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Saccharomyces cerevisiae Fermentation of 28 Barley and 12 Oat Cultivars

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Abstract: As barley and oat production have recently increased in Canada, it has become prudent to investigate these cereal crops as potential feedstocks for alcoholic fermentation. Ethanol and other coproduct yields can vary substantially among fermented feedstocks, which currently consist primarily of wheat and corn. In this study, the liquified mash of milled grains from 28 barley (hulled and hull-less) and 12 oat cultivars were fermented with *Saccharomyces cerevisiae* to determine concentrations of fermentation products (ethanol, isopropanol, acetic acid, lactic acid, succinic acid, α-glycerylphosphorylcholine (α-GPC), and glycerol). On average, the fermentation of barley produced significantly higher amounts of ethanol, isopropanol, acetic acid, succinic acid, α-GPC, and glycerol than that of oats. The best performing barley cultivars were able to produce up to 78.48 g/L (CDC Clear) ethanol and 1.81 g/L α-GPC (CDC Cowboy). Furthermore, the presence of milled hulls did not impact ethanol yield amongst barley cultivars. Due to its superior ethanol yield compared to oats, barley is a suitable feedstock for ethanol production. In addition, the accumulation of α-GPC could add considerable value to the fermentation of these cereal crops.

Keywords: barley; oats; α-glycerylphosphorylcholine; fermentation; ethanol; *Saccharomyces cereivisae*

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1. Introduction

Barley and oats are major crops grown in Canada, with production of 10.4 and 4.2 Mt in 2019, respectively [1]. Barley and value-added barley products return over CAD $2 billion in Canadian exported goods alone [2]. Demand for these grains continues to be strong, with an increase of 14.0% and 18.1% acres planted in 2019 compared to the prior year for barley and oats, respectively [3]. These crops are primarily used as animal feed, but are also used to produce food and alcohol [2–4]. Oats and barley are excellent sources of carbohydrates and fibre (e.g., β-glucans) [5,6], and have an abundance of starch (>60% of the grain dry weight) [7,8].

The abundant starch in these grains is suitable for renewable fuel production via alcoholic fermentation by the yeast *Saccharomyces cerevisiae*. With increasing barley and oats production in Canada, it is beneficial to investigate these cereal crops as feedstocks for producing ethanol, as lower grades and damaged crops can still be suitable for fermentation. In addition to ethanol, valuable organic solutes can also be coproduced during fermentation [9]. Recovery of these compounds could increase the profitability of ethanol production by fermentation [9–11]. These coproducts include isopropanol, acetic acid, succinic acid, α-glycerylphosphorylcholine (α-GPC), and glycerol [12,13]. Therefore, monitoring for these coproducts is essential in optimizing fermentation conditions and increasing ethanol product yields.

n-Propanol is naturally synthesized from amino acids and simple sugars during fermentation processes (e.g., Ehrlich pathway reactions) [14–16]. Isopropanol can also be

produced through acetone reduction by lactic acid bacteria [17], a common contaminant of fermentation [18]. The presence of nuisance organisms can also result in increased production of acetic acid [19] and lactic acid [20]. Production of succinic acid is also gaining attention due the potential of converting this compound to a range of industrial chemicals (e.g., plastics and organic solvents) [21]. However, successful biological production of succinic acid requires the selection/development of succinic acid producing microorganism [22,23], selection of feedstock, specific productivity of the fermenters, and the development of efficient recovery processes [24]. Nonetheless, the production and purification of succinic acid from renewable feedstocks could potentially create supplemental value.

Glycerol is also coproduced during anaerobic fermentation via *Saccharomyces cereivisae* [20,21]; however, it is relatively inexpensive. Glycerol is produced by yeast to maintain the balance between the NAD^+/NADH ratio during cell growth [25]. However, this compound is also produced under osmotic stress conditions, as a means to protect the cells against lysis [26–28]. Therefore, fermentation conditions can play an important role in decreasing glycerol production and improving ethanol yield [29]. Nonetheless, the glycerol in the fermentation mash can be upgraded through conversion to 1,3-propanediol (a more valuable compound) using lactobacilli [10,11].

Finally, α-GPC is a biosynthetic precursor of the neurotransmitter acetylcholine, as well as membrane phospholipids [30]. This compound can improve cognitive abilities [31] and isometric strength [32], and appears to have benefits for various other physical and mental performance tasks [33]. More importantly, α-GPC is marketed as a nootropic nutraceutical and pharmaceutical for the treatment of Alzheimer's disease [34]. It is estimated that by 2050, more than 130 million people will be diagnosed with Alzheimers [35]. The potential to treat neurodegenerative diseases using α-GPC has increased the value of this compound substantially [36–38]. Therefore, there is great potential in developing alternative, inexpensive, and sustainable means for commercial production to supply this compound.

In this study, 28 cultivars of barley and 12 cultivars of oats were subjected to *Saccharomyces cerevisiae* fermentation to identify which cultivars produced optimum yields of ethanol and organic solutes (α-GPC, acetic acid, ethanol, succinic acid, glycerol, isopropanol, and lactic acid).

2. Materials and Methods

2.1. Fermentation Conditions

Barley and oat cultivars (Table 1) were obtained from the Crop Development Centre, University of Saskatchewan (Saskatoon, SK, Canada). Commercial enzymes, yeast *(Saccharomyces cerevisiae*), and urea were obtained from Terra Grain Fuels, Belle Plaine, SK, Canada. Whole barley and oat kernels were milled to a coarse flour using a Glen Mills Type C/11/1 tabletop grinder/disc mill, with the coarseness set to 18 (Clifton, NJ, USA). Milled whole barley and oat flour were gelatinized with boiled distilled water (36%, *w/v*) and incubated at 130 °C for 15 min using a VWR Constant Temperature Oven (Model 1350GM; Mississauga, ON, Canada). Saccharification was initiated by adding α-amylase (0.2%, *v/v*) and the mash was incubated at 80 °C for 60 min. A 1:3 mixture of glucanase:xylanase was then added to the mash (0.01%, *v/v*) which was then heated for an additional 30 min at 55 °C. The mash was stirred every 15 min during heating after enzyme additions. Samples were then cooled to 37 °C and glucoamylase (0.1%, *v/v*), liquid yeast (0.5%, *v/v*; *Saccharomyces cerevisiae*), and liquid urea (0.05%, *v/v*) were added, and a gas trap was fitted to the fermenter. The total liquified fermentation volume was 1 L. Each fermentation broth was incubated at 37 °C until completion at 72 h. An aliquot of 500 µL was collected for analysis at 0, 24, 48, and 72 h.

Table 1. Barley cultivars (28 cultivars; all 2-row except for 6-row CDC Clyde) and oats (12 cultivars) subjected to *Saccharomyces cerevisiae* fermentation.

Barley Cultivars		Oat Cultivars	
Cultivar	**Hulled vs. Hull-Less**	**Cultivar**	**Hulled**
AAC Synergy	Hulled	CDC Arborg	Hulled
AC Metcalfe	Hulled	CDC Dancer	Hulled
CDC Austenson	Hulled	CDC Morrison	Hulled
CDC Bow	Hulled	CDC Nasser	Hulled
CDC Clear	Hull-less	CDC Norsemen	Hulled
CDC Clyde	Hulled	CDC Seabiscuit	Hulled
CDC Copeland	Hulled	OT3071	Hulled
CDC Cowboy	Hulled	OT3087	Hulled
CDC Fibar	Hull-less	OT3102	Hulled
CDC Fraser	Hulled	OT3103	Hulled
CDC Hilose	Hull-less	OT3104	Hulled
CDC Kindersley	Hulled	OT3105	Hulled
CDC Maverick	Hulled		
CDC McGwire	Hull-less		
CDC Meredith	Hulled		
CDC Rattan	Hull-less		
Champion	Hulled		
Claymore	Hulled		
FB207	Hulled		
FB208	Hulled		
HB16337	Hull-less		
Oreana	Hulled		
Sirish	Hulled		
TR14150	Hulled		
TR16156	Hulled		
TR17163	Hulled		
TR17166	Hulled		
TR17167	Hulled		

2.2. NMR Spectroscopy

Immediately following yeast inoculation, an aliquot (2 mL) was taken from each fermentation mash using a VWR® disposable transfer pipets (Mississauga, ON, Canada), with additional samples taken every 24 h. Aliquots were dispensed into VWR microcentrifuge tubes (2 mL) and centrifuged at 10,000 rpm for 10 min, using a Labnet Spectrafuge 24D Digital Microcentrifuge (NJ, USA). The samples were then filtered using a 3 mL BD syringe (New Jersey, USA) equipped with a VWR 0.45 µm nylon membrane syringe filter (25 mm; Mississauga, ON, Canada). After filtration, an aliquot (500 µL) was dispensed into a nuclear magnetic resonance (NMR) tube containing deuterium oxide (50 µL; EMD Millipore, Oakville, ON, Canada) and pyrazine (40 µL of 20 mg/µL; Sigma Millipore, Oakville, ON, Canada) as an internal standard. Double-pulse field gradient spin echo ^{1}H-NMR spectroscopy was conducted according to Ratanapariyanuch et al. [39] Spectra were recorded at 500 MHz (AMX 500, NMR Bruker, Mississauga, ON, Canada) with 16 scans

per spectrum. Spectroscopy data collection and analyses were conducted with TopSpin™ 3.8 software (Bruker BioSpin GmbH, Billerica, MA, USA).

2.3. Statistical Analysis

Statistical analyses of organic solute content, determined via ^{1}H-NMR, were performed using the Statistical Package for the Social Sciences (SPSS) version 25.0 (IBM Corp., Armonk, NY, USA). A Pearson coefficient correlation test was used to identify significance between average oat and barley fermentation. Significant differences were reported at the 95% confidence interval ($p < 0.05$).

3. Results

The concentrations of ethanol, isopropanol, acetic acid, succinic acid, α-GPC, and glycerol found during barley and oat fermentations are reported in Figures 1 and 2. Fermentation was deemed complete after 72 h, as observed glucose in the barley and oat mash was largely consumed (≤14% remaining). The average concentrations of compounds found in barley and oat mashes over 72 h were calculated (Figure 3). Concentrations of glucose were also monitored throughout the barley and oat fermentations (Figures 1G and 2G).

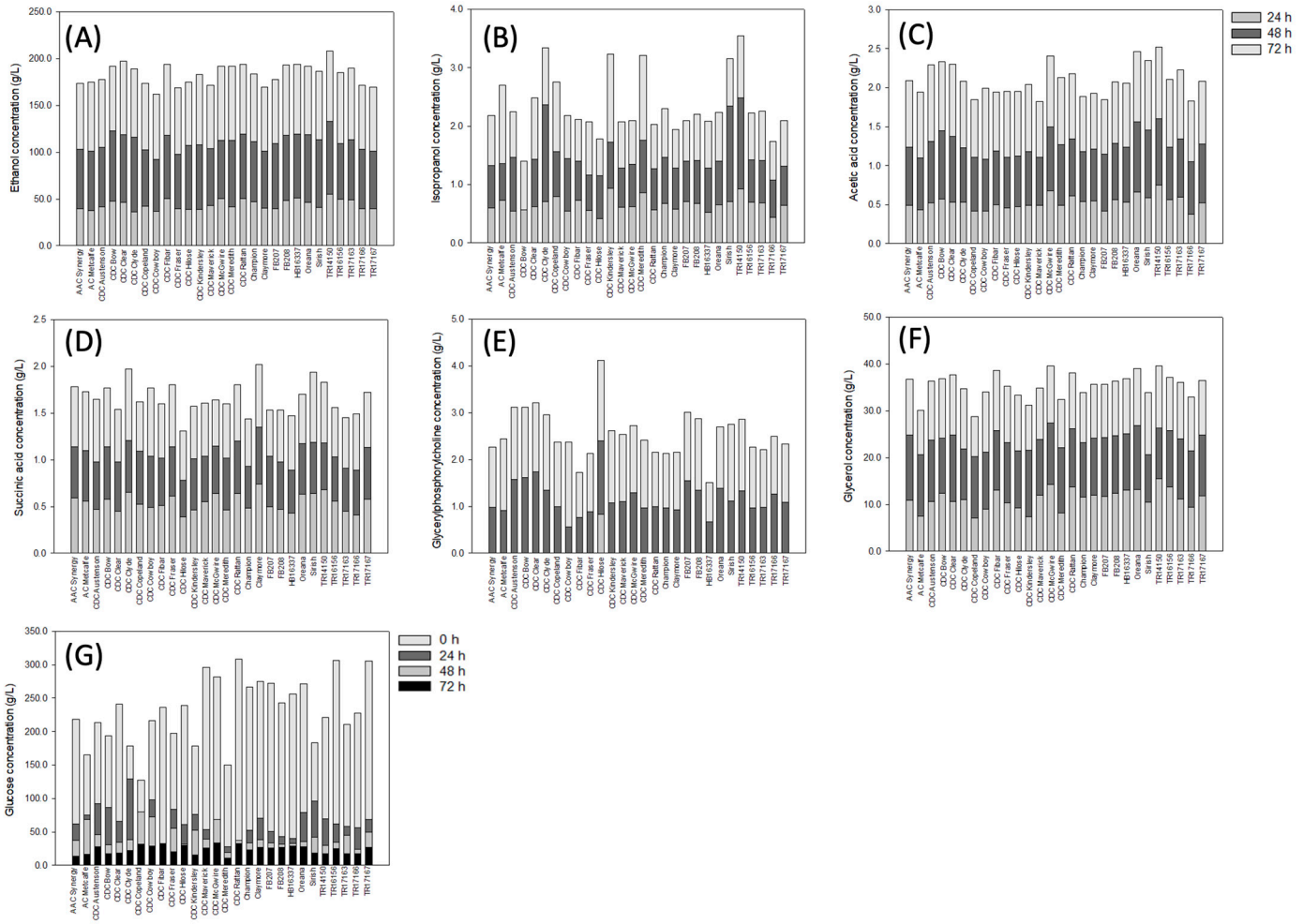

Figure 1. Concentration of ethanol (**A**), isopropanol (**B**), acetic acid (**C**), succinic acid (**D**), α-glycerylphosphorylcholine (**E**), glycerol (**F**) and glucose (**G**) in mash sampled every 24 h during yeast fermentation of different barley cultivars.

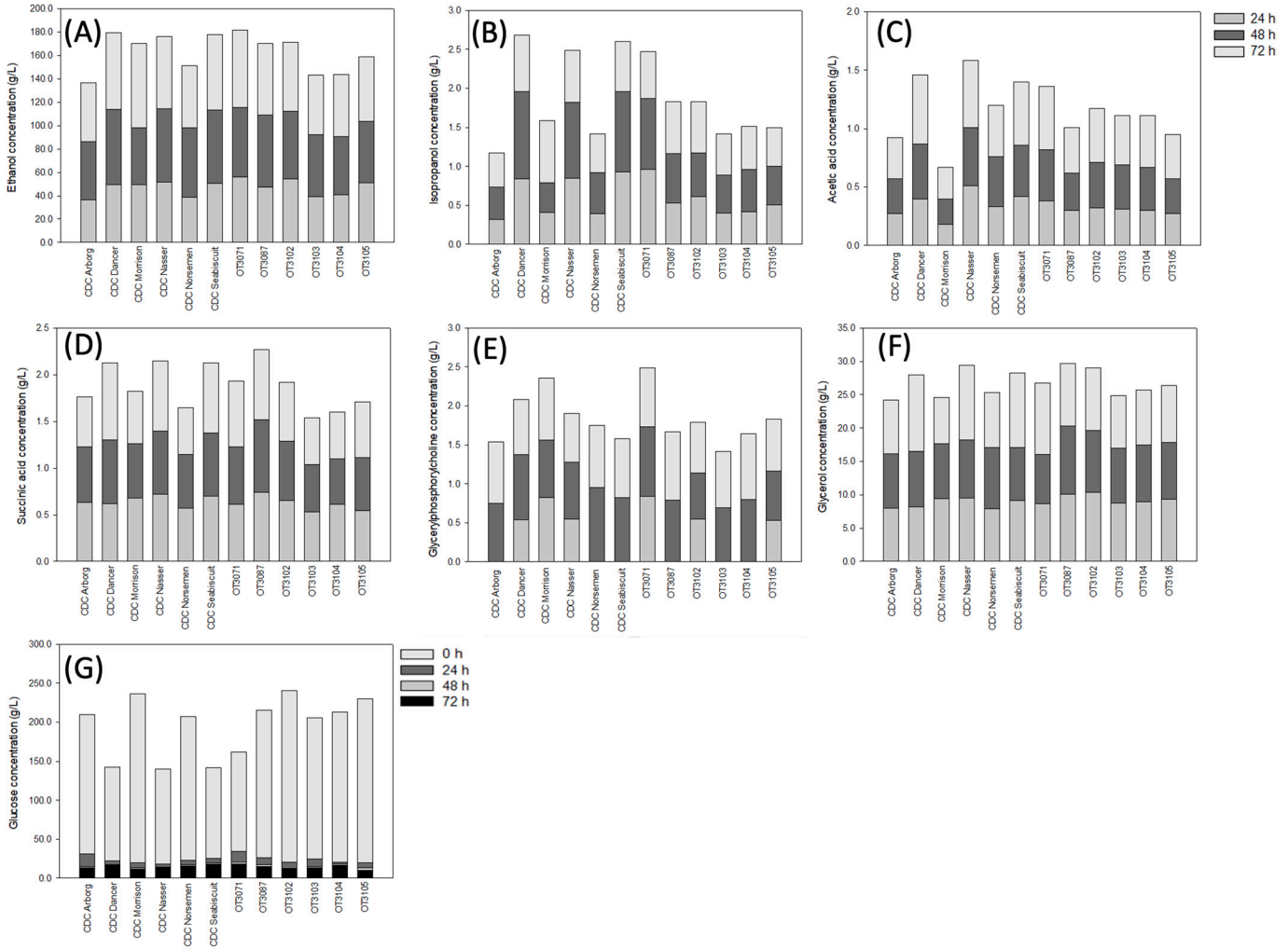

Figure 2. Concentration of ethanol (**A**), isopropanol (**B**), acetic acid (**C**), succinic acid (**D**), α-glycerylphosphorylcholine (**E**), glycerol (**F**) and glucose (**G**) in mash sampled every 24 h during yeast fermentation of different oat cultivars.

3.1. Concentration of Organic Solutes in Barley

Glucose content after saccharification (Figure 1G) varied between 191.7 (CDC Morrison) and 366.3 g/L (Claymore) with an average of 318.8 ± 55.2 g/L (Figures 3A, 4A and 5A). Average ethanol content after fermentation of the various barley cultivars was 72.7 ± 3.4 g/L (Figure 3A) after 72 h (Figure 4A), with CDC Hilose exhibiting the lowest yield at 68.0 g/L and CDC McGwire achieving the highest yield at 78.5 g/L (Figure 1A). The average production of isopropanol, acetic acid, and succinic acid was 0.89 ± 0.23, 0.83 ± 0.07, and 0.60 ± 0.07 g/L, respectively (Figure 3B–D). The highest concentrations for isopropanol, acetic acid, and succinic acid were found in CDC Kindersley, CDC Austenson, and CDC Clyde, respectively, with the lowest concentrations found in Claymore, FB207, and CDC McGwire, respectively (Figure 1B–D). Interestingly, α-GPC was observed in all barley cultivars, with an average α-GPC across cultivars at 72 h of 1.38 ± 0.22 g/L (Figure 3E). The difference in α-GPC concentration between barley cultivars was considerable, with the concentration at 72 h varying from 0.84 g/L for HB13667 to 1.81 g/L for CDC Cowboy (Figure 1E). In contrast, glycerol content was more similar among barley cultivars, with a range of 8.54 (CDC Copeland) to 13.23 g/L (TR14150), and an average across cultivars of 11.69 ± 1.15 g/L after 72 h of fermentation (Figure 3F). Furthermore, glycerol content plateaued after 24 h of fermentation (Figure 5A). The average yield of all measured fermentation products was similar between cultivars with hulls and those without (Figure 6). Barley mash had significantly higher average concentrations of ethanol

($p < 0.01$), isopropanol ($p < 0.05$), acetic acid ($p < 0.01$), α-GPC ($p < 0.05$), and glycerol ($p < 0.01$) when compared to oats.

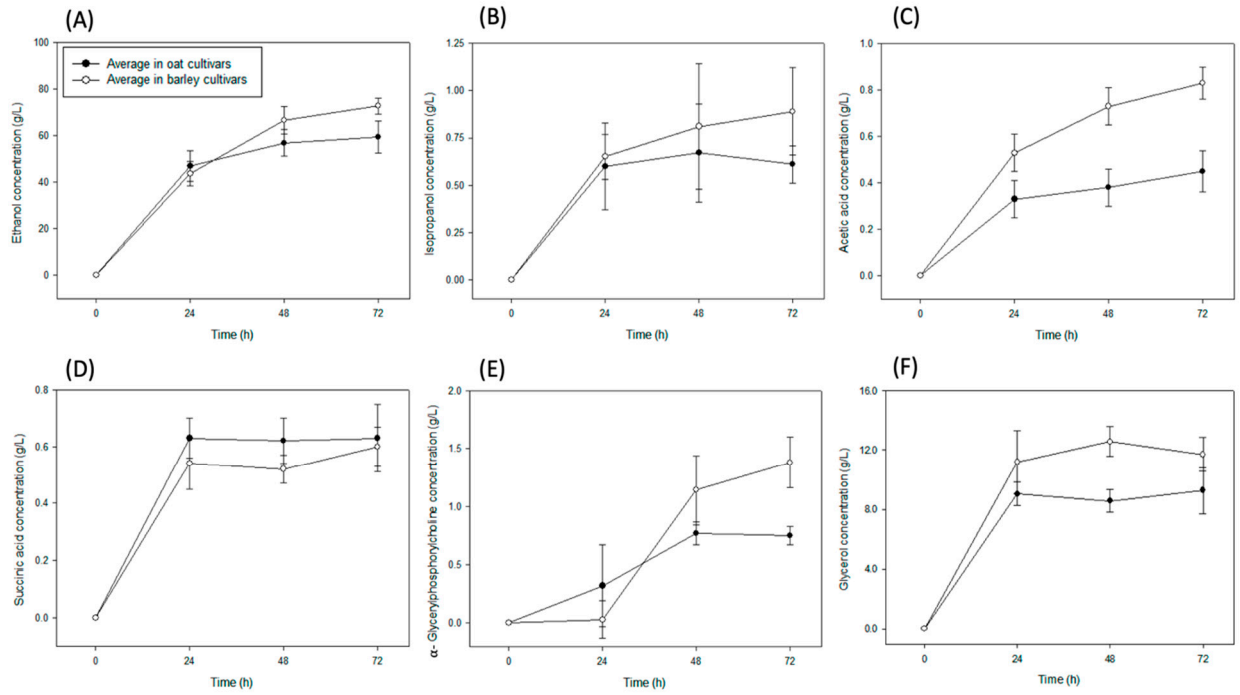

Figure 3. Average barley and oat fermentation product accumulation among cultivars over 72 h for ethanol (**A**), isopropanol (**B**), acetic acid (**C**), succinic acid (**D**), α-glycerylphosphorylcholine (**E**), and glycerol (**F**).

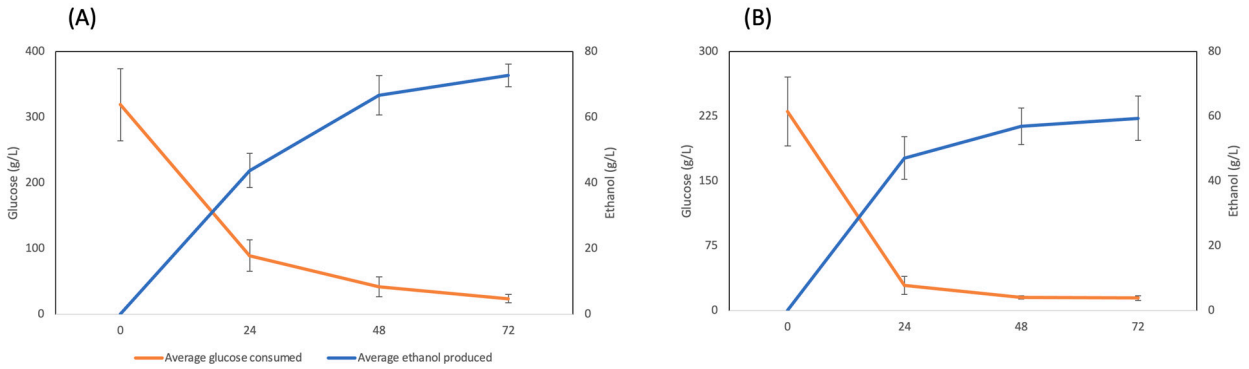

Figure 4. Average consumption of glucose and average production of ethanol by *Saccharomyces cerevisiae* fermentations of barley (**A**) and oats (**B**).

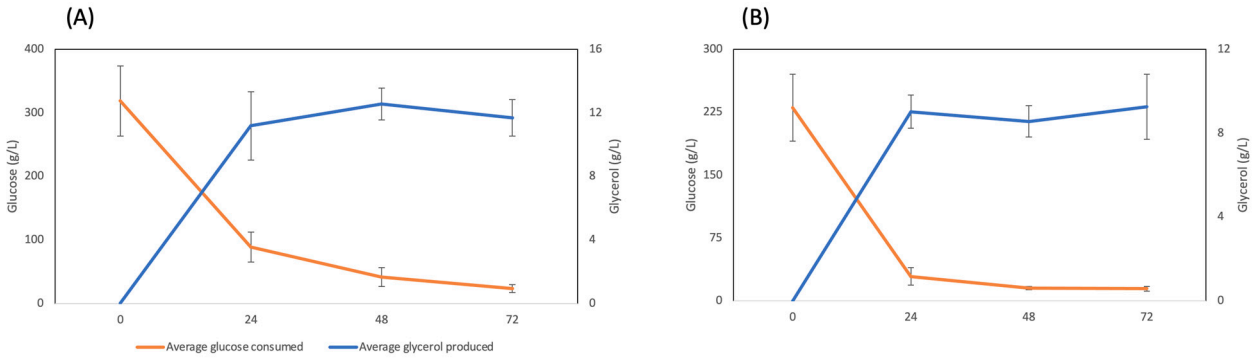

Figure 5. Average consumption of glucose and average production of glycerol for barley (**A**) and oats (**B**).

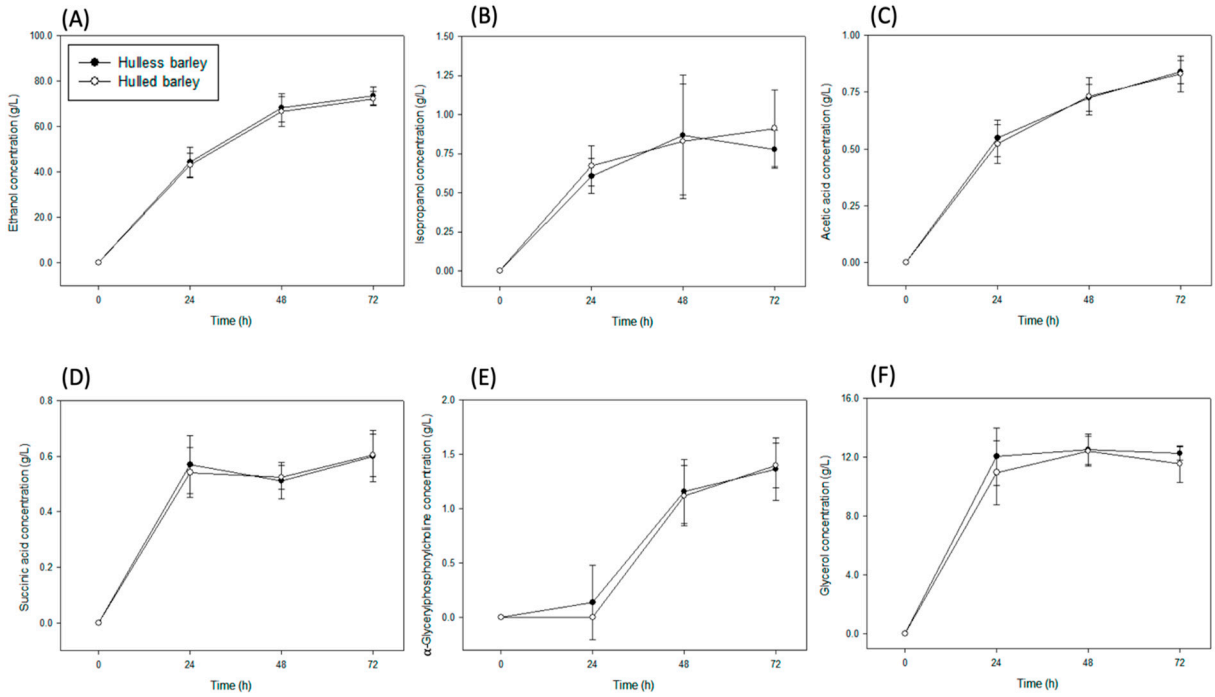

Figure 6. Average fermentation product yields for ethanol (**A**), isopropanol (**B**), acetic acid (**C**), succinic acid (**D**), α-glycerylphosphorylcholine (**E**), and glycerol (**F**) for barley with and without hulls.

3.2. Concentration of Organic Solutes in Oat Cultivars

Compared to the average product accumulation observed in the fermented barley mash, oat mash accumulated less ethanol, isopropanol, acetic acid, α-GPC, and glycerol (Figure 3A–C,E,F). However, succinic acid production was similar between mash from the two crops (Figure 3D). Average ethanol content for mash from the 12 oat cultivars was 59.4 ± 6.9 g/L after 72 h of fermentation (Figure 4B), with a range of 50.6 (CDC Arborg) to 72.0 g/L (CDC Morrison) (Figure 2A). Meanwhile, oat glucose content (Figure 2G) after saccharification was substantially less than in barley, varying between 169.3 (CDC Nasser) and 265.3 g/L (OT3104) with an average of 230.3 ± 39.9 g/L (Figure 4B). Similar to the barley mash, the isopropanol, acetic acid, and succinic acid content were lower than ethanol, with averages of 0.61 ± 0.10, 0.45 ± 0.09 and 0.63 ± 0.12 g/L, respectively (Figure 3B–D). Yeast fermentation of milled oats also produced mash with α-GPC, although the concentrations were much lower than for barley mash (Figure 3E). The average accumulation of α-GPC was 0.75 ± 0.08 g/L, with CDC Nasser accumulating the lowest concentration at 0.62 g/L and OT3087 the highest at 0.88 g/L (Figure 2E). Finally, average glycerol in oat mash was 9.26 ± 1.54 g/L, varying between 6.89 (CDC Morrison) and 11.48 g/L (CDC Dancer) (Figure 2F). Similar to barley, the average glycerol content plateaued after 24 h of fermentation (Figure 5B). Interestingly, average succinic acid concentration in oat mash was significantly greater than that observed in barley ($p < 0.01$).

4. Discussion

Ethanol production is a billion-dollar industry [40], with important implications for the food, pharmaceutical, and fuel industries. In the United States, corn is the primary feedstock used in the production of fuel ethanol, due to its low price and abundance [41]. Both corn and wheat are routinely used in the production of ethanol in Canada [42]. These crops can produce variable yields of ethanol, with some wheat cultivars producing between 59.9 and 71.8 g/L of ethanol after 72 h of fermentation [9]. Fermentation of barley produced greater amounts of ethanol in this study (up to 78.5 g/L). The presence of milled hulls did not appear to impact ethanol yield. However, hull-less barley has been previously

observed to ferment faster than wheat mashes, as well as producing higher yields of ethanol compared to wheat mashes [43].

The differences in ethanol and glycerol production among individual cultivars could be attributed to differences in starch content among grain type, variety and the environment in which the crop was grown [44–46]. This was observed in the differences in glucose content among cultivars, after saccharificiation. However, the use of ^{1}H-NMR spectroscopy has limitations in accurately quantifying the concentration of sugars, as the C-H units of the carbohydrate backbone can lead to "accidental overlap" [47]. Furthermore, measurement of glucose in a complex solution with changing pH can be difficult [48] and as this molecule undergoes mutarotation [49] accurate measurement of glucose can be complicated using these methodologies. Nonetheless, on average, barley observed substantially higher amounts of glucose than in oats.

In contrast, oat cultivars did not perform nearly as well, owing to the lower glucose content observed in oats. The average ethanol content in oats was observed to be 59.4 $\pm$ 6.8 g/L. Only the cultivar CDC Morrison yielded >70 g/L of ethanol when fermented. Corn bioethanol production is even lower at 20–25 g/L when using solid state fermentation [50,51] and liquid state fermentation [52]. However, pre-treatment processes and immobilization techniques can increase ethanol production while reducing process costs [53]. The fermentation of corn meal via the immobilization of yeast can result in approximately 90 g/L of ethanol [54]; unfortunately, the industrial use of immobilized cells is still limited [55].

Isopropanol, acetic acid, and succinic acid were minor components produced during the fermentation process. Acetic acid is a normal by-product of alcoholic fermentation by *Saccharomyces cerevisiae*, and of contaminating lactic acid and acetic acid bacteria [56–59]. In fact, acetic acid typically does not surpass 0.4 g/L in bacteria-free fermentations [60]. In typical alcoholic fermentations, 0.2 to 0.6 g/L acetic acid does not appear to impair fermentation [59]. The average concentrations of acetic acid observed after fermentation of barley and oats were 0.83 $\pm$ 0.07 and 0.45 $\pm$ 0.09 g/L, respectively. The relatively low accumulation of acetic acid in oat mash suggests minimal contamination from acetic or lactic acid bacteria. However, the significantly higher acetic acid accumulation in barley mash ($p < 0.01$) might be attributed to endogenous acidogenic bacteria. Furthermore, the hydrolysis of lignocelluloses in these cereal crops may have also contributed to the formation of acetic acid [61,62]. Therefore, the concentration of acetic acid does not appear to suggest consequential negative effects on the progression of fermentation in oat mash [59]. Although barley mash showed a somewhat elevated acetic acid content, the fermentations did not stagnate or halt, which can result with high acetic acid levels [59].

Glycerol is also produced during alcoholic fermentation, and is the main solute produced by *Saccharomyces cerevisiae* in response to osmotic stress, in order to prevent dehydration [11]. Increased glycerol production by yeast can result in decreased ethanol [63] and carbon dioxide production [62] through the redirection of the yeast's carbon metabolism [64]. Consequently, minimizing glycerol production can result in increased ethanol yields [65]. The sudden increase in glycerol in oats and barley at 24 h can most likely attributed to the efficiency for yeast to adapt to the osmotic stress [12] in the fermentation medium. After 24 h, it appears that glycerol production ceased (concentration plateaued) and ethanol production increased, suggesting that the consumption of glucose was primarily due to the production of ethanol.

Glycerol content in barley and oat mash (11.7 $\pm$ 1.2 and 9.3 $\pm$ 1.5 g/L, respectively) were comparable to wheat (~10 g/L) [9]. Mash produced from some barley cultivars (i.e., TR14150) accumulated up to 13 g/L glycerol. Through metabolic and stress management, decreased glycerol and increased ethanol production during fermentation can be attained [11]. Conversely, glycerol found in the thin stillage by-product could also be used in a second fermentation with lactobacilli [10,11]. These organisms can convert inexpensive glycerol into higher value products, such as 1,3-propanediol, which is used in the manufacturing of textiles. The presence of these organisms can also increase protein

content in distillers' grains [10,11], which can then be used as domestic feed. In regard to protein content, hull-less barley cultivars contain similar amounts to wheat and are typically higher than hulled barley cultivars [43].

Alpha-glycerylphosphorylcholine was another important substance found in the barley and oat mash. This compound is a naturally produced endogenous choline derivative; however, it is rarely found at high concentrations in nature. Therefore, there is great potential in developing alternative, inexpensive, and sustainable means for commercial production to supply this compound. Production of α-GPC can be catalyzed chemically or enzymatically through phosphatidylcholine (PC) hydrolysis [13,36–38]. It can also be produced through the condensation of glycerol derivatives [13,66,67], although published methods require the use of toxic substrates such as trimethylamine, strong acids and harmful solvents [68]. As a result, enzymatic hydrolysis of PC [69–71] is preferred as it avoids the use of harmful substrates and is relatively inexpensive.

This compound has previously been observed in wheat mash, with concentrations ranging between 1.03 and 1.34 g/L [9]. Similarly, most of the barley mash produced more than 1 g/L of α-GPC, including CDC Cowboy, which produced the highest amount of α-GPC observed in this study. The compound α-GPC has considerable value [36,37], and can be used as a supplement to treat cognitive disorders (e.g., Alzheimer's disease) and improve muscle strength [29,30].

Increases in phosphatidylcholine have been observed in plant cells deprived of phosphate [72]. Deprivation of phosphate results in a decrease in the phospholipid content of plants leading to mobilization in the phosphate reserve, and an increase in the production of non-phosphorous membrane lipids (e.g., digalactosyldiacylglycerol) [72]. In this study, CDC Cowboy mash accumulated similar concentrations of ethanol to wheat [9], while also accumulating considerably more α-GPC. Therefore, future studies should investigate pre-treatment methods to increase PC content, followed by developing methods to decrease metabolic and osmotic stress during yeast fermentation. This could provide optimum conditions to increase α-GPC and ethanol yields, while minimizing glycerol accumulation.

5. Conclusions

Overall, barley mash accumulated greater concentrations of ethanol, isopropanol, acetic acid, α-GPC, and glycerol than oat mash. Alpha-GPC for mash prepared from the barley cultivars such as CDC Cowboy exceeded the amounts previously found from wheat mash. The isolation and purification of this compound can create new opportunities for commercial growth in the food and health industries. The use of barley as a feedstock for bioethanol production may therefore be appealing, due to its affordability, abundance, and comparable ethanol yields to wheat. The optimization of ethanol and α-GPC production via the minimization of glycerol production and phosphate deprivation, respectively, should be investigated to fully maximize the economic return of barley fermentation. Like wheat thin stillage, barley thin-stillage could also undergo a two-stage fermentation process with lactobacilli organisms to convert the relatively high yield of inexpensive glycerol into a more valuable product [10,11]. Overall, barley appears to be a suitable replacement for wheat in fermentation for ethanol, producing mash with similar or higher ethanol yields and increased α-GPC concentrations after 72 h of anaerobic fermentation.

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Conflicts of Interest: Martin J. T. Reaney is the founder of, and has an equity interest in, Prairie Tide Diversified Inc. (PTD, Saskatoon, SK, Canada: previous company name is Prairie Tide Chemicals Inc.).

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