For: Bioresource Technology

Title: Production of single cell oil from *Lipomyces starkeyi* ATCC 56304 using biorefinery by-products.

Authors: KYLE V. PROBST¹ and PRAVEEN V. VADLANI²

¹*IGERT in Bioprocessing, ²Bioprocessing and Renewable Energy Lab, Departments of Grain Science and Industry & Chemical Engineering, Kansas State University, BIVAP Building, 1980 Kimball Ave., Manhattan, Kansas 66506, United States*

Corresponding Author:

Kyle V. Probst (Corresponding Author) instance

Grain Processing Corporation

1600 Oregon St.

Muscatine, IA 52761

Tel: (812) 327-4814

Email: kyle.probst@grainprocessing.com

Abstract

Single cell oil (SCO) is a valuable noncrop-based renewable oil source. Hemicellulose derived sugars can be utilized to produce SCO using the oleaginous yeast *Lipomyces starkeyi* ATCC 56304. Bran by-products were tested as hemicellulose-rich feedstocks for the production of SCO. Whole and destarched corn and wheat bran hydrolysates were produced using hydrothermal and dilute sulfuric acid (0, 0.5, 1.0%, v/v) pretreatment along with enzymatic hydrolysis. Whole bran hydrolysates produced from hydrothermal pretreatment generated the highest average oil yields of 126.7 and 124.3 mg oil/g sugar for both wheat and corn bran, respectively. 1.0% acid pretreatment was effective for the destarched bran generating a hemicellulose hydrolysis efficiency of 94 and 84% for wheat and corn bran, respectively, resulting in the highest oil yield of 70.7 mg oil/g sugar. The results indicate pretreated corn and wheat bran hydrolysates can serve as viable feedstocks for oleaginous yeast SCO bioconversion.

Keywords: bran, pretreatment, fermentation, oleaginous yeast, single cell oil

1. Introduction

Single cell oil (SCO) from oleaginous yeast can serve as a noncrop-based renewable replacement for petroleum-based fuels, chemicals, and power (Probst et al., In Press). Currently, oilseed crops serve as the major renewable oil source but, as the world population increases, edible oil sources will be needed to feed the growing population. Oleaginous yeast have potential as a viable alternative oil-source but manufacturing for commodity-based products is cost-prohibitive mainly due to the high costs of feedstocks. Oleaginous yeast are capable of generating SCO from a variety of low-valued inedible plant-based feedstocks including corn cobs (Huang et al., 2012; Gao et al., 2014), corn residue (Hu et al., 2011; Liu et al., 2012; Galafassi et al., 2012; Sitepu et al., 2014), corn fiber (Liang et al., 2014), rice straw (Huang et al., 2009), sorghum bagasse (Liang et al., 2012), sugarcane bagasse (Tsigie, 2011), and wheat straw (Yu et al., 2011). The oleaginous yeast *Lipomyces starkeyi* has considerable potential as a viable SCO producer due to its ability to produce high amounts of oil from hemicellulose-derived sugars including xylose (Anschau et al., 2014). For the commercialization of cellulosic ethanol, efficient use of the hemicellulose portion remains to be a challenge because *Saccharomyces cerevisiae* is unable to naturally produce ethanol from pentose sugars. Since hemicellulose may comprise up to a third of plant-derived biomass (Saha, 2003), using oleaginous yeast to produce SCO as a value-added bioconversion platform could provide additional revenue streams for future biomass-based biorefineries.

Corn and wheat bran are readily available feedstocks containing up to 30-35% hemicellulose on a dry wt basis (Saulnier and Thibault, 1999; Saha, 2003). Wheat bran is a by-product of the flour milling industry with approximately 7×10^6 dry tons produced annually in the US and sold as an animal feed (known as middlings) for \$70-150 per ton (Blasi et al., 1998;

Horner and Sexten, 2015). Corn bran and corn fiber are by-products of the corn dry and wet milling industries, respectively, with a total production of 3×10^6 dry tons per year (Rose, Inglett and Liu, 2010). Often, corn bran is combined with steeping solids, a by-product of corn wet milling, to produce corn gluten feed often priced \$100-160 per ton (Horner and Sexten, 2015). Outside of the animal feed market, opportunities exist for developing value-added uses of bran such as feedstocks for SCO fermentation.

Commercialization of yeast SCO fermentation has been a challenging endeavor. Production has been limited due to feedstock costs and competition from lower priced oilseed crops. A recent economic analysis of yeast SCO production determined that the raw material costs (including feedstocks) can account for roughly 40% of the total costs; use of low- to minimum-value feedstocks will be compulsory for economical SCO manufacture (Koutinas et al., 2014). Based on the study performed by Koutinas et al. (2014), the cost to produce SCO at a best case scenario including a zero feedstock cost was \$1.76 per kg. The co-location of yeast SCO bioconversion platforms near existing wheat and corn biorefineries in the US Midwest may help reduce feedstock costs by providing a readily available source of bran. Furthermore, competition from lower priced oilseed crops such as soybean oil priced at \$0.55-0.65 per kg limit targeting commodity biofuels such as biodiesel (Ash, 2015). SCO could be targeted to additional markets outside of fuels such as the manufacture of higher-value fatty-acid-based oleochemicals, a \$18.3 billion global market in 2014 expected to grow to \$25.7 billion by 2019 (BCC Research, 2015).

Consideration of non-edible feedstocks as inputs for renewable oils will be imperative for a growing population. Bran from corn and wheat contain a considerable amount of non-nutritional polysaccharides including cellulose, arabinoxylans and β -glucans that can be

converted into fermentable sugars, up to 50% (dry basis). Since bran often contains minimal lignin (< 5%, dry wt basis), dilute sulfuric acid (0.5-1%, v/v) at moderate temperatures (120-130°C) combined with enzymatic hydrolysis can achieve high sugar conversion yields with minimal inhibitor generation (Saha and Bothast, 1999; Saha, 2003; Choteborska et al., 2004). The mild pretreatment conditions make the use of bran hydrolysates an attractive fermentable feedstock for the production of SCO.

The objective of this study was to evaluate hemicellulose-rich bran as feedstocks for the production of SCO using the oleaginous yeast *Lipomyces starkeyi* ATCC 56304. We tested the fermentability of hydrothermal and acid pretreated bran hydrolysates from two types of grains, corn and wheat, using both whole (as-is) and destarched bran for the production of SCO. To our knowledge, no work has been done using hydrolysates from acid pretreated wheat and corn bran to produce SCO using oleaginous yeast. This study demonstrates the use of widely available bran by-products as viable source of hemicellulose-derived sugars as a low-cost feedstock for SCO production.

2. Materials and methods

2.1 Microorganisms, media and chemicals

Lyophilized culture of *Lipomyces starkeyi* (ATCC 56304) was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and propagated in yeast mold (YM) broth (Difco Laboratories, Detroit, MI, USA): 10 g/L glucose, 5 g/L peptone, 3 g/L maltose, 3 g/L yeast extract at 200 rpm at 25°C for 8 days. Cultures were preserved on YM agar plates at 3°C and re-cultivated monthly to maintain fresh culture. All chemicals were obtained from certified suppliers and were of analytical reagent grade (Fisher Scientific, Pittsburg, PA, USA).

2.2 Hemicellulose-rich bran feedstocks

Wheat bran and corn bran were generously provided by Phibro Animal Health Corporation, Manhattan, KS, USA and by LifeLine Foods, St. Joseph, MO, USA, respectively. Each were milled (Fitz-Mill, Fitzpatrick Company, Elmhurst, IL, USA) to pass through a 2.36 mm screen, dried in a forced-air oven at 40°C for 72 h and stored at room temperature. Geometric mean diameter (ASABE S319.4) was determined by sieving through a Ro-Tap shaker (Model RX-29, W.S. Tyler Inc., Mentor, OH, USA).

For preparation of destarched bran, commercial-grade alpha-amylase (Liquozyme®, Novozymes Inc. Franklinton, NC, USA) and glucoamylase (GC480, Genencor International Inc. Palo Alto, CA, USA) were generously provided by a dry-grind corn ethanol facility (MGP Ingredients, Atchison, KS, USA) and used for liquefaction and saccharification. Bran was slurried in deionized water (dH₂O) at a 15% (dry w/v) solid loading and the pH was adjusted to 5.5 via hydrochloric acid (3M). Liquefaction was performed using alpha-amylase (1 µL/g starch) at 80°C for 1 h; saccharification was performed using glucoamylase (5 µL/g starch) at 65°C for 2 h. Destarched bran was washed with dH₂O and dried at 40°C for 72 h.

2.3 Acid pretreatment and enzymatic hydrolysis

Bran was slurried in deionized water (dH₂O) with the required amount of sulfuric acid (0-1.0%, v/v) at a 15% (dry w/v) solid loading in shake-flasks. Pretreatment was performed at 121°C for 1 h. After pretreatment, the pH was neutralized and adjusted to 5.5 using sodium hydroxide (10M) prior to addition of commercial-grade cellulolytic (Cellic® CTec2, Novozymes) and hemicellulolytic (Cellic® HTec2, Novozymes) enzymes. A 3% (w/w of dry biomass) enzyme dosage was used with a 9:1 ratio of CTec2:HTec2. Enzymatic hydrolysis was performed at 55°C at 150 rpm for 48 h. After hydrolysis, solids were removed via centrifugation

at 4000 rpm (2300 x g) for 20 min (Sorvall Super T21, Thermo Fisher Scientific Inc., Waltham, MA, USA) and filter sterilized (0.22 µm) prior to being aseptically transferred to a pre-sterilized shake-flask for fermentation.

2.4 Fermentation

A 10% (v/v) inoculum, sub-cultured 2x in YM broth at 26°C and 200 rpm for 48 h was concentrated to $\sim 5 \times 10^8$ cells/mL and used for all fermentations. Batch shake-flask fermentations were carried out in 250 mL Erlenmeyer shake-flasks using 10% (v/v) of hydrolysate to provide sufficient surface area for aeration. Flasks were incubated at 26°C and 200 rpm for 120 h unless otherwise noted.

2.5 Analytical methods

2.5.1 Composition analysis

Composition analysis was conducted on corn bran and wheat bran including: proximate (moisture (AOAC Official Method 934.01), crude fat (acid hydrolysis, AOAC 954.02), crude protein (Kjedahl, AOAC 984.13 (A-D)), crude fiber (AOAC 978.10), and ash (AOAC 942.05)), acid detergent fiber (ADF, AOAC 973.18 (A-D)), neutral detergent fiber (NDF, AOAC 973.18 (A-D)), starch (AACC Approved Methods No. 76-13), acid detergent lignin (AOAC 973.18 (A-D)), and hydrolysis monosaccharide profile (glucose, xylose, arabinose, mannose, fucose, galactose, ribose). Representative samples were collected in triplicate and analyzed at an external laboratory (Agricultural Experimental Station Chemical Laboratories, University of Missouri, Columbia, MO, USA).

2.5.2 Dry cell weight determination

Dry cell weight (DCW) was determined gravimetrically after washing cells 2x with dH₂O and drying the wet cells at 80°C for 12 h.

2.5.3 HPLC analysis of sugars and inhibitors

Sugars (D-glucose, D-xylose, L-arabinose, and alpha-maltose) and inhibitors (acetic acid, 5-hydroxymethylfurfural (HMF), and furfural) were identified and quantified using high performance liquid chromatography (HPLC, Prominence LC20AB, Shimadzu Scientific Instruments, Columbia, MD, USA). Samples were diluted 10x with dH₂O and filtered (0.22 µm membrane) before injection. Sugars were separated using an ion exclusion column (RezexTM RCM-monosaccharide 300 x 7.8 mm, Phenomenex, Torrance, CA, USA) and quantified using a refractive index detector (RID) with an isocratic mobile phase of dH₂O (Direct Q, Millipore Inc., USA) pumped at a rate of 0.6 mL/min at a column temperature of 80°C. Inhibitors were separated using an ion exclusion column (RezexTM ROA-Organic Acid 150 x 7.8 mm, Phenomenex) with an isocratic mobile phase of 0.005N sulfuric acid pumped at a rate of 0.6 mL/min at a column temperature of 80°C and quantified with a photodiode array detector (PDA); acetic acid at 199 nm and HMF/furfural at 254 nm. LCsolution software (Version 1.25, Shimadzu) was used to identify and quantify each analyte from retention times and calibration curves generated from known standards of glucose, xylose, arabinose, maltose, acetic acid, HMF, and furfural.

2.5.4 Lipid extraction and fatty acid analysis

The lipid extraction was performed on concentrated yeast cells (~5x10⁸ cells/mL) prepared by harvesting the yeast via centrifugation (2500 x g) for 15 min, washing 2x with dH₂O, lysed using a bead homogenizer and subsequent extraction using a modified Bligh and Dyer method (Bligh and Dyer, 1959) Bead lysis was performed in a 2.5 mL polypropylene microvial with O-ring sealed cap; the contents of the microvial included 0.5 mL concentrated yeast cells, 0.5 mL of methanol, 0.5 of chloroform and 1 mL of 0.5 mm cubic zirconia beads.

Lysis was achieved using a Beadbeater homogenizer (Mini-Beadbeater-24, BioSpec Products, Inc., Bartlesville, OK, USA) operated in 45 sec intervals with intermittent cooling on ice for 10 min for a total of six cycles. The cell lysate was transferred to 7 mL glass test-tube with PTFE-lined caps after washing two times with equal parts chloroform and methanol to ensure all contents of the microvial were transferred to the test-tube. Additional chloroform:methanol:water were added to achieve a ratio of 1:2:0.8, respectively, vortexed and centrifuged ($1500 \times g$) for 15 min to achieve phase separation. The lower chloroform layer containing the lipids of interest was removed using a Pasteur pipette and transferred into a clean test-tube; extractions were repeated a total of 3x and the combined chloroform layer was filtered using a 0.2 μm PTFE filter and washed using 1M potassium chloride solution, and dried down under nitrogen gas at 40°C , vacuum dried for 30 min to remove any residual solvent and weighed; this was known as the total lipid extract and used for calculating the oil content. The total lipid extract was re-dissolved in 1 mL of chloroform and stored at -80°C until further analysis.

Fatty acids were transesterified into fatty acid methyl esters (FAMES) using 3M methanolic HCL at 78°C for 30 min followed by addition of water and extraction with hexane and identified using a gas chromatograph (GC-2014, Shimadzu Scientific Instruments, Columbia, MD, USA). 1 μL of sample was injected at 220°C with a split ratio of 30:1, separated on a capillary column (Zebron ZB-Wax_{plus} 30m x 0.25mm x 0.25 μm , Phenomenex, Torrance, CA, USA) at a column temperature of 160°C with a column flow of 3mL/min and identified using a flame ionization detector (FID) set at 250°C . A FAME mixture (Supleco 37 Component FAME Mix) was used to determine the relative retention time for each FAME species. An internal standard of 25 nmol of pentadecanoic acid (C15:0) was added prior to transesterification and used for calculating the fatty acid profile.

2.6 Experimental design and statistical analysis

Separate experiments were performed for whole as-is bran and destarched bran to test the effect of bran source (corn bran and wheat bran) and sulfuric acid concentration (0, 0.5, 1.0%, v/v) on sugar hydrolysis, inhibitor generation and fermentation performance of SCO production. Design Expert software (Design Expert 8.0.7.1, Stat-Ease Inc., Minneapolis, MN, USA) was used to generate factorial designs. Three replicates for each treatment was performed.

Statistical software SAS (SAS v9.1, SAS Institute, Cary, NC, USA) was used to perform PROC GLM to determine means and Tukey's Honestly Significant Difference test for comparison between treatments ($\alpha=0.05$).

3. Results and discussion

3.1 Bran composition

The composition of each bran feedstock is shown in Table 1. Both corn bran and wheat bran have three major types of polysaccharides present: starch, cellulose and hemicellulose which account for about 65% (w/w) of the bran. Corn bran had a considerable amount of crude fat (9.7%, w/w) indicating the presence of germ; oil pooling on the surface of hydrolysates was noticeable and became problematic during the filter sterilization step. Starch was evident in both feedstocks. When completely removed and re-calculated, hemicellulose content increased to 30.9 and 29.2% (w/w) for destarched corn bran and wheat bran, respectively (Table 1). The low lignin content (<4%, w/w) of the bran sources is an added benefit preventing the need for harsher pretreatment conditions.

3.2 Bran pretreatment and hydrolysis

For a schematic overview of the pretreatment process, please refer to Figure 1. To summarize, both as-is whole (scheme B) and destarched (scheme A) wheat bran and corn bran

were subjected to varying degrees of sulfuric acid pretreatment (0, 0.5, and 1.0%, v/v) followed by enzymatic hydrolysis using cellulases and hemicellulases.

Hydrothermal (0%) and acid (0.5, 1.0%) pretreatment with enzyme hydrolysis were able to hydrolyze the starch, hemicellulose, and cellulose fractions of the corn bran and wheat bran. From the results shown in Figure 2 and Figure 3, the use of acid pretreatment is critical for generating efficient sugar hydrolysis across both whole bran and destarched bran sources.

3.2.1 Whole bran

Starch hydrolysis was evident in the whole bran and contributed to the presence of glucose in the hydrolysates, even under the hydrothermal pretreatment suggesting the hemicellulases/cellulases have a certain degree of glucoamylase activity (Saha and Bothast, 1999). As reported by others, use of hydrothermal pretreatment and enzyme hydrolysis did not readily hydrolyze the hemicellulose portion indicated by the low xylose and arabinose yields (Figure 2). This is most likely due to the naturally high resistance of hemicellulose to enzymatic action (Rose, Inglett and Liu, 2010). Acid treatment was much more effective. 0.5% acid treatment significantly increased the combined xylose and arabinose content of corn bran and wheat bran from 2.6 to 14.0 g/100 g bran and 4.4 to 13.7 g/100 g bran, respectively. During acid pretreatment, glucose degradation, as a result of the residual starch, was problematic generating up to 0.4 and 0.8 g/L of HMF for wheat bran and corn bran, respectively (Figure 4).

3.2.2 Destarched bran

To reduce the formation of HMF and increase the amount of pentose sugars in the bran hydrolysates, the starch was removed from both corn bran and wheat bran using alpha-amylase and glucoamylase prior to pretreatment (Figure 1). Results from the destarched bran sugar hydrolysis are shown in Figure 3 which, again, indicate the presence of acid is imperative for

hemicellulose hydrolysis. As expected, increasing the acid content from 0% to 0.5% showed a greater release of combined xylose and arabinose from 2.8 to 27.6 g/100 g and 6.8 to 21.8 g/100 g for corn bran and wheat bran, respectively. The continuing trend of poor hydrolysis for corn bran using hydrothermal and enzyme hydrolysis is, again, most likely due to the resistance of corn bran to enzymatic breakdown (Saha, 2003; Rose, Inglett and Liu, 2010; Agger, Vikso-Nielsen and Meyer, 2010). Corn bran arabinoxylans are highly cross-linked via di-ferulic acid bridges, which further resists attack from degrading enzymes (Agger, Vikso-Nielsen and Meyer, 2010). Addition of acid facilitates the breakdown of these crosslinks, exposing the xylan backbone for enzymatic attack and greater sugar hydrolysis. Starch removal substantially reduced the glucose content by roughly 3-fold compared to the whole bran hydrolysates. Furthermore, acid pretreatment also improved cellulose hydrolysis from the destarched bran, generating 10.5 g/100 g and 16.3 g/100 g of glucose for wheat bran and corn bran, respectively.

Starch removal was able to minimize HMF formation but furfural, produced from pentose sugar degradation, increased substantially up to 165.3 and 219.3 mg/L for wheat bran and corn bran, respectively (Figure 5). Compared to the whole bran hydrolysates, the presence of acetic acid also became readily apparent in the destarched bran hydrolysates, increasing 2-fold from approximately 0.5 to 1.3 g/L and 1.5 to 3 g/L for wheat bran and corn bran, respectively. Acetic acid is naturally present in both wheat bran and corn bran arabinoxylans as esterified linkages to the xylan backbone (up to 3-5% in corn bran); this may account for its increased presence in the destarched hydrolysates (Agger et al., 2010).

It should be noted that the content of arabinose in the acid treated wheat bran hydrolysates for both the whole and destarched bran was higher than the theoretical value (Figure 2 and 3). This may be due to the different methods of analysis used for quantifying

arabinose in the hydrolysates versus the composition of arabinose in the wheat bran. It may also suggest that the calculated values of arabinose in the destarched bran may be different from the actual composition. Composition testing of the destarched bran would be needed to confirm this observation.

3.3 Fermentation performance

Fermentation performance using corn bran and wheat bran hydrolysates varied considerably under different pretreatment conditions (Table 2). Fermentation proceeded un-aided for hydrolysates generated from the hydrothermal pretreatment but was inhibited when using acid pretreatment for both whole bran and destarched bran (with the exception of 0.5% acid for destarched wheat bran). To allow the fermentation to proceed, the hydrolysates were diluted 2x with dH₂O, filter sterilized and re-fermented.

L. starkeyi ATCC 56304 was capable of utilizing, on average, 95-98% of the total sugars (glucose, xylose, arabinose, and maltose). Maltose, a disaccharide present due to incomplete starch hydrolysis, was readily utilized by *L. starkeyi* which had not been previously recorded in our lab. Additionally, arabinose was also utilized, though some residual amounts remained that were unconsumed during fermentation. Contradictory to earlier findings, this suggests that *L. starkeyi* ATCC 56304 is capable of utilizing arabinose from bran hydrolysates. It is not readily apparent why this occurred because an earlier study found that arabinose was not readily utilized when provided as the sole carbon source. One possibility is that arabinose may only be consumed for lipid accumulation (i.e. during stationary phase) albeit at very low rates once cell growth has been established. Further experimental testing would be needed to confirm this hypothesis. The ability of *L. starkeyi* to utilize arabinose from bran hydrolysates is an exciting find indicating that a greater portion of the hemicellulose can be used for SCO production.

3.3.1 Whole bran

As shown in Table 2, whole bran hydrolysates from both corn bran and wheat bran generated higher oil contents and yields compared to destarched bran hydrolysates. Within whole bran, hydrolysates produced from the hydrothermal pretreatment generated the highest oil yields of 126.7 and 124.3 mg oil/g sugar for corn bran and wheat bran, respectively. The hydrothermal pretreatment was able to generate high amounts of glucose (~50-60 g/L) from the highly water soluble starch portion with little to no inhibitor formation; this provided the necessary conditions for improved oil accumulation. It is apparent the oil yield was significantly affected by the use of acid pretreatment due to the presence of inhibitors. Dilution did not remove HMF and acetic acid, it only reduced the concentration; thus, growth and lipid accumulation were most likely still affected. Furthermore, dilution also limited the amount of available sugar that could be utilized for lipid accumulation, resulting in lower oil yields.

3.3.2 Destarched bran

The fermentation performance of destarched bran hydrolysates was poor compared to whole bran hydrolysates. The presence of furfural and acetic acid along with less fermentable sugar as a result of starch removal were the plausible reasons for this performance. Unlike the whole bran hydrolysates, 1.0% (v/v) acid pretreated destarched bran hydrolysates resulted in the highest oil yield of 70.7 mg oil/g sugar for both corn bran and wheat bran. Even after dilution and the presence of furfural and acetic acid, acid pretreated bran generated the best fermentation performance. Hydrothermal pretreatment of destarched corn bran was ineffective producing the lowest oil yield of 32.4 mg oil/g sugar while the more water soluble wheat bran had a significantly higher oil yield of 66.5 mg oil/g sugar. The use of acid pretreatment was an

effective method for the destarched bran leading to improved sugar hydrolysis and oil production.

3.3.3 Fermentation comparison

Compared to batch shake-flask fermentation of pure sugar sources, both the whole and destarched bran hydrolysates, on average, generated higher oil yields compared to pure glucose (58 mg oil/g glucose). Oil yields produced from the whole bran hydrothermal pretreated hydrolysates (126.7 mg oil/g sugar) were comparable to pure xylose (148.8 mg oil/g xylose). These results compare well to another study using *L. starkeyi* grown on non-detoxified wheat straw hydrolysate (Yu et al., 2011). The authors were able to produce an oil yield of 160 mg oil/g sugar by using dilute sulfuric acid pretreatment without enzyme hydrolysis. This technique generated higher proportion of xylose by minimizing the formation of glucose which improved oil yields. This further reinforces the notion that xylose utilization during lipid accumulation is key for generating high oil yields.

Overall, it is evident that fermentation performance was affected by the amount of available sugar and the presence of inhibitory compounds. The availability of surplus sugar (i.e. higher solid loading) for enhancing SCO production is a key consideration (Liang et al. 2014). While higher solid loading would be beneficial, the co-generation of inhibitory compounds from the pretreatment step often limits production. The deleterious effect of inhibitory compounds including acetic acid, HMF, and furfural on oleaginous yeast growth and lipid accumulation has been well-documented by others (Huang et al., 2011; Hu et al., 2009; Chen et al., 2009) and confirmed by our study. While the effect of each inhibitor may vary across oleaginous yeast species and cultivation conditions, multiple studies have reported that furfural has a higher degree of inhibition compared to HMF (Huang et al., 2011; Chen et al., 2009; Zhao et al., 2012;

Hu et al., 2009). This may account for the lower oil yields obtained using destarched hydrolysates which had a higher level of furfural compared to the whole bran hydrolysates (Figure 5). To minimize the effect of inhibitors, a detoxification step after pretreatment can be used, often using liming and/or activated carbon (Zhao et al., 2012; Yu et al., 2011). While detoxification can improve fermentation performance, sugar losses can occur, which we observed in our laboratory. The dilution approach used in this experiment proved to be effective for allowing fermentation to proceed by reducing the inhibitor concentration, but the reduced sugar concentration is not desirable. Thus, development of pretreatment and hydrolysis techniques to generate high-quality sugar streams with minimal inhibitor formation would be beneficial for viable industrial fermentation platforms including SCO from yeast. On the other hand, identifying robust oleaginous yeast strains such as *Rhodotorula glutinis* ATCC 204091, shown to be capable of metabolizing acetic acid, HMF and furfural in undetoxified corncob hydrolysate, is equally important toward developing economical SCO platforms (Liu et al., 2015).

3.4 Fatty acid analysis

The major fatty acid species produced from *L. starkeyi* included palmitic acid (C16:0) and oleic acid (C18:1). The fatty acid profile remained constant for both the whole bran and destarched bran hydrolysates (Figure 6). Oleic acid accounted for about 70% of the total fatty acid profile for *L. starkeyi*. The large abundance of oleic acid may provide additional value for SCO produced from *L. starkeyi* ATCC 56304. Recently, there has been considerable interest in high oleic oils for biofuels with improved oxidative stability and cold-flow properties, and as an input for many renewable polymers, lubricants, coatings, and elastomers (Corbett, 2003; Durrett et al., 2008; Probst et al., In Press).

4. Conclusions

This study demonstrated that corn bran and wheat bran can be utilized to produce SCO using *L. starkeyi* ATCC 56304. The highest oil yields of 126.7 and 124.3 mg oil/g sugar were produced from the whole corn and wheat bran hydrolysates, respectively, using hydrothermal pretreatment with enzyme hydrolysis; an effective method for hydrolyzing the remaining starch portion and limiting inhibitor formation. Acid pretreatment was effective for the destarched bran producing the highest oil yield of 70.7 mg oil/g sugar for both corn and wheat bran, but the presence of inhibitors limited fermentation performance when compared to the whole bran hydrolysates.

Acknowledgements

This work was supported by National Science Foundation Grant # 0903701: “Integrating the Socioeconomic, Technical, and Agricultural Aspects of Renewable and Sustainable Biorefining Program awarded to Kansas State University.” Contribution no. 16-095-J from the Kansas Agricultural Experimental Station. Special thanks to: Mr. Charles Fahrenholz of Phibro Animal Health Corp. for donating the wheat bran, Dr. Matt Gibson of LifeLine Foods, LLC for donating the corn bran, and Mr. Ray Brown of MGP Ingredients, Inc. for donating the alpha-amylase and glucoamylase enzymes. Authors are also grateful to the Lortscher Endowment and KSU for funding the project and Mr. Yadhu N. Guragain for his valuable suggestions on pretreatment methods.

References

1. Agger, J., Vikso-Nielsen, A., Meyer, A.S., 2010. Enzymatic Xylose Release from Pretreated Corn Bran Arabinoxylan: Differential Effects of Deacetylation and Deferuloylation on Insoluble and Soluble Substrate Fractions, *J. Agric. Food Chem.* 58, 6141-6148.
2. Anschau, A., Xavier, M.C.A., Hernalsteens, S., Franco, T.T., 2014. Effect of feeding strategies on lipid production by *Lipomyces starkeyi*, *Bioresour. Technol.* 157, 214-222.

3. Ash, M., 2015. Oil crops outlook. Economic Research Service, USDA. Situation and Outlook Report OCS-15h. Available: <http://www.ers.usda.gov/media/1881268/ocs15h.pdf> Accessed Online August 24, 2015.
4. BCC Research, 2015. Global markets for oleochemical fatty acids: report overview. Report ID: CHM062B.
5. Blasi, D.A., Juhl, G.L., Drouillard, J.S., Reed, C.R., Trigo-Stockli, D.M., Behnke, K.C., Fairchild, F.J., 1998. Wheat middlings: composition, feeding value, and storage guidelines. KSU Agricultural Experiment Station and Cooperative Extension Service, Manhattan, KS. MF-2353.
6. Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification, Can. J. of Biochem. and Phys. 37, 911-917.
7. Chen, X., Li, Z., Zhang, X., Hu, F., Ryu, D.D.Y., Bao, J., 2009. Screening of oleaginous yeast strains tolerant to lignocellulose degradation compounds, Appl. Biochem. Biotechnol. 159, 591-604.
8. Choteborska, P., Palmarola-Adrados, B., Galbe, M., Zacchi, G., Melzoch, K., Rychtera, M., 2004. Processing of wheat bran to sugar solution, J. Food Eng. 61, 561-565.
9. Corbett, P., 2003. It's time for an oil change! Opportunities for high-oleic vegetable oils. INFORM - International News on Fats, Oils and Related Materials. 14, 480-481.
10. Durrett, T.P., Benning, C., Ohlrogge, J., 2008. Plant triacylglycerols as feedstocks for the production of biofuels, Plant J. 54, 593-607.
11. Galafassi, S., Cucchetti, D., Pizza, F., Franzosi, G., Bianchi, D., Compagno, C., 2012. Lipid production for second generation biodiesel by the oleaginous yeast *Rhodotorula graminis*, Bioresour. Technol. 111, 398-403.
12. Gao, Q., Cui, Z., Zhang, J., Bao, J., 2014. Lipid fermentation of corncob residues hydrolysate by oleaginous yeast *Trichosporon cutaneum*, Bioresour. Technol. 152, 552-556.
13. Horner, J., Sexten, J., 2015. By-product feed price listing. University of Missouri Extension. Available: <http://agebb.missouri.edu/dairy/byprod/bplist.asp>. Accessed Online August 24 2015.
14. Hu, C., Zhao, X., Zhao, J., Wu, S., Zhao, Z.K., 2009. Effects of biomass hydrolysis by-products on oleaginous yeast *Rhodospiridium toruloides*, Bioresour. Technol. 100, 4843-4847.
15. Huang, C., Chen, X., Xiong, L., Chen, X., Ma, L., 2012. Oil production by the yeast *Trichosporon dermatis* cultured in enzymatic hydrolysates of corncobs, Bioresour. Technol. 110, 711-714.
16. Huang, C., Wu, H., Liu, Q., Li, Y., Zong, M., 2011. Effects of aldehydes on the growth and lipid accumulation of oleaginous yeast *Trichosporon fermentans*, J. Agric. Food Chem. 59, 4606-4613.
17. Huang, C., Zong, M., Wu, H., Liu, Q., 2009. Microbial oil production from rice straw hydrolysate by *Trichosporon fermentans*, Bioresour. Technol. 100, 4535-4538.

18. Koutinas, A., Chatzifragkou, A., Kopsahelis, N., Papanikolaou, S., Kookos, I., 2014. Design and techno-economic evaluation of microbial oil production as a renewable resource for biodiesel and oleochemical production, *Fuel*. 116, 566-577.
19. Liang, Y., Perez, I., Goetzelmann, K., Trupia, S., 2014. Microbial lipid production from pretreated and hydrolyzed corn fiber, *Biotechnol. Prog.* 30, 945-951.
20. Liang, Y., Tang, T., Umagiliyage, A.L., Siddaramu, T., McCarroll, M., Choudhary, R., 2012. Utilization of sorghum bagasse hydrolysates for producing microbial lipids, *Appl. Energy*. 91, 451-458.
21. Liu, Y., Wang, Y., Liu, H., Zhang, J., 2015. Enhanced lipid production with undetoxified corncob hydrolysate by *Rhodotorula glutinis* using a high cell density culture strategy, *Bioresour. Technol.* 180, 32-39.
22. Liu, W., Wang, Y., Yu, Z., Bao, J., 2012. Simultaneous saccharification and microbial lipid fermentation of corn stover by oleaginous yeast *Trichosporon cutaneum*, *Bioresour. Technol.* 118, 13-18.
23. Probst, K.V., Schulte, L.R., Durrett, T.P., Rezac, M.E., Vadhani, P.V. In Press. Oleaginous yeast: a value-added platform for renewable oils, *Crit. Rev. Biotechnol* DOI:10.3109/07388551.2015.1064855.
24. Rose, D.J., Inglett, G.E., Liu, S.X., 2010. Utilisation of corn (*Zea mays*) bran and corn fiber in the production of food components, *J. Sci. Food Agric.* 90, 915-924.
25. Saha, B.C., 2003. Hemicellulose bioconversion, *J. Ind. Microbiol. Biotechnol.* 30, 279-291.
26. Saha, B.C., Bothast, R.J., 1999. Pretreatment and enzymatic saccharification of corn fiber, *Appl. Biochem. Biotechnol.* 76, 65-77.
27. Saulnier, L., Thibault, J.F., 1999. Ferulic acid and diferulic acids as components of sugar-beet pectins and maize bran heteroxylans, *J. Sci. Food Agric.* 79, 396-402.
28. Sitepu, I.R., Jin, M., Fernandez, J.E., Sousa, L.d.C., Balan, V., Boundy-Mills, K.L., 2014. Identification of oleaginous yeast strains able to accumulate high intracellular lipids when cultivated in alkaline pretreated corn stover, *Appl. Microbiol. Biotechnol.* 98, 7645-7657.
29. Tsigie, Y.A., 2011. Lipid production from *Yarrowia lipolytica* Po1g grown in sugarcane bagasse hydrolysate, *Bioresour. Technol.* 102, 9216-9222.
30. Yu, X., Zheng, Y., Dorgan, K.M., Chen, S., 2011. Oil production by oleaginous yeasts using the hydrolysate from pretreatment of wheat straw with dilute sulfuric acid, *Bioresour. Technol.* 102, 6134-6140.
31. Zhao, X., Peng, F., Du, W., Liu, C., Liu, D., 2012. Effects of some inhibitors on the growth and lipid accumulation of oleaginous yeast *Rhodospiridium toruloides* and preparation of biodiesel by enzymatic transesterification of the lipid, *Bioproc Biosyst Eng.* 35, 993-1004.

Table 1. Composition of whole and destarched corn bran and wheat bran on a dry mass basis.

Component (% w/w)	Whole bran		Destarched bran^C	
	Corn bran^A	Wheat bran^B	Corn bran	Wheat bran
Moisture	3.7 ± 0.2	10.5 ± 0.3	-	-
Crude protein*	11.6 ± 0.2	16.5 ± 0.1	17.2	23.9
Crude fat	9.7 ± 0.1	2.3 ± 0.4	14.3	3.4
Crude fiber	10.0 ± 1.4	8.2 ± 0.6	14.7	11.9
Ash	3.4 ± 0.03	7.4 ± 0.04	5.0	10.8
Cellulose†	9.6 ± 0.2	8.0 ± 0.3	14.2	11.6
Hemicellulose ^s	25.8 ± 1.3	23.5 ± 1.0	38.0	34.1
Starch	32.2 ± 3.3	31.1 ± 1.0	-	-
Acid detergent lignin	1.4 ± 0.1	2.5 ± 0.1	2.1	3.6
Sugars (% w/w)‡				
Glucose	34.2 ± 3.1	35.4 ± 1.2	15.8	12.8
Xylose	13.5 ± 1.1	12.6 ± 0.6	19.9	18.3
Arabinose	8.8 ± 0.7	3.4 ± 0.2	13.0	5.0
Galactose	1.8 ± 0.2	-	2.7	-

^AGeometric mean diameter of 417±12µm

^BGeometric mean diameter of 440±15µm

^CCalculated based on complete starch removal.

*By Kjeldahl (%N x 6.25) w/w% = grams per 100 grams of sample

†Cellulose (calculated) = acid detergent fiber (ADF) - acid detergent lignin (ADL)

^sHemicellulose (calculated) = neutral detergent fiber (NDF) - acid detergent fiber (ADF)

‡Hydrolysis sugar profile.

Table 2.SCO fermentation performance of whole and destarched bran hydrolysates.

	Sugar utilized	Dry cell wt. (g/L)	Oil content (%,	Y _{ps} (mg oil/g
Whole bran	(g/L)†		dry wt.)	sugar)
Wheat, 0% Acid	51.3 ± 1.6 ^A	17.1 ± 1.0 ^A	37.3 ± 1.3 ^A	124.3 ± 12.1 ^A
*Wheat, 0.5% Acid	40.5 ± 2.9 ^B	12.7 ± 0.9 ^B	31.7 ± 0.8 ^B	99.7 ± 2.4 ^{BC}
*Wheat, 1.0% Acid	39.4 ± 1.1 ^B	10.9 ± 0.3 ^B	31.3 ± 0.3 ^B	87.1 ± 0.4 ^{CD}
Corn, 0% Acid	61.7 ± 1.9 ^C	23.5 ± 0.1 ^C	33.3 ± 1.1 ^{AB}	126.7 ± 7.0 ^A
*Corn, 0.5% Acid	37.2 ± 4.5 ^B	12.3 ± 0.1 ^B	32.5 ± 2.8 ^B	108.5 ± 12.0 ^{AB}
*Corn, 1.0% Acid	47.4 ± 7.0 ^B	12.9 ± 2.4 ^B	25.4 ± 1.5 ^C	68.8 ± 3.4 ^D
Destarched bran				
Wheat, 0% Acid	22.4 ± 5.5 ^{ZY}	6.4 ± 0.6 ^Z	22.6 ± 1.3 ^Z	66.5 ± 8.1 ^Z
Wheat, 0.5% Acid	38.2 ± 12.6 ^{YX}	9.6 ± 1.2 ^Z	24.5 ± 6.6 ^Z	62.4 ± 1.3 ^{ZY}
*Wheat, 1.0% Acid	29.5 ± 2.4 ^{ZYX}	7.5 ± 0.3 ^{ZY}	27.5 ± 1.8 ^Z	70.7 ± 7.8 ^Z
Corn, 0% Acid	16.7 ± 0.15 ^Z	5.5 ± 0.5 ^Z	9.8 ± 0.6 ^Z	32.4 ± 4.4 ^Y
*Corn, 0.5% Acid	40.3 ± 6.0 ^X	10.1 ± 1.0 ^X	28.0 ± 1.0 ^Z	70.7 ± 6.3 ^Z
*Corn, 1.0% Acid	35.5 ± 0.4 ^{YX}	10.5 ± 0.6 ^X	22.9 ± 6.5 ^Y	68.1 ± 22.5 ^Z
Pure sugars				
Glucose				58.0 ± 12.5
Xylose				148.8 ± 12.9

N=3, mean values with the same letters (A-D for whole bran and W-Z for destarched bran) in the same column are not significantly different ($P \geq 0.05$), values after $\pm$ are standard deviation.

*Samples diluted 2x with dH₂O before fermentation.

†Included glucose, xylose, maltose, and arabinose. Over 95-98% of sugars were completely utilized during fermentation across all treatments.

figure 1

figure 2

figure 3

figure 4

figure 5

figure 6