, 11.5% cellulose remained for *A. thermocellus*, suggesting the pure culture had not fully utilized available cellulose at this after this extended duration, and similar results have been reported for higher substrate loading with these organisms [46].

It is interesting to note that at the higher substrate loading, *A. thermocellus* shows not only slower and less complete cellulose utilization but also an extended lag phase of at least 24 h with no observable hydrolysis (Figure 3b). However, when *A. thermocellus* was cultivated in Hungate bottles, 38.5 ± 5.4% had already been achieved by this point (Figure 1b). Furthermore, the consortium shows no lag phase in reactor studies (Figure 3a) and showed markedly faster cellulose utilization compared to *A. thermocellus* in Hungate bottles (Figure 1a,b). As no reducing agent was added during cultivation in both Hungate bottles and bioreactor studies, the lag demonstrated by *A. thermocellus* could possibly be due to the lack of sufficient reducing conditions in the bioreactor, which would be exacerbated in bioreactors compared to cyclically vacuum sparged Hungate bottles. However, for the consortium, the lack of this lag effect and generally increased rates of cellulose utilization suggests enhanced robustness possibly due to higher tolerance to these conditions compared to the pure culture. This tolerance may be due to the presence of *T. thermosaccharolyticum* and other organisms, contributing to the rapid generation of reduced conditions conducive to anaerobic cellulose hydrolysis. Thus, the relationship between the cellulolytic consortium and the Oxidation Reduction Potential (ORP) should be characterized further to assess the degree of aerotolerance and its causes. The robust aerotolerance of this microbial consortium could potentially help to overcome the challenges in operating anaerobic bioprocesses.

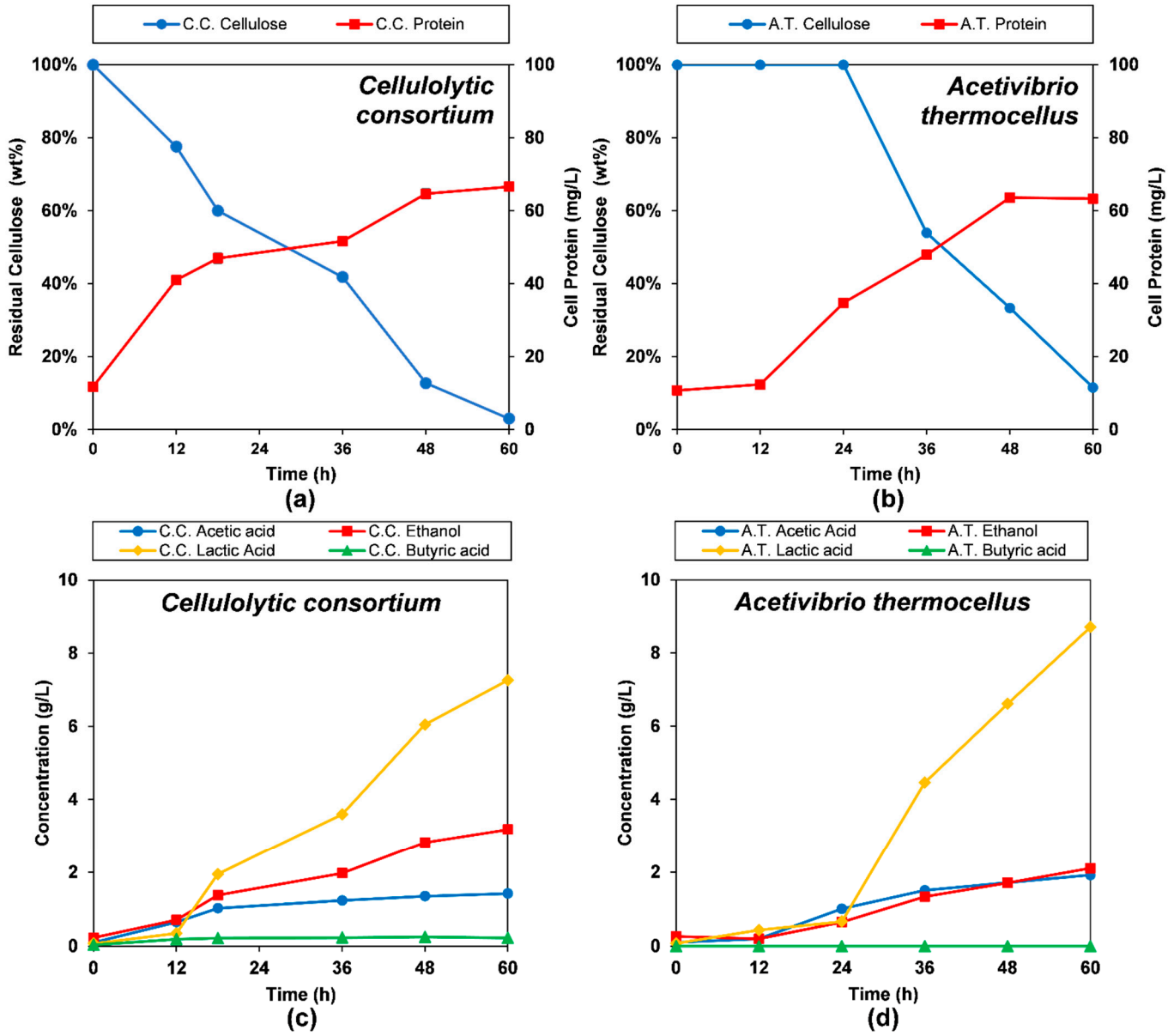


Figure 3. Conversion of cellulose to end-products in 2 L bioreactors with 20 g/L cellulose by a cellulolytic consortium (C.C.) and *A. thermocellus* (A.T.): (**a**) cellulose utilization and Bradford protein for C.C. cultures; (**b**) cellulose utilization and Bradford protein for A.T. cultures; (**c**) end-products for C.C. cultures; (**d**) end-products for A.T. cultures.

The cellulolytic consortium cultivated in bioreactors produces a similar end-product profile as *A. thermocellus*, comprised mainly of lactic acid, also with acetic acid and ethanol, but no butyric acid (Figure 3c). While both *A. thermocellus* and the consortium produced lactic acid predominantly, the consortium was observed to produce slightly less lactic acid (7.2 g/L), acetic acid (1.4 g/L) and more ethanol (3.2 g/L), compared to *A. thermocellus* (8.7, 1.9 and 2.1 g/L, respectively), while also producing some butyric acid (0.2 g/L). The cellulolytic consortium has a lower total yield of soluble products of 0.62 g/g, compared to 0.72 g/g for *A. thermocellus* (Table 1), possibly due to differences in production of gaseous products, such as H_2, CO_2 and CH_4. Although lactic acid is not typically the predominant end-product from *A. thermocellus* fermentation [46], the production is thermodynamically favourable and can reach up to 15.7 g/L under certain conditions [47]. It is also interesting to note that the consortium shifted from butyric to lactic acid production at higher substrate loadings and controlled pH, and the pronounced shift in both the pure culture and the

consortium to lactic acid suggests this behaviour is a result of electron overflow with an excess of substrate leading to the formation of lactic acid to enable regeneration of NADH. Regardless, when considering these yields of total soluble end-products ($Y_{S/Cellulose}$), the values are notably higher than the theoretical yield of ethanol from the cellulose of 0.50 g/g [48], undoubtedly because the values reported here include all by-products. This could have important ramifications on bioprocessing costs, which have historically limited cellulosic bioconversion for ethanol production, especially if this cocktail of end-products can be demonstrated to be suitable for use as a substrate for subsequent fermentation.

Table 1. Yield coefficients for cellulose bioconversion, end-products bioconversion and combined two-stage bioconversion to single-cell protein and oil (SCPO) on a dry weight basis (dwb).

Cellulose Bioconversion Titre, Rate and Yield Coefficients			
Yield on cellulose (dwb)	*A. thermocellus*	*Cellulolytic consortium*	
Acetic acid yield ($Y_{Acetic\ acid}$)	0.11 g/g	0.07 g/g	
Butyric acid yield ($Y_{Butyric\ acid}$)	0.00 g/g	0.01 g/g	
Lactic acid yield ($Y_{Lactic\ acid}$)	0.49 g/g	0.37 g/g	
Ethanol yield ($Y_{Ethanol}$)	0.12 g/g	0.16 g/g	
End-products yield ($Y_{S/Cellulose}$) [1]	0.72 g/g	0.62 g/g	
End-products to SCPO bioconversion titre, rate and yield (TRY) coefficients			
TRY on end-products (dwb)	*C. jadinii*	*Y. lipolytica*	*C. glutamicum*
Biomass titre (x)	2.47 ± 0.03 g/L	5.20 ± 0.14 g/L	3.15 ± 0.41 g/L
Biomass rate (q_X)	0.10 ± 0.00 g/L/h	0.22 ± 0.01 g/L/h	0.13 ± 0.02 g/L/h
Biomass yield ($Y_{X/S}$) [2]	0.20 ± 0.003 g/g	0.43 ± 0.012 g/g	0.26 ± 0.034 g/g
Two-stage bioconversion of cellulose to SCPO yield coefficients			
Yield on cellulose (dwb)	*C. jadinii*	*Y. lipolytica*	*C. glutamicum*
Biomass yield ($Y_{X/Cellulose}$) [3]	0.13 ± 0.002 g/g	0.27 ± 0.007 g/g	0.16 ± 0.021 g/g
Protein yield ($Y_{P/Cellulose}$)	0.07 ± 0.001 g/g	0.11 ± 0.003 g/g	0.09 ± 0.012 g/g
Lipid yield ($Y_{L/Cellulose}$)	0.010 ± 0.000 g/g	0.023 ± 0.001 g/g	0.013 ± 0.002 g/g

[1] $Y_{S/Cellulose}$: total end-products yield from cellulose. [2] $Y_{X/S}$: biomass yield from end-products. [3] $Y_{X/Cellulose}$: biomass yield from cellulose in two-stage bioprocess.

3.4. End-Product Bioconversion to Single-Cell Protein and Oil (SCPO)

Two-carbon compound substrates such as acetic acid and ethanol have already been investigated as sustainable fermentation feedstocks [49]. However, while previous research has demonstrated cellulolytic consortia's ability to effectively solubilize cellulose into such compounds, few studies have investigated using the total cocktail of end-products as a substrate for microbial growth. Here, the direct utilization of cellulosic fermentation broth was used as a substrate in a secondary aerobic stage, using three food-grade microorganisms: *Cyberlindnera jadinii*, *Yarrowia lipolytica* and *Corynebacterium glutamicum*.

C. jadinii is also known as *Candida utilis* in its anamorph state or as *Torula utilis*, a name given when first isolated from German yeast factories for food and feed in 1926 [21]. In the food and feed industry, *C. jadinii* is often referred to as "Torula yeast" and used for flavouring on account of its high protein content and richness in glutamic acid with flavour-enhancing properties [5]. *C. jadinii* has potential as a functional protein source in aquafeed and has been shown to modulate immunoregulatory pathways in the zebrafish intestine [50]. *Y. lipolytica*, also known as *Candida lipolytica* in its anamorph state, is an oleaginous yeast capable of producing over 40% lipids on CDW [22]. *Y. lipolytica* has been used as a protein source for animal feed, a production host for eicosapentaenoic acid production, and in biofuel production, with the protein-rich residual biomass approved for use in food by the European Food and Safety Authority (EFSA) since 2019 [51]. *C. glutamicum*

is the microbial chassis of choice for the fermentative production of most amino acids, including L-glutamate and L-lysine, for both the food and feed industry [23].

These organisms demonstrated varied growth on end-products from the fermentation of 20 g/L cellulose by the soil consortium, as reported in Figure 4, showing the growth and utilization of these end-products as substrates by the three microorganisms. A distinct preference with respect to end-products can be observed, with *Y. lipolytica* showing the rapid and complete utilization of both acetic acid and ethanol within 12 h, followed by a steady and partial consumption of lactic acid over the span of 24 h. *C. glutamicum* showed a rapid utilization of lactic acid and acetic acid, followed by a more prolonged utilization of ethanol. Conversely, *C. jadinii* showed a prolonged utilization of lactic acid and ethanol but did not utilize acetic acid. *C. jadinii* was reported to grow well on ethanol through the conversion to acetyl-CoA with acetic acid as a by-product, which explains the apparent lack of acetic acid utilization [52]. Similarly, *Y. lipolytica* is capable of metabolizing both ethanol and acetic acid [53], and *C. glutamicum* possesses both L- and D-lactate dehydrogenase for oxidation to pyruvate [54]. These substrate preferences help explain the partial utilization of the different end-products, as reported in Figure 4, and differences in growth, but further studies should be conducted to carefully characterize the kinetics and metabolism using tools such as transcriptomics and tracer techniques.

The total cell dry weight biomass achieved for each organism was 2.5 ± 0.03 g/L with *C. jadinii*, 3.1 ± 0.4 g/L for *C. glutamicum* and 5.2 ± 0.14 g/L with *Y. lipolytica*. By comparing the starting concentration of cellulose (i.e., 20 g/L), residual cellulose, end-product concentrations and the final cell dry weight biomass, the yield coefficients from end-product substrates and even cellulose can be calculated (Table 1). Apparent SCPO biomass yields from cellulose of 0.13 ± 0.002 g/g, 0.27 ± 0.007 g/g and 0.16 ± 0.021 g/g can be achieved with *C. jadinii*, *Y. lipolytica* and *C. glutamicum*, respectively. For comparison, yields up to 0.35 ± 0.01 g/g from glucose have been obtained with *Y. lipolytica* in media mimicking lignocellulosic biomass hydrolysates and up to 0.42 ± 0.03 g/g in defined media [55]. Thus, especially in the case of *Y. lipolytica*, promising conversion yields of cellulose to single-cell protein and oil are achievable, especially considering the substrate studied here is recalcitrant cellulose rather than simple sugars from enzymatic hydrolysis.

Recent work has proposed the use of whole-cell biomass as a feed ingredient in aquaculture, thus obviating the need for the downstream fractionation of biomass components [1,2]. The composition of the cell biomass generated here from cellulose has been characterized. Importantly, crude protein contents reached 55.6%, 42.6% and 57.9% for *C. jadinii*, *Y. lipolytica* and *C. glutamicum*, respectively, demonstrating that not only growth but also protein content varies across organisms when cultivated on cellulose-derived fermentation end-products (Table 2). The crude protein contents are generally comparable with those from the literature, with *C. jadinii* having 46.3 [3], *Y. lipolytica* 30.5% to 56.4% [51] and *C. glutamicum* 60.9% crude protein [56]. The relatively high protein content of *C. jadinii* of 55.6% cultivated on end-products reported here is similar to that from the cultivation of sugar beet molasses of 53.8% to 55.1%, with only 2.3% to 2.4% non-protein nitrogen [57]. With further improvement in the biomass yield on cellulose ($Y_{X/Cellulose}$) of 0.13 ± 0.002 g/g, the two-stage bioconversion process could potentially compete with sugar beet molasses for the economic cultivation of *C. jadinii* for aquafeed application ($Y_{X/S}$ = 0.51 g/g) [58].

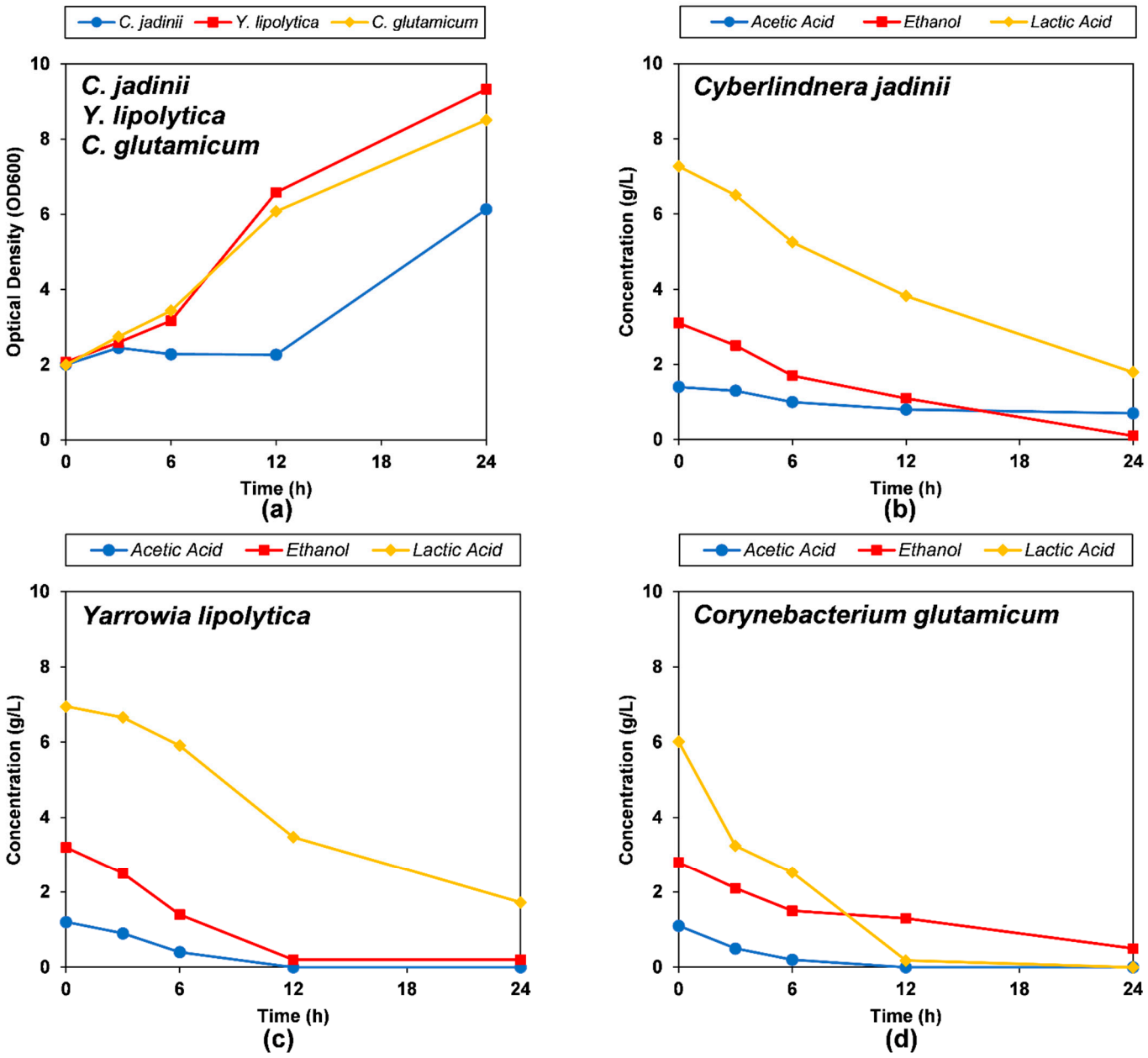


Figure 4. Growth and utilization of cellulose-derived end-products produced by a cellulolytic consortium in a 500 mL bioreactor: (**a**) optical density at 600 nm (OD600); (**b**) utilization of end-products by *C. jadinii*; (**c**) utilization of end-products by *Y. lipolytica;* (**d**) utilization of end-products by *C. glutamicum*.

Table 2. Amino acids on a protein basis and crude protein on a dry weight basis for *C. jadinii*, *Y. lipolytica* and *C. glutamicum* cultivated on soluble products generated by a cellulolytic consortium.

Amino Acid (Protein Basis)	*C. jadinii*	*Y. lipolytica*	*C. glutamicum*
L-Aspartic acid	9.4%	9.1%	10.3%
L-Glutamic Acid	15.2%	17.9%	15.5%
Serine	5.1%	4.4%	4.5%
L-Histidine	2.8%	2.3%	2.9%
L-Glycine	4.7%	13.2%	5.4%
L-Threonine	5.6%	3.4%	5.3%
L-Arginine	5.6%	5.2%	7.2%

Table 2. *Cont.*

Amino Acid (Protein Basis)	*C. jadinii*	*Y. lipolytica*	*C. glutamicum*
L-Alanine	6.5%	7.8%	9.2%
L-Tyrosine	5.0%	1.9%	2.5%
L-Cysteine	1.2%	1.1%	0.6%
L-Valine	6.5%	4.1%	6.5%
L-Methionine	1.8%	1.2%	2.2%
L-Tryptophan	1.2%	0.6%	1.2%
L-Phenylalanine	4.5%	3.0%	4.0%
L-Isoleucine	4.7%	3.1%	4.5%
L-Leucine	7.7%	4.7%	7.9%
L-Lysine	8.3%	5.5%	5.3%
L-Proline	4.2%	11.6%	4.9%
Σ EAA [1]	43.2%	27.8%	39.9%
Σ NEAA [2]	56.8%	72.2%	60.1%
Crude protein (dwb [3])	55.6%	42.5%	57.9%

[1] EAAs: essential amino acids. [2] NEAAs: non-essential amino acids. [3] Dry weight basis.

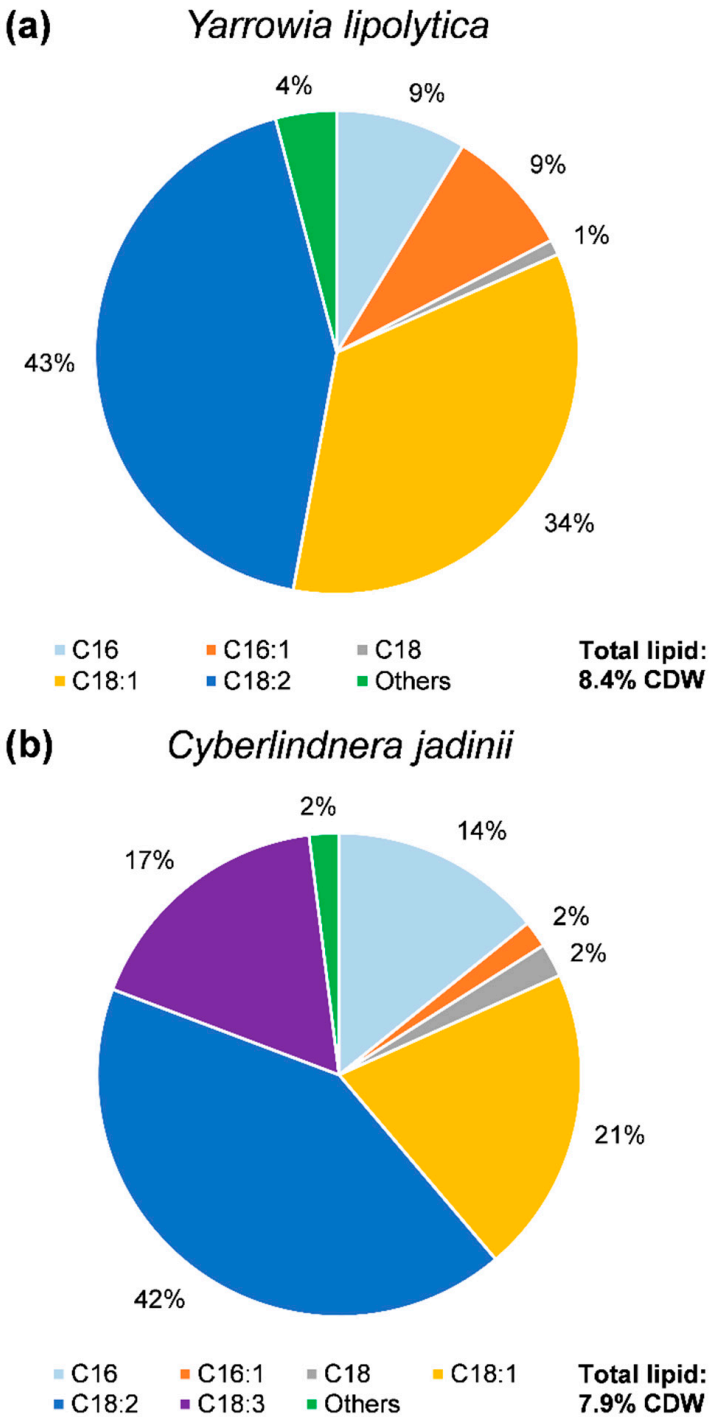
A marked difference in the essential amino acid content of the different microorganisms can be observed, with *C. jadinii* demonstrating 43.2% EAA content in available protein, while *Y. lipolytica* achieved 27.8% and *C. glutamicum* 39.9% EAA. The amino acid profiles and EAA contents are similar to those reported in the literature when cultivated on glucose as a substrate, with *C. jadinii* having an average of 46.8% EAA [3], *Y. lipolytica* 13.1% to 41.4% EAA [51] and *C. glutamicum* 38.3% EAA [59]. The medium composition and bioprocess conditions influence the amino acid composition, and further work to optimize these values is needed while also considering economical inputs for nitrogen and phosphorus, likely through the valorization of nitrogenous organic sidestreams.

Interestingly, in this study, microorganisms were observed to produce substantial amounts of accumulated microbial lipids, reaching values of 7.8 to 8.4% of total cell biomass. This is a marked change from previous work with *C. jadinii* cultivated on spruce sugars, which achieved only 0.4–1.8% lipids [1]. Thus, compared to when simple sugars are used as a substrate, low-molecular-weight fermentation end-products such as lactic acid, acetic acid and ethanol may be more readily diverted into lipid synthesis. The relatively high degree of lipid accumulation achieved using a two-stage approach through the utilization of cellulose-derived fermentation end-products has the potential to increase the nutritional value of whole-cell biomass through enhanced lipid accumulation. Lipid yield coefficients of up to 0.023 ± 0.001 g/g from cellulose and 0.036 ± 0.001 g/g from total substrates were achieved for *Y. lipolytica* in the two-stage bioconversion (Table 1), which can be compared to 0.16 g/g lipid yield from acetate [60], 0.005 to 0.011 g/g from a mixture of volatile fatty acids [61] and 0.17 ± 0.01 g/g from fatty acid distillate [62] as reported in the state-of-the-art. Lipid accumulation in oleaginous yeasts is triggered by low nitrogen contents, but the optimal carbon-to-nitrogen ratio depends on the yeast species, fermentation feedstock and bioprocess conditions [63]. Further optimization is required to effectively modulate the lipid yield from the cocktail of cellulose-derived end-products.

The analyzed lipid composition shows variability across tested strains as well (Figure 5). *Y. lipolytica* demonstrated an unsaturated lipid content of 89%, which was comprised mainly of C16:1 (9%), C18:1 (34%) and C18:2 (43%). *C. jadinii* also demonstrated a predominant (i.e., 83%) unsaturated lipid content comprised mainly of C18:1 (21%), C18:2 (42%) and C18:3 (17%), and thus showing a higher degree of polyunsaturated lipid content (59%) compared to *Y. lipolytica* (43%). Conversely, in the case of *C. glutamicum*, a relatively lower unsaturated lipid content was observed, comprised exclusively of monounsaturated C18:1 (49%), with a relatively high saturated content of C16 (43%), in agreement with

previously reported lipidomic profiling [64]. These results suggest that yeasts accumulate a higher fraction of diverse polyunsaturated lipids, while bacteria accumulate more saturated and mono-unsaturated lipids. This could have important implications in feed nutrition, warranting further exploration of medium optimization and cultivation conditions for enhanced polyunsaturated lipid production such as nitrogen limitation.

Titre, rate and yields of the two-stage bioconversion processes with *C. jadinii*, *Y. lipolytica* and *C. glutamicum* have been compared, and *Y. lipolytica* was found to be the statistically best-performing microorganism in terms of titre, rate and yield for biomass, protein and lipid production (Figure 6).



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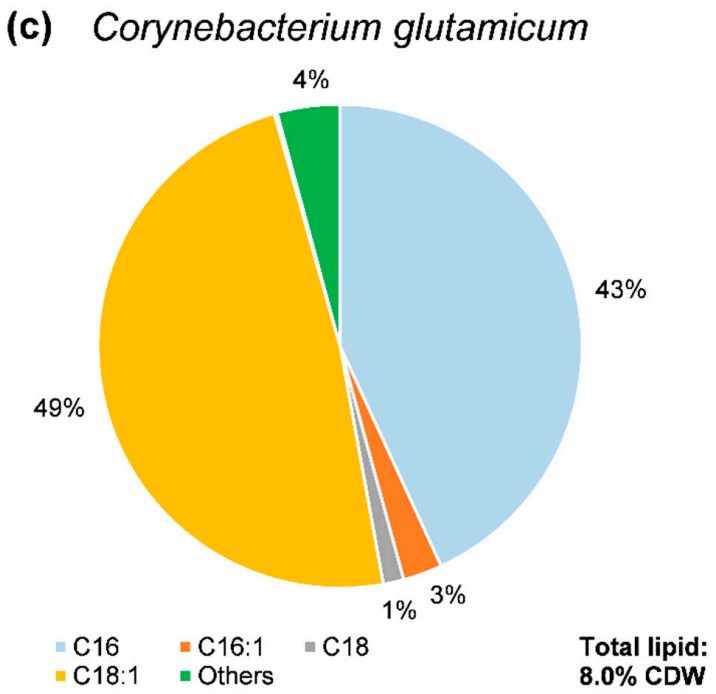

Figure 5. Lipid fatty acid composition for (**a**) *Y. lipolytica*, (**b**) *C. jadini*, and (**c**) *C. glutamicum* cultivated on end-products derived from the bioconversion of 20 g/L cellulose by a cellulolytic consortium.

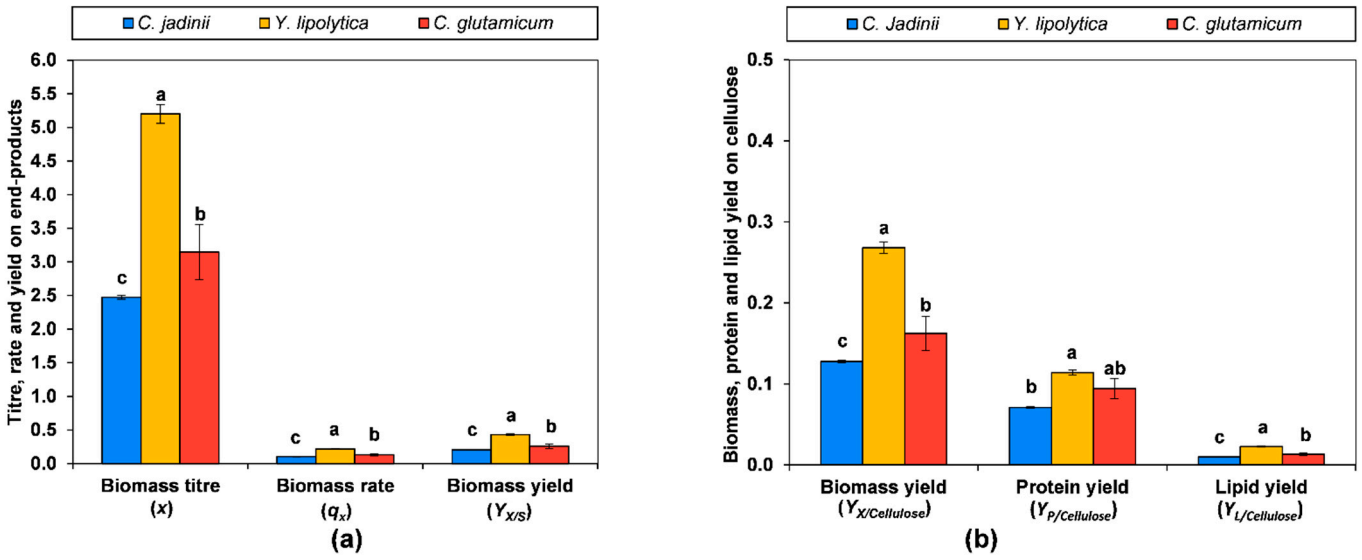

Figure 6. Titre, rate and yield of the two-stage bioconversion of cellulose with utilization of end-products by *C. jadinii*, *Y. lipolytica* and *C. glutamicum*: (**a**) biomass titre (x), biomass rate (q_x) and biomass yield ($Y_{X/S}$) on end-products; (**b**) biomass yield ($Y_{X/Cellulose}$), protein yield ($Y_{P/Cellulose}$) and lipid yield ($Y_{L/Cellulose}$). Means for each parameter not sharing any letter are significantly different by the Holm–Bonferroni test with $\alpha = 0.05$.

4. Conclusions

This work demonstrates that practical amounts of nutritious microbial biomass, known as single-cell protein and oil, can be generated from a recalcitrant cellulosic material using a consolidated bioprocess leveraging a naturally occurring cellulolytic consortium. The cellulolytic consortium was characterized by metagenomic sequencing and consisted mainly of *Thermoanaerobacterium thermosaccharolyticum*, *Sporolactobacillus* spp. and *Clostridium sensu stricto*. The cellulose hydrolysis capability of the cellulolytic consortium was compared to

Acetivibrio thermocellus (formerly *Clostridium thermocellum*) in bottles and bioreactors, and an end-product cocktail consisting of acetic acid, butyric acid, lactic acid and ethanol obtained.

Three microorganisms used extensively in food and feed, *Cyberlindnera jadinii*, *Yarrowia lipolytica* and *Corynebacterium glutamicum*, were successfully cultured on the end-products, producing biomass with up to 0.268 ± 0.007 g/g biomass yield from cellulose, with a high protein content of 42.5% to 57.9% and essential amino acid contents of 27.8% to 43.2%. Lipid contents of 7.9% to 8.4% were obtained, with *C. jadinii* and *Y. lipolytica* having a high proportion of unsaturated fatty acids of 83% and 89%, respectively. The produced single-cell protein and oil thus has a high nutritional value for animal feed application.

This establishes a proof of concept for a consolidated bioprocess consisting of a two-stage bioconversion of cellulose for microbial biomass production, which can increase food security and sustainability and contribute towards a biomass-based circular bioeconomy. This work aims to establish cellulolytic consortia as a platform for cellulosic bioconversion by producing mixed fermentation end-products and, subsequently, single-cell protein and oil. Future studies will apply this promising low-cost two-stage approach to diverse real-world residues while assessing techno-economic feasibility, environmental footprint and food production implications.

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

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