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Coprocessing Corn Germ Meal for Oil Recovery and Ethanol Production: A Process Model for Lipid-Producing Energy Crops

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Abstract: Efforts to engineer high-productivity crops to accumulate oils in their vegetative tissue present the possibility of expanding biodiesel production. However, processing the new crops for lipid recovery and ethanol production from cell wall saccharides is challenging and expensive. In a previous study using corn germ meal as a model substrate, we reported that liquid hot water (LHW) pretreatment enriched the lipid concentration by 2.2 to 4.2 fold. This study investigated combining oil recovery with ethanol production by extracting oil following LHW and simultaneous saccharification and co-fermentation (SSCF) of the biomass. Corn germ meal was again used to model the oil-bearing energy crops. Pretreated germ meal hydrolysate or solids (160 and 180 °C for 10 min) were fermented, and lipids were extracted from both the spent fermentation whole broth and fermentation solids, which were recovered by centrifugation and convective drying. Lipid contents in spent fermentation solids increased 3.7 to 5.7 fold compared to the beginning germ meal. The highest lipid yield achieved after fermentation was 36.0 mg lipid g^{-1} raw biomass; the maximum relative amount of triacylglycerol (TAG) was 50.9% of extracted oil. Although the fermentation step increased the lipid concentration of the recovered solids, it did not improve the lipid yields of pretreated biomass and detrimentally affected oil compositions by increasing the relative concentrations of free fatty acids.

Keywords: oil-bearing energy crops; corn germ meal; oil recovery; cellulosic ethanol

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1. Introduction

The need to reduce greenhouse gas emissions provides a strong impetus for the production of renewable fuels. Biofuels produced from dedicated biomass crops and agricultural residues are considered as future sustainable alternatives to petroleum-based fuels. Biodiesel, currently produced mainly from vegetable oils, is one of the most widely used biofuels. Its production results in the release of fewer greenhouse gases and pollutants into the atmosphere than fossil fuels while yielding comparable engine performance [1,2]. It can be integrated into the existing infrastructure for fueling diesel vehicles. In addition, its production process has been improved technically and economically via the development of bio-based catalysts for the transesterification reaction [3] and application of silica in the purification of crude biodiesel [4]. However, soybean yields, the major crop for biodiesel, constrain production and add costs to biodiesel. In the US, 57.5 billion gal of distillate fuel was consumed in 2020, while only 1.82 billion gal of biodiesel was produced—61% using soybean oil [5,6]. Biodiesel retailed at a national average price of 2.36 to 3.51 \$ gal^{-1} during

the first quarter of 2020, which is generally higher than the average retail price of diesel fuel at 2.43 \$ gal^{-1} [7,8]. The manufacturing cost of biodiesel is determined by the cost of bio-oil [2,9].

The development of energy crops that accumulate lipids in their vegetative tissues can overcome the oil supply barrier because they can be grown on marginal lands and/or provide higher production yields compared to traditional oil seed crops (e.g., soybean). Recent advances in plant biotechnology have led to the successful engineering of lipid production in tobacco, sugarcane and potato plants, albeit at low lipid levels [10–16]. This allows for the simultaneous production of biodiesel from plant lipids and high-value fuels or chemicals from cellulosic residues. A coproduction scheme for oil-bearing energy crops affords an opportunity to boost production and lower the cost for biodiesel. Technoeconomic analysis [17] predicted that biodiesel production costs from lipidcane containing 2% to 20% lipid content in plant stems (0.59 to 0.89 \$ L^{-1}) were less than soy oil (1.08 \$ L^{-1}). A major technical challenge to utilizing an oil-bearing energy crop is developing an efficient process to recover oil from vegetative tissues and convert recalcitrant lignocellulosic biomass into fermentable sugars [18,19].

Due to the insufficient availability of engineered oil-bearing crop samples, corn germ meal, with 2.3% oil on a dry basis [20], was used as a model lignocellulosic energy crop. Corn germ meal, the solid residue of corn germ after oil extraction from wet-milled corn, is a low-value product rationed in ruminant animal diets [21,22]. Liquid hot water (LHW) pretreatment, an essential step to enhance the enzymatic recovery of fermentable sugars, solubilizes hemicellulose and partially removes lignin. Previously, corn germ meal was used as a model feedstock to optimize LHW pretreatment conditions for the lipid enrichment of solids [20]. At the optimal pretreatment severity, lipid content in residual solids increased 2.2 to 4.2 fold.

Having established the compatibility of LHW for oil recovery [20], the goal of this study was to ferment the pretreated germ meal and investigate the fate of oil during hydrolysis and fermentation. Simultaneous saccharification and co-fermentation (SSCF) is a method that combines the enzymatic hydrolysis of carbohydrates to monosaccharides and their fermentation to ethanol by yeast; metabolically modified yeast *Saccharomyces cerevisiae* [23–26] can be used to ferment glucose (from the hydrolysis of cellulose) and xylose (from the hydrolysis of hemicellulose) together. SSCF is expected to increase ethanol conversion efficiencies and final ethanol titers compared to other common fermentation schemes (e.g., separate enzymatic hydrolysis and fermentation, and simultaneous saccharification and fermentation) [14,27,28]. A higher final ethanol titer reduces distillation costs and energy consumption [29]. Removal of lipids post-fermentation would be advantageous because it would streamline the entire process. This study also examined the fractionation of lipids between the liquid and residual solids following fermentation.

2. Materials and Methods

2.1. Corn Germ Meal Samples

Corn germ meal samples were collected from a commercial wet milling facility and dried in an oven maintained at 45 °C for 24 h to reduce moisture content to less than 5% on a wet basis. Dried samples were stored at 4 °C. Corn germ meal is composed of 13.6% water/ethanol extractives, 31.0% glucan, 22.4% xylan, 5.1% acid-insoluble lignin, 8.4% acid-soluble lignin, and 0.03% ash, on a dry basis [20].

2.2. Hot Water Pretreatment

Corn germ meal samples and deionized water were mixed to form a slurry at 20% ($w\ w^{-1}$) solid content; slurry was transferred into 50 mL stainless steel tube reactors (316 stainless steel tubing, 1.905 cm outer diameter × 0.165 cm wall × 10.478 cm length, SS-T12-S-065–20, Swagelok, Chicago Fluid System Technologies, Chicago, IL, USA). Reactors were capped with stainless steel caps (316 stainless steel, SS-1210-C, Swagelok, Chicago Fluid System Technologies, Chicago, IL, USA). Reactors were immersed in a fluidized sand

bath (IFB-51 Industrial Fluidized Bath, Techne Inc., Burlington, NJ, USA) and heated and held at 160 °C or 180 °C for 10 min (Figure 1). The reaction temperature was monitored by a thermocouple (Penetration/Immersion Thermocouple Probe Mini Conn (temperature ranges from −250 °C to 900 °C), McMaster-Carr, Robbinsville, NJ, USA) inserted into one tube reactor and connected to a data logger (HH306/306A, Datalogger Thermometer, Omega, Stamford, CT, USA). After pretreatment, tube reactors were submerged in cold water to quench the reaction.

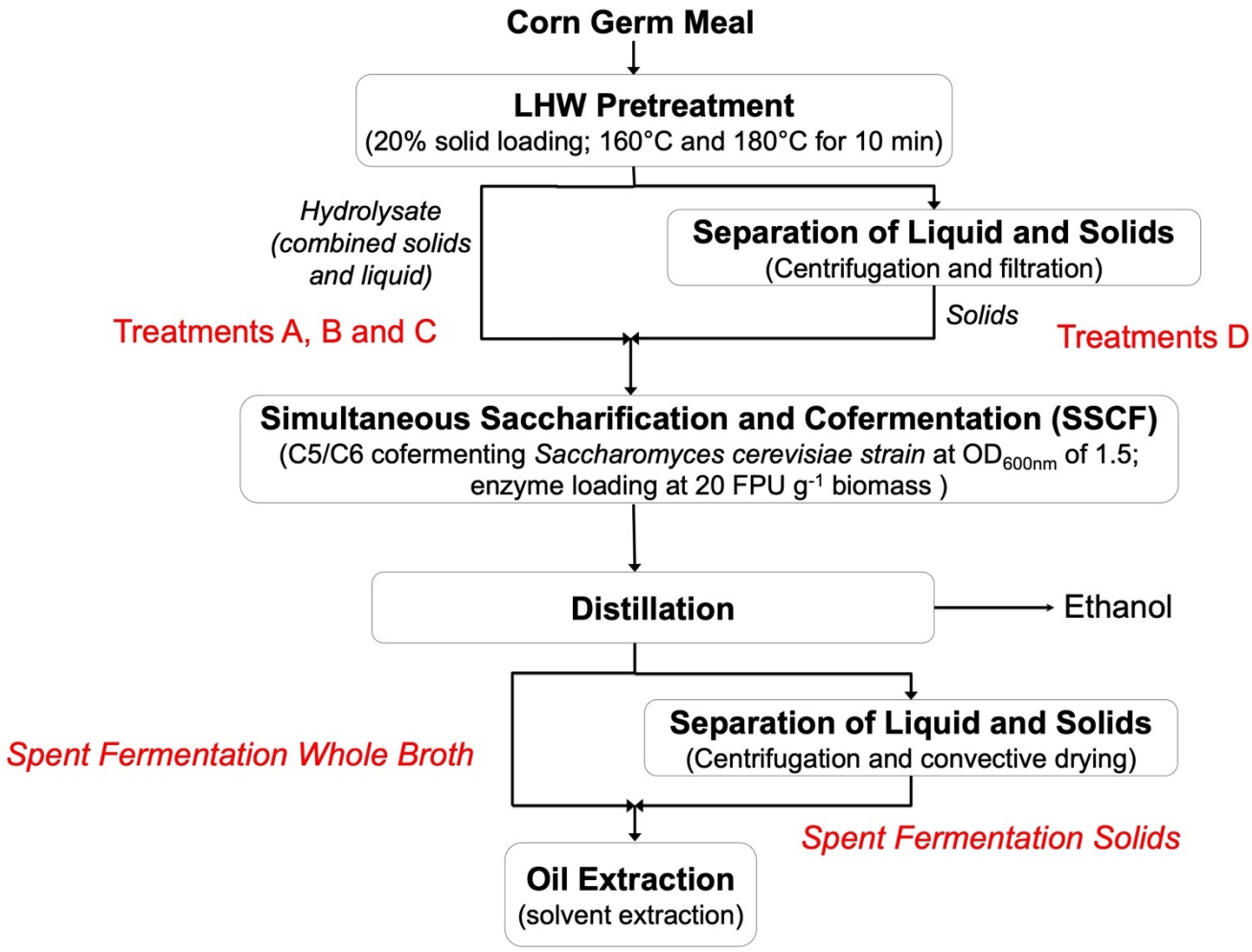

Figure 1. Flow diagram of experimental process to produce lipids and ethanol from corn germ meal. Corn germ meal was pretreated. Pretreated hydrolysate or solids were fermented for 96 h to produce ethanol. After distillation, lipids were extracted from spent fermentation whole broth or solids.

The severity parameter R_o is defined by Equation (1) [18,30–32]. Severity of LHW pretreatment was measured by the log severity factor, represented by Log R_o. The log severity factor is a common method for describing pretreatment conditions [18,32–36].

$$R_o = t \times \exp[(T - 100)/14.75] \quad (1)$$

where R_o is the severity parameter, t is the residence time (min), and T is the pretreatment temperature (°C).

For the fermentation of pretreated solids only, pretreated hydrolysate was centrifuged at 15,000× *g* for 15 min to collect pretreated solids. Liquid was separated immediately and collected for HPLC (Bio-Rad Aminex HPX 87H, Biorad, Hercules, CA, USA) to measure levels of inhibitors and acids generated from pretreatment.

2.3. Yeast Culture Preparation

A co-fermenting *Saccharomyces cerevisiae* yeast strain provided by DSM (Heerlen, The Netherlands) was used. The yeast was prepared according to Wang et al. [32]. Seed culture was obtained by inoculating a single colony of the yeast strain in 3 mL YPD media (2% $w\ v^{-1}$ glucose, 2% $w\ v^{-1}$ Bacto™ peptone and 1% $w\ v^{-1}$ yeast extract) at 32 °C and 225 rpm for 24 h. One mL of seed culture was mixed with 25 mL of YPD media, and the yeast culture was incubated at 32 °C and mixed at 225 rpm; the optical density (OD_{600nm}) of the culture was measured using a spectrophotometer set to 600 nm (Evolution 60S, Thermo Scientific, Waltham, MA, USA). When the culture reached an OD_{600nm} of 4, it was centrifuged at 2305× g and the cell pellet resuspended in phosphate-buffered saline to an OD_{600nm} of 50. Resuspended yeast culture was preserved for later use during fermentation.

2.4. Simultaneous Saccharification and Co-Fermentation (SSCF)

Pretreated biomass was fermented using procedures adapted from Wang et al. [32]. Biomass samples were mixed with deionized water to achieve desired solid content, 1 M citrate buffer (pH 6.0) to achieve a final buffer concentration of 0.05 M and YP medium (2% $w\ v^{-1}$ bacto peptone and 1% $w\ v^{-1}$ yeast extract). The pH was adjusted to pH at 6.0 by adding calcium hydroxide solution. A mixture of cellulases and hemicellulases (NS22,257, Novozymes North America, Inc., Franklinton, NC, USA) with an enzymatic activity of 231 FPU mL^{-1} was added at 20 FPU g^{-1} dry substrate and the culture was inoculated to a beginning OD_{600nm} of 1.5 in the final mixture. For raw or pretreated solids (treatment A and D), SSCF was conducted at 20% $w\ w^{-1}$ solid content. For LHW pretreated hydrolysate at 160 °C or 180 °C at 10 min (treatment B and C), SSCF was conducted at 15% and 14% ($w\ w^{-1}$), respectively. These are the solid contents of LHW pretreated hydrolysate after addition of YP media, buffer, yeast culture, enzyme, and carbon hydroxide solution [37]. Enzyme blanks and samples in duplicate were incubated at 32 °C and 150 rpm. Samples (0.5 mL) taken at 0, 3, 6, 9, 24, 48, 72 and 96 h were analyzed using HPLC (Bio-Rad Aminex HPX-87H, Biorad, Hercules, CA, USA) for sugar and ethanol concentrations. Three treatments (A, B, C) of SSCF for LHW pretreated corn germ meal were first conducted (Table 1, Figure 1): in treatment A, which served as the control, raw germ meal was fermented at 20% ($w\ w^{-1}$) solid content; in treatment B, 160 °C LHW/10 min pretreated germ meal hydrolysate (combined solid and liquid fractions) was fermented at 15% ($w\ w^{-1}$) solid content; in treatment C, 180 °C LHW/10 min pretreated hydrolysate (combined solid and liquid fractions) was fermented at 14% ($w\ w^{-1}$) solid content. Treatment C was expected to have higher ethanol conversion efficiency than treatment B because of its higher pretreatment temperature [18,20,38]. However, as discussed in detail in Section 3.1, this expectation was not met, which could be due to inhibitors released into the liquid portion of pretreated germ meal mixture during pretreatment. Therefore, treatment D was conducted to improve treatment C, whereby the liquid proportion of the pretreated hydrolysate (180 °C LHW/10 min) was separated, and the solid fraction was fermented at 20% ($w\ w^{-1}$) solid content. After fermentation, ethanol was removed from the spent fermentation whole broth by heating the broth at 90 °C for 1 h [39]. Ethanol conversion efficiency (%, $w\ w^{-1}$) was calculated based on beginning glucan and xylan contents in untreated germ meal. Beginning glucan and xylan contents were calculated based on compositions of untreated germ meal, including 31.0% glucan and 22.4% xylan, obtained from Jia et al. [20]. Solid content of pretreated germ meal hydrolysate was determined to calculate amounts of beginning untreated germ meal used for treatments B and C. Solid recovery rate (36.8%) of 180 °C LHW/10 min [20] was used to calculate amounts of beginning untreated germ meal used for treatment D.

Table 1. Treatments of SSCF for LHW [1] pretreated corn germ meal.

Treatment	Pretreatment Condition	Log Severity Factor (Log R_o)	Solid Content (%, $w\ w^{-1}$)
A [2]	Untreated	0	20
B [3]	160 °C LHW/10 min	2.8	15 [4]
C [5]	180 °C LHW/10 min	3.4	14 [4]
D [6]	180 °C LHW/10 min	3.4	20

[1] LHW = liquid hot water pretreatment. [2] A: Raw germ meal was fermented at 20% $w\ w^{-1}$ solid content. [3] B: 160 °C LHW/10 min pretreated germ meal hydrolysate (solid and liquid fraction) fermented at 15% $w\ w^{-1}$. [4] Highest possible solid content was calculated when no additional deionized water was added with addition of YP medium, buffer, resuspended yeast culture, enzyme, and carbon hydroxide solution. [5] C: 180 °C LHW/10 min pretreated germ meal hydrolysate fermented at 14% $w\ w^{-1}$. [6] D: Liquor fraction of 180 °C LHW/10 min pretreated germ meal hydrolysate was removed prior to fermentation. Solid fraction was separated and fermented at 20% $w\ w^{-1}$.

2.5. Lipid Extraction

Spent fermentation whole broth was centrifuged at 15,000× *g* for 15 min to remove supernatant, and the residual spent fermentation solids were dried for 24 h at 45°C using a convection oven. Lipids were extracted from spent fermentation whole broth and solids using extraction procedures outlined by Huang et al. [39]. Briefly, spent fermentation whole broth or solids were mixed with hexane (15 mL) and isopropanol (10 mL). The mixture was homogenized twice (1 min each time) using a homogenizer (LabGen 700, Cole Parmer, Vernon Hills, IL, USA) and mixed for 10 min using a wrist action shaker (HB-1000 Hybridizer, UVP LLC, Upland, CA, USA). Next, 16 mL of sodium sulfate solution (6.7% $w\ v^{-1}$) was added to the sample and was mixed for another 10 min. The mixture was centrifuged at 5× *g* for 20 min and supernatant was transferred to a tared centrifuge tube for evaporation under nitrogen. The tube was reweighed after lipids were dried. Extractable lipid yield (mg lipid g^{-1} raw biomass) was calculated as the ratio of extracted lipids and beginning untreated germ meal. Lipid concentrations of spent fermentation solids were calculated as the ratio of extracted lipids and weight of solid samples from which lipids were extracted.

2.6. Lipid Analyses

For lipid composition analysis, dried oil extracts were redissolved in hexane to a concentration of 10 mg mL^{-1}. They were filtered (0.45 µm PTFE) into HPLC vials. Samples were analyzed using a HPLC system (LC-20AT, Shimadzu, Columbia, MD, USA) equipped with a photodiode array (PDA) (SPD-M20A, Shimadzu, Columbia, MD, USA) and evaporative light scattering defectors (ELSD) (Model-LTII, Shimadzu, Columbia, MD, USA) [20]. External standard curves were developed with the ELSD for triacontane (hydrocarbon, HC), behenyl behenate (wax ester, WE), cholesteryl oleate (steryl ester, SE), monoolein (monoacylglycerol, MG), diolein (diacylgycerol, DG), triolein (triacylglycerol, TAG), oleic acid (free fatty acid, FFA) and cholesterol (sterol, ST), and the PDA set at 320 nm for oryzanol (steryl ferulate, SF). Calibrations were linear for all components except for SE, which was fitted a with second-order polynomial, all with $R^2 \geq 0.99$. Method limits of quantitation were 0.5% ($w\ w^{-1}$) for HC, WE, SE, DG, MG, TAG and ST and 0.05% ($w\ w^{-1}$) for SF.

Fatty acid profiling of extracted lipids was performed using procedures outlined by Quarterman et al. [40]. Dried oil extracts were trans-esterified using 2 mL of hexane and 0.2 mL of 2M KOH in methanol. The resulted fatty acid methyl esters (FAME) were analyzed by gas chromatography with flame ionization detection (GC-FID) (Agilent Technologies, Santa Clara, CA, USA) using an Agilent HP-88 capacity column (30 m × 0.25 mm). A commercial standard with 31 known FAMEs (Nu-Chek Prep, Inc., Elysian, MN, USA, cat. No. GLC-411) was used to identify retention times of FAMEs in the samples. Corn oil was analyzed as a control. Percent of total fatty acids was calculated based on the sum of all FAME peak areas identified by GC-FID.

2.7. HPLC Analysis of Sugars, Organic Acids, and Ethanol

Pretreated hydrolysate from LHW and samples from SSCF were centrifuged at 15,000× *g* for 15 min. Supernatants were filtered through a 0.2 µm PTFE filter and analyzed by HPLC (Bio-Rad Aminex HPX-87H, Biorad, Hercules, CA, USA) to determine concentrations of monosaccharides, organic acids, common inhibitors, and ethanol.

2.8. Statistical Analysis

Analysis of variance (ANOVA) and Tukey's honest significant difference (HSD) test were performed using R software (V.3.6.1, R Foundation for Statistical Computing, Vienna, Austria) to compare means of final ethanol titers, ethanol conversion efficiencies, inhibitor concentrations, lipid yields, and lipid concentrations. All experiments were analyzed using two replicates. The significance level for difference was chosen as 5% ($p < 0.05$).

3. Results and Discussion

3.1. Ethanol and Sugar Profile during SSCF

The corn germ meal was pretreated at three severities (treatment A, B, and C; Table 1). For treatments A, B, and C, glucose and xylose were consumed as fast as they were released after 24 h of fermentation (Figure 2). The glucose and xylose concentrations peaked at 3 h into the fermentations. Ethanol was produced rapidly as glucose and xylose were consumed. Treatments A, B, and C resulted in final ethanol titers of 29.3, 32.5, and 27.9 g L^{-1} and ethanol conversion efficiencies of 38.9%, 60.1%, and 49.9%, *w* w^{-1}, respectively (Figure 3). Therefore, treatment B resulted in the greatest ethanol titer and ethanol conversion efficiency among these three treatments ($p < 0.05$).

Although more severe pretreatment conditions were previously observed to aid the release of monosaccharides [18,38], ethanol conversion efficiency did not increase uniformly with pretreatment severity in this study. This can be explained by the inhibitor concentrations in the LHW pretreated hydrolysates (Table 2): LHW at 160 °C for 10 min had lower amounts of 5-hydroxymethylfurfural (HMF) and furfural in the pretreated liquid (0.16 and 0.70 g L^{-1}, respectively) compared to LHW at 180 °C for 10 min (0.62 and 2.31 g L^{-1} HMF and furfural, respectively). Other inhibitors in the pretreated liquid, including lactic acid (formation of lactic acid is explained in Appendix A), formic acid, and acetic acid, were observed at such low concentrations that they could not cause inhibition [41–44]. The HMF and furfural concentrations were higher than the inhibitor concentrations observed in pretreated hydrolysates of sugarcane bagasse and biomass sorghum [18,32,34]. It is possible that the inhibitors present in the hydrolysate used in treatment C led to the lower ethanol titer. Microbial inhibitors are soluble; therefore, treatment C was modified (in treatment D) by removing the pretreatment liquor. The solid content was increased to 20% in SSCF. These changes led to an increased final ethanol titer of 32.4 g L^{-1}, which demonstrated that inhibitors were responsible for the poorer fermentation results for treatment C. It might be supposed that removing the liquid prior to fermentation (treatment D) would be preferable; however, this led to a decreased ethanol conversion efficiency of 15.7%, *w* w^{-1}, which was caused by the significant loss of fermentable sugars released during pretreatment by discarding pretreated liquor [20]. Thus, fermenting pretreated solids instead of the whole hydrolysate (combined solid and liquid portion) was disadvantageous for fermentation. The ethanol titer for treatment A was surprisingly high given that it had received no pretreatment. This is due to the lower recalcitrance of seed cell walls compared to stems and leaves [45–47]. Therefore, it is anticipated that pretreatment will be essential for SSCF of plants engineered for oil production stored in stems.

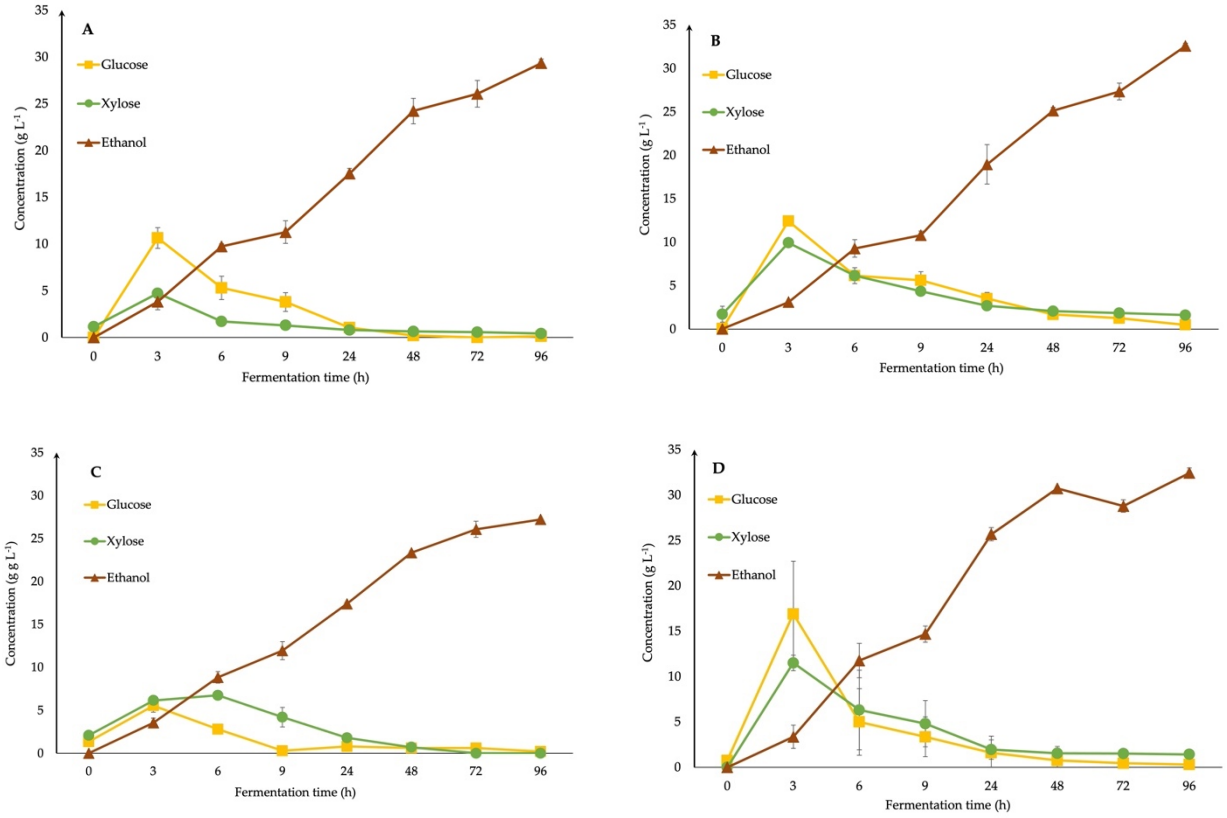

Figure 2. Glucose, xylose, and ethanol concentrations during 96 h of SSCF in treatments A, B, C, and D. Error bars represent ± one standard deviation. Two replicates per mean were used. (**A**) Treatment A: raw germ meal fermented at 20% $w\ w^{-1}$ solid content. (**B**) Treatment B: 160 °C LHW/10 min pretreated germ meal hydrolysate (solid and liquid fraction) fermented at 15% $w\ w^{-1}$. (**C**) Treatment C: 180 °C LHW/10 min pretreated germ meal hydrolysate fermented at 14% $w\ w^{-1}$. (**D**) Treatment D: solid fraction from 180 °C LHW/10 min pretreated germ meal hydrolysate was separated and fermented at 20% $w\ w^{-1}$.

It is notable that treatment B had the highest ethanol conversion efficiency (Figure 3) and its final ethanol concentration was comparable to that for treatment D, where the liquid/inhibitors had been removed prior to fermentation. The final ethanol titers for treatments B and D were 32.5 and 32.4 g L^{-1}, respectively. These ethanol titer results compare favorably to past results: [48] reported an ethanol concentration of 29.5 g L^{-1} after SSCF of dilute acid pretreated corn stover; [49] reported an ethanol concentration of 36.5 g L^{-1} after SSCF of aqueous ammonia pretreated corn stover; [50] observed an ethanol concentration of 32.6 g L^{-1} after SSCF of alkali pretreated wheat straw. The final ethanol titers of treatments B and D were in the same range of reported values from the co-fermentation of lignocellulosic feedstocks, although further improvement is desired [29,48–51].

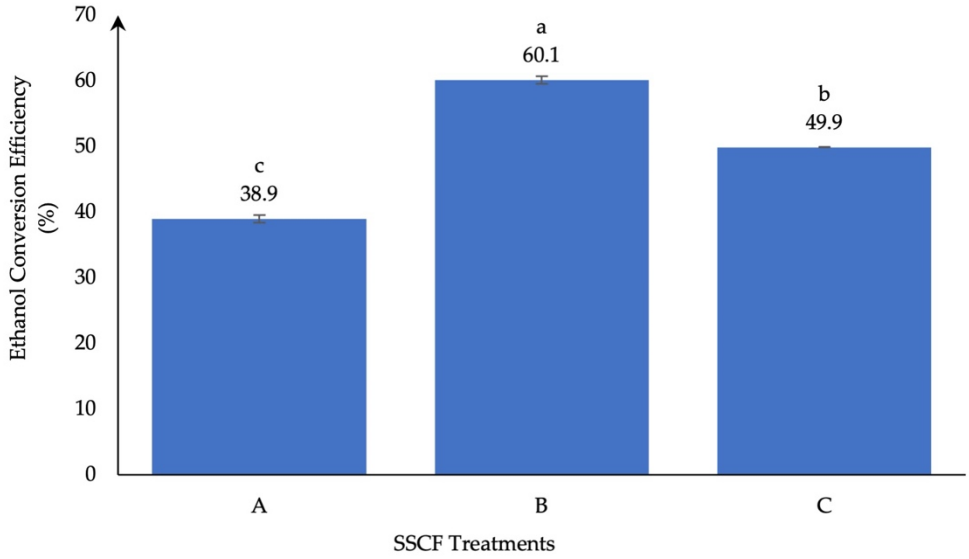

Figure 3. Mean ethanol conversion efficiency (%, $w\ w^{-1}$) at end of 96 h of SSCF in treatments A, B, and C. Error bars represent ± one standard deviation. Means without same letters were different. Two replicates per mean were used. Treatment A: raw germ meal fermented at 20% $w\ w^{-1}$ solid content. Treatment B: 160°C LHW/10 min pretreated germ meal hydrolysate (solid and liquid fraction) fermented at 15% $w\ w^{-1}$. Treatment C: 180 °C LHW/10 min pretreated germ meal hydrolysate fermented at 14% $w\ w^{-1}$.

Table 2. Inhibitor concentrations of LHW [1] pretreated liquid sample [2].

Inhibitors (g/L)	Treatments	
	B (160 °C LHW/10 min)	C (180 °C LHW/10 min)
Lactic acid	0.39 ± 0.04 a	0.36 ± 0.01 a
Formic acid	0.43 ± 0.52 a	0.86 ± 0.01 a
Acetic acid	0.38 ± 0.37 a	1.19 [3] a
Levulinic acid	BDL [4]	BDL
HMF [5]	0.16 ± 0.04 b	0.62 ± 0.01 a
Furfural	0.70 ± 0.16 b	2.31 ± 0.02 a

[1] LHW = liquid hot water pretreatment. [2] Results are represented as mean ± 1 standard deviation. Two replicates per mean were used. Means without same letters within one row were different. [3] Standard deviations below 0.01 were not displayed. [4] BDL = below the detectable limit (0.01 g/L). [5] HMF = 5-hydroxymethylfurfural.

The ethanol conversion efficiency of pretreated corn germ meal was relatively low compared to typical lignocellulosic feedstocks that are plant stems or leaves, such as sugarcane bagasse, hemp, corn stover, reed, and switchgrass. Ethanol conversion efficiencies of fermentation using LHW-pretreated typical cellulosic biomass ranged from 75% to 94% (Table A1 in Appendix B) [32,52–55]. Typical cellulosic feedstocks contain cell walls with greater recalcitrance, explaining why their glucan content was higher than germ meal and increased or remained the same after LHW, and they were pretreated with more severe conditions without significant loss of solids or carbohydrates [32,52–56]. Germ meal, however, contains cell walls with lower recalcitrance, and the recalcitrance was reduced during the wet milling process as well [22,45–47,57]. Significant loss of solids and sugars after 160 °C LHW/10 min was observed for germ meal [20], which could be related to its low ethanol conversion efficiency.

3.2. Lipid Recovery after SSCF

SSCF of LHW pretreated biomass was expected to extract cell wall carbohydrates during enzymatic hydrolysis and promote lipid recovery from the spent fermentation whole broth (both solids and liquid) and spent fermentation solids. Treatment C resulted

in the highest lipid yield of 36.0 mg lipid g^{-1} raw biomass (Figure 4). Extractable lipid yields after fermentation increased with increased pretreatment severity. However, following pretreatment and before fermentation, the highest lipid yield achieved by extraction from pretreated germ meal was 34.0 mg lipid g^{-1} raw biomass [20]. No difference was detected between the highest lipid yield after fermentation and the highest lipid yield from pretreated germ meal ($p > 0.05$), indicating that SSCF did not further improve the lipid yields of pretreated biomass.

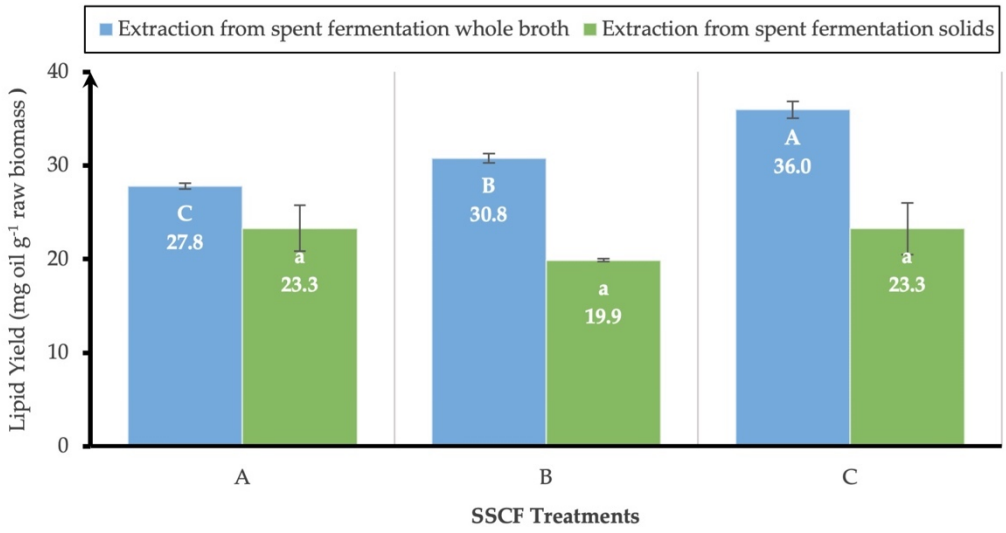

Figure 4. Extractable lipid yields (mg lipid g^{-1} raw biomass) for treatments A, B, and C using SSCF and LHW pretreated germ meal. Error bars represent ± one standard deviation. Two replicates per mean were used. Data within one series without same letters were significantly different ($p < 0.05$): the uppercase letters were designated for extraction from spent fermentation whole broth; the lowercase letters were designated for extraction from spent fermentation solids. Treatment A: raw germ meal fermented at 20% $w\ w^{-1}$ solid content. Treatment B: 160°C LHW/10 min pretreated germ meal hydrolysate (solid and liquid fraction) fermented at 15% $w\ w^{-1}$. Treatment C: 180 °C LHW/10 min pretreated germ meal hydrolysate fermented at 14% $w\ w^{-1}$.

While the extractable lipid yield was always greater for spent whole broth versus solids, the difference increased with pretreatment severity for A, B, and C (4.5, 10.9, and 12.7 mg lipid g^{-1} raw biomass, respectively), which largely reflects lipids/fatty acids that migrated to the liquid section. This means that more lipids were released to the fermentation liquor during SSCF with increased severity. In previous studies [58–60] using corn germ, 90% of lipids were stored in the scutellum of the germ, a storage organ mainly composed of tissues, in the form of lipid droplets (LD), in which they are protected by a phospholipid layer and oleosin molecules. To extract lipids within LDs, LDs need to be dissociated from the storage tissue matrix and then chemically or physically disrupted to release the lipids [61–63]. SSCF following pretreatment theoretically could liberate LD to release lipids into the fermentation broth. Due to the absence of pretreatment in treatment A, fewer cell wall carbohydrates were hydrolyzed, and thus fewer LD were released from the tissues into the fermentation broth compared to treatments B and C. As expected, lipid released from the solids increased with increased pretreatment severity. However, the observed trend could be also partially related to the convective drying of spent fermentation solids. Some lipids are sensitive to thermal decomposition [64–67] and thus might have partially degraded in spent fermentation solids.

Lipid concentrations of spent fermentation solids from treatments A, B, and C were 9.7%, 12.0%, and 13.2%, $w\ w^{-1}$, respectively (Figure 5). Lipid concentrations of spent fermentation solids increased 4.2 to 5.7 fold compared to original germ meal (2.3%) [20]. Compared to a 2.2- to 4.2-fold increase in the lipid content of germ meal after LHW

pretreatment reported in [20], SSCF further improved the lipid concentrations. Treatments B and C both had the highest lipid concentrations of spent fermentation solids (Figure 5).

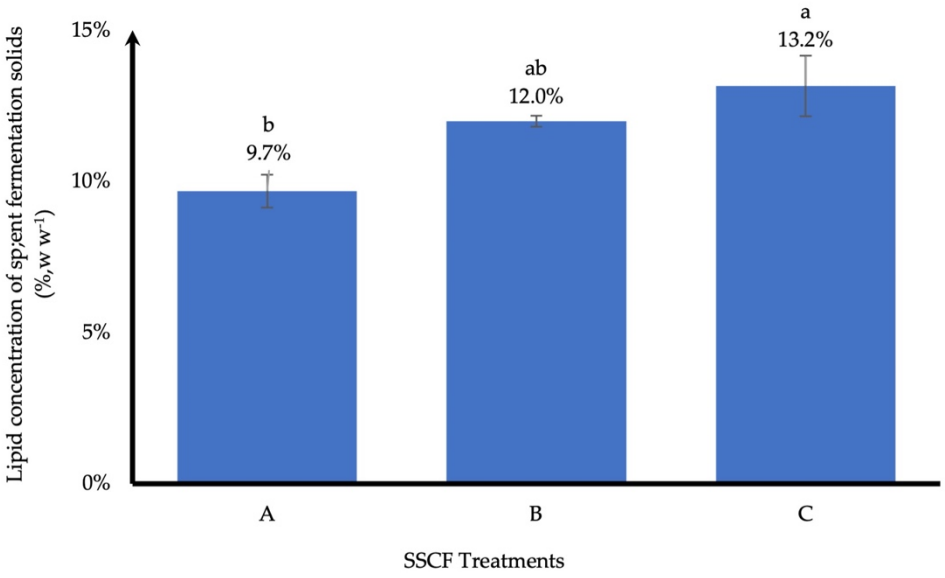

Figure 5. Lipid concentrations of spent fermentation solids (%, $w\ w^{-1}$) from treatments A, B, and C. Error bars represent ± one standard deviation. Means denoted with different letters are different. Two replicates per mean were used. Treatment A: raw germ meal fermented at 20% $w\ w^{-1}$ solid content. Treatment B: 160 °C LHW/10 min pretreated germ meal hydrolysate (solid and liquid fraction) fermented at 15% $w\ w^{-1}$. Treatment C: 180°C LHW/10 min pretreated germ meal hydrolysate fermented at 14% $w\ w^{-1}$.

For treatment D, lipid yields from spent fermentation whole broth and solids were 15.2 and 12.9 mg lipid g^{-1} raw biomass, respectively; the lipid concentration of spent fermentation solids was 12.9%. Lipid yields decreased compared to treatment C because of the significant loss of biomass by removing the pretreated liquor prior to fermentation. Therefore, removing pretreated liquor prior to fermentation (treatment D) was disadvantageous for lipid recovery.

3.3. Compositions of Oil and FAME from Oil

The recovered lipids were examined for composition (Figure 6). The largest fraction of lipids was TAGs, constituting 31.7% to 50.9% of extracted oil (Figure 6). Removing the liquor prior to fermentation (treatment D) lowered the TAG content of the spent whole fermentation broth from 41.3% to 31.7% (Figure 6), indicating another disadvantage of removing the liquor prior to fermentation. The maximum TAG content (50.9%) was observed in spent fermentation solids for treatment B. DG contents were up to four-fold higher in oil from treatments A and C compared to oil from treatments B and D (Figure 6). FFA contents constituted 8.1% to 16.1%, $w\ w^{-1}$ oil (Figure 6), so a neutralization step would be required to prevent FFA from hindering the transesterification reaction if the oil was used for biodiesel production [68–70]. MG, SE, SF, and HC contents were minor constituents (Figure 6), together constituting up to 18.0%, $w\ w^{-1}$ oil.

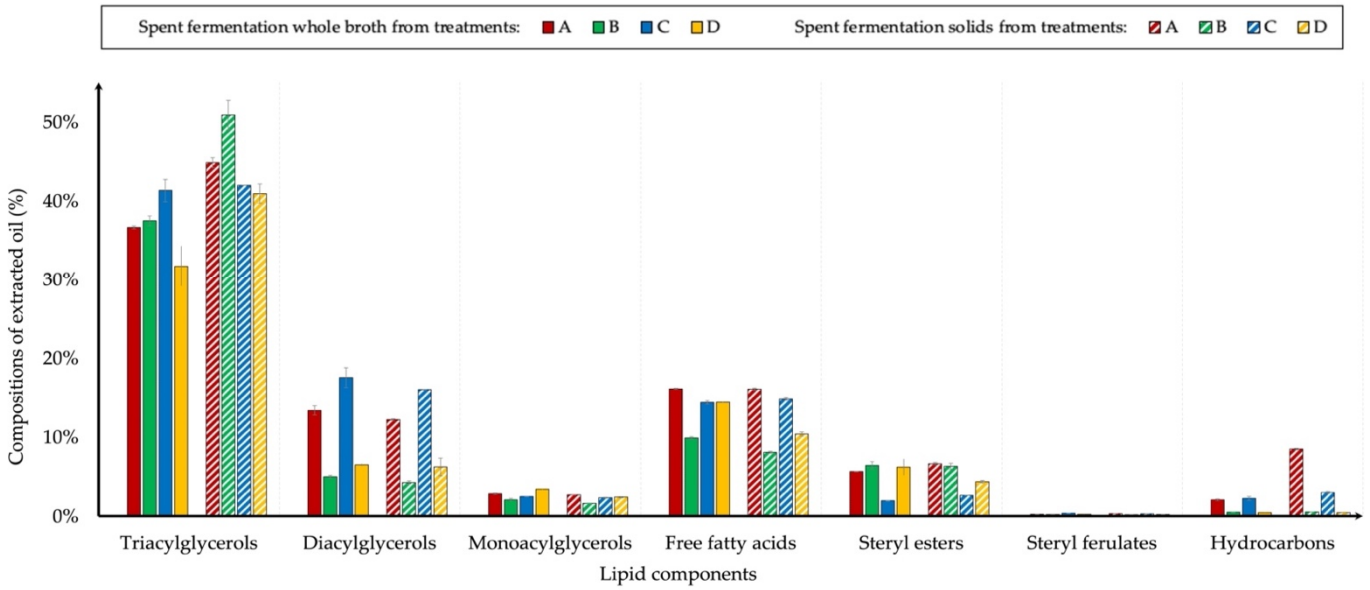

Figure 6. Compositions (%, $w\ w^{-1}$ oil) of extracted oil from spent fermentation whole broth and spent fermentation solids from treatments A, B, C, and D. Error bars represent ± one standard deviation. Two replicates per mean were used. Treatment A: raw germ meal fermented at 20% $w\ w^{-1}$ solid content. Treatment B: 160 °C LHW/10 min pretreated germ meal hydrolysate (solid and liquid fraction) fermented at 15% $w\ w^{-1}$. Treatment C: 180 °C LHW/10 min pretreated germ meal hydrolysate fermented at 14% $w\ w^{-1}$. Treatment D: solid fraction from 180 °C LHW/10 min pretreated germ meal hydrolysate was separated and fermented at 20% $w\ w^{-1}$.

Following pretreatment with LHW and before SSCF, the LHW pretreated corn germ contained 71.6% of TAG, 0.3% of FFA, and 6.2% of minor constituents [20]. Therefore, SSCF led to a substantial increase in the amount of relative FFAs (Figure 6), presumably because of the saponification of TAGS during SSCF. Increased FFA content is undesirable [65,66].

In the fatty acids analysis, palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), alpha-linoleic acid (C18:3), and arachidic acid (C20:2) were identified. Linoleic acid (C18:2) was the major component of all recovered oil extracts, constituting 53.1% to 54.4% of total fatty acids (Figure 7). Compositions of total FAME obtained from the extracted oil across treatments were similar, indicating that SSCF did not affect the fatty acid composition of TAGs. Moreover, the fatty acid compositions were similar to the corn oil control sample, including 54.4% linoleic acid, 29.2% oleic acid, and 12.7% palmitic acid.

Overall, SSCF detrimentally affected the compositions of oil from LHW pretreated germ meal.

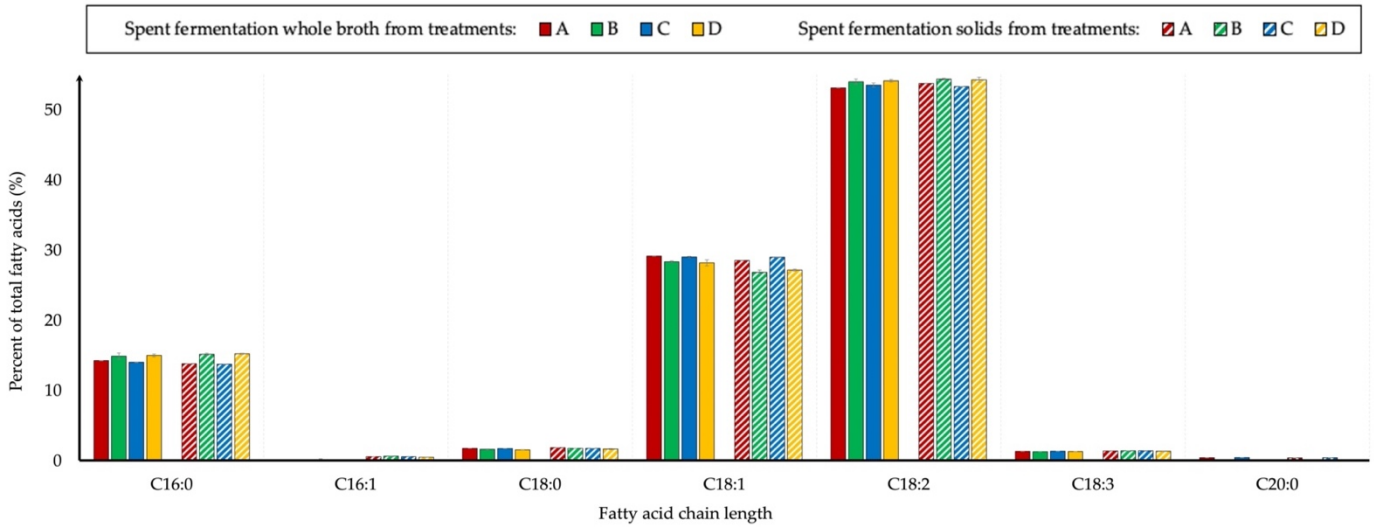

Figure 7. Compositions (%) of total FAME obtained from extracted oil from spent fermentation whole broth and spent fermentation solids from treatments A, B, C, and D. Error bars represent ± one standard deviation. Two replicates per mean were used. Treatment A: raw germ meal fermented at 20% $w\ w^{-1}$ solid content. Treatment B: 160 °C LHW/10 min pretreated germ meal hydrolysate (solid and liquid fraction) fermented at 15% $w\ w^{-1}$. Treatment C: 180 °C LHW/10 min pretreated germ meal hydrolysate fermented at 14% $w\ w^{-1}$. Treatment D: solid fraction from 180 °C LHW/10 min pretreated germ meal hydrolysate was separated and fermented at 20% $w\ w^{-1}$.

4. Conclusions

After fermentation, the highest lipid yield was 36.0 mg lipid g^{-1} raw biomass, the maximum relative amount of TAG was 50.9% of extracted oil, and the highest lipid concentration of spent fermentation solids was 12.0%. Compared to lipid recovery and compositions following pretreatment and before fermentation, the fermentation step did not improve the lipid yield of pretreated biomass and detrimentally affected oil compositions by increasing the relative concentrations of free fatty acids.

Glucose and xylose fermentation profiles during SSCF and compositions of FAME were similar across all treatments. Treatment B appears to be the most favorable as it resulted in the maximal ethanol titer (32.5 g L^{-1}), maximal ethanol conversion efficiency (60.1%), the highest lipid concentration (12.0%) of spent fermentation solids, and maximum relative amounts of TAG (50.9% of extracted oil).

5. Patents

A provisional patent application has been filed from this work (U.S. Patent Application No.: 62/945,438).

Author Contributions: Y.J. conducted all the experiments, analyzed the data, and prepared the manuscript; D.K. helped formulate the study and edited the manuscript; J.K.W.-M. conducted oil composition analyses and edited the manuscript; B.D. conducted FAME analyses and edited the manuscript; K.R. and M.E.T. helped formulate the study and edited manuscript; V.S. is the PI of this project, helped formulate the study, and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

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Appendix A

Although the formation of lactic acid during pretreatment has been observed for various lignocellulosic feedstocks, including sorghum, corn stover, and sugarcane bagasse [19,32,71], it is unclear how lactic acid is formed during pretreatment. Possible reasons suggested by researchers [44,71–73] include wet oxidation of sugar and lignin compounds, endwise truncation of polysaccharides, and contamination of lactic-acid-producing bacteria in biomass.

Appendix B

Table A1. Ethanol conversion efficiencies of fermentation using LHW [1] pretreated lignocellulosic feedstocks [2].

Feedstock	Pretreatment Conditions	Fermentation	Ethanol Conversion Efficiency (%)	Reference
Sugarcane bagasse	180 °C LHW/10 min	SSCF [2]	94%	[32]
Hemp	170 °C LHW/30 min	SSF [3]	75%	[53]
Corn stover	190 °C LHW/15 min	SSCF [2]	88%	[52]
Reed	210 °C LHW/20 min	SSF [3]	86%	[55]
Switchgrass	200 °C LHW/10 min	SSF [3]	86%	[54]

[1] LHW = liquid hot water pretreatment. [2] SSCF = simultaneous saccharification and co-fermentation. [3] SSF = simultaneous saccharification and fermentation.

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