Industrial Crops and Products 62 (2014) 53–60

Contents lists available at ScienceDirect

Industrial Crops and Products

journal homepage: www.elsevier.com/locate/indcrop

Simultaneous saccharification and fermentation of industrial sweetpotatoes for ethanol production and anthocyanins extraction

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ARTICLE INFO

Article history:
Received 8 April 2014
Received in revised form 30 June 2014
Accepted 18 July 2014

Keywords:
Ipomoea batatas
Kluyveromyces marxianus
Saccharomyces cerevisiae
Antioxidants
Fermentation
Starch
Sweet potato

ABSTRACT

A simultaneous saccharification fermentation (SSF) system was studied for ethanol production in flour industrial sweetpotato (ISP) feedstocks (lines: white DM02-180 and purple NC-413) as an integrated cost saving process, and to examine the feasibility of extracting anthocyanins from flour purple ISPs under a simultaneous extraction and fermentation (SEF) system. Furthermore, a separate hydrolysis fermentation (SHF) configuration was carried out to establish a baseline in sugar consumption and ethanol production from the ISP lines. The thermotolerant ethanol producing yeast strain *Kluyveromyces marxianus* NCYC 851 and the mesophilic *Saccharomyces cerevisiae* Ethanol Red were evaluated, using commercial alpha amylases for hydrolysis of available ISP starch to sugars. Fermentation by *S. cerevisiae* during SHF had an ethanol yield of 0.32 g/g dry ISP, a 1.1-fold increase above that produced by *K. marxianus*. Subsequent studies showed that ethanol yield could be increased in a SSF system with a maximum ethanol yield of 0.39 g/g dry ISP achieved, a 15% increase compared with using a SHF system when *S. cerevisiae* was used. Simultaneous extraction and fermentation of flour purple-fleshed NC-413 ISPs was studied to evaluate the effect of pH on extraction of total monomeric anthocyanins (TMA) and ethanol production. On average, maximum ethanol yield ranged from 0.31 to 0.34 g/g dry ISP and TMA concentration ranged from 45 to 64 mg cyanidin-3-glu/100 g dry powder (10–22 mg/100 g fresh weight) with the greatest ethanol production coming from non-adjusted pH fermentations. The highest anthocyanin recovery, 64 mg cyanidin-3-glu/100 g dry powder was obtained at 35 °C and pH 4.5 using *S. cerevisiae* Ethanol Red. This study showed the feasibility of extracting anthocyanins and producing ethanol simultaneously in one unit operation without the need of purified solvents.

1. Introduction

Demand for biodegradable and renewable resources to generate bioenergy and bioproducts has been increasing due to the necessity to rely less on petroleum and develop sustainable, eco-friendly materials and products. Bioethanol production is derived primarily from sugar crops (i.e. sugar cane) and starch-based food crops (i.e. corn). However, in the United States of America the use of cereal starches to produce bioethanol competes with agricultural land needed for food/feed production (Schenk et al., 2008; Naik et al., 2010). Therefore, it is important to find alternative renewable feedstock sources for valuable fuel and chemical commodities.

Sweetpotato (*Ipomoea batatas*) is an important starch-producing crop grown around the world primarily in the tropics and subtropics. The storage roots store energy in the form of carbohydrates representing around 80–90% of its dry matter and consisting mainly of starch and sugars (Aina et al., 2012). Sweetpotatoes bred for high dry matter content (30–40%, of which 60–90% is starch) in the US are not considered a food crop and have significant potential in industrial applications. These industrial sweetpotatoes (ISPs) are an excellent substrate for alcohol fermentation since they contain large amounts of starch, high starch yield per unit of land cultivated (12–14 MG dry starch/ha (10,926–12,297 lb dry starch/acre)), and have advantages in production including low requirements of fertilizer and pesticides, drought tolerance, and feasible growth on marginal lands (Duvernay et al., 2013; Kim and Hamdy, 1985).

Several studies have examined sweetpotato conversion to ethanol and valued-added products using enzymes (Duvernay et al., 2013; Kim and Hamdy, 1985; Bridgers et al., 2010; Zhang

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http://dx.doi.org/10.1016/j.indcrop.2014.07.032
0926-6690/

et al., 2011; Srichuwong et al., 2012). These studies have primarily focused on optimizing process conditions (e.g. substrate loading, enzyme loading, temperature, and yeast concentration) and on reducing sweetpotato viscosity during processing to improve ethanol production yield. Separate hydrolysis and fermentation (SHF) is a common process used to convert sugars to ethanol because optimal temperatures can be set in each unit, but hydrolysis product (glucose) is accumulated in the reactor and can lead to enzyme inhibition as glucose concentration increases (Drissen et al., 2009; Ohgren et al., 2007). Challenges with enzyme inhibition in a SHF system could be resolved using a simultaneous saccharification fermentation system (SSF). SSF is an alternative approach to produce ethanol and offers advantages of minimizing enzyme inhibition (reduction of glucose accumulation) and investment cost (single unit operation and vessel), and supports shorter processing times (Ohgren et al., 2007; Wyman, 1994; Isci et al., 2008; Olofsson et al., 2008).

Recent studies in SSF on sweetpotatoes have focused on evaluating process conditions, such as liquefaction temperature, enzyme loading, substrate loading, for ethanol production using sweetpotato lines with low gelatinization temperatures (line Kyushu 159) and the use of cellulases in very high gravity sweetpotato mashes to allow viscosity reduction prior to liquefaction (Zhang et al., 2011; Srichuwong et al., 2012; Cao et al., 2011). The primary objective of these research efforts was to decrease energy consumption during liquefaction, but little work has been done to assess performance of yeast and SSF operating temperatures to maximize rate of starch hydrolysis, improve sugar consumption rates, and enhance ethanol production.

Since the optimal operating temperature for glucoamylase enzymes used for hydrolysis is around 60 °C, it would be advantageous to use a temperature tolerant microorganism capable of growth and ethanol production at higher temperatures. *Kluyveromyces marxianus* is one of the most used thermotolerant yeast reported in the literature with maximum growth temperatures reported up to 52 °C and alcohol production at temperatures above 40 °C, using sugars from substrates including switchgrass, barley straw, and cellobiose (Banat et al., 1998). The use of a thermophilic microorganism in a SSF system has the potential to enhance conversion of industrial high dry matter sweetpotatoes to ethanol.

In addition, purple-fleshed ISPs produce high quantities of starch and antioxidants in the form of anthocyanins (Bridgers et al., 2010). Anthocyanins have been extensively reported to help reduce the risk of cardiovascular disease, chronic disease, and neuronal degeneration (Wu et al., 2008; Bridle and Timberlake, 1997), with industrial applications as a natural food and textile colorant and as a nutraceutical ingredient (Deroles, 2009; Wegener et al., 2009). Production and concentration of anthocyanins can be affected by environmental factors such as pH, UV radiation, temperature, and presence of nitrogen and phosphorous compounds (Wu et al., 2008; Castañeda-Ovando et al., 2009; Pascual-Teresa and Sanchez-Ballesta, 2008). Generally, at low pH (~4) the flavylium cation (red in color) is the frequent form of the anthocyanin, but the increase in pH can cause its denaturation. Extraction of anthocyanins is typically performed with solvent (acetone, ethanol, methanol, acidified methanol, and acidified ethanol) at specific pH levels (Bridgers et al., 2010; Brown et al., 2005; Steed and Truong, 2008; Cevallos-Casals and Cisneros-Zevallos, 2003). SSF on purple-fleshed ISPs under acidic pH can be further examined as an approach to simultaneously extract anthocyanins with reduced inputs and generate in a single step two value-added product streams (ethanol and anthocyanins).

This study investigated the potential fermentability of sugars from ISPs using the mesophilic yeast *Saccharomyces cerevisiae* and the thermophilic yeast *K. marxianus*. The aim was to establish SSF parameters for ethanol fermentation using flour preparations of ISPs (lines NC-413 and DM02-180) and examine the feasibility of extracting anthocyanins from flour purple-fleshed ISPs (line NC-413) during ethanol fermentation through a SEF system while considering the effect of pH.

2. Materials and methods

2.1. Substrate preparation

Two different sweetpotato (swp) clones, purple-fleshed ISP line NC-413 and white-fleshed ISP line DM02-180, were selected from the sweetpotato breeding program at NCSU (Raleigh, NC) and used in experiments. Sweetpotato line NC-413 was grown and harvested during the 2011 cropping season from the Cunningham Research Station at Kinston, NC (35.298° N, 77.575° W). Sweetpotato line DM02-180 was harvested during the 2011 cropping season from the Horticultural Crops Research Station at Clinton, NC (35.023° N; 78.278° W). After harvest, the roots were cured and stored (14 °C, 85% rh, 9 months). All roots were selected at the same time and used within 2 months. Roots were washed to remove soil, dirt, and then stored (14 °C, 2 days) before use. Sweetpotato roots were randomly selected from a stored batch and flour preparations were prepared by placing sliced (~2 mm thickness) roots in an oven at 70 °C for 60 h and dried samples were ground (using a Wiley mill) through a 2 mm mesh screen. Flour samples were stored in sealed plastic bags in the refrigerator (4 °C) until use.

2.2. Solvents, enzymes, and yeasts

Ethyl alcohol (Cat #E190, Pharmco-AAPER, 95%) used in starch alcohol insoluble solids (AIS) determination was of ACS/USP grade. Commercial sources of α-amylase and glucoamylase were used in the hydrolysis experiments. The α-amylase used for liquefaction was Liquozyme SC (Novozymes, North America, stored at 4 °C, density 1.25 g/ml) with optimal temperature of 85 °C, optimal pH of 5.5, and activity of 120 KNU-S/g enzyme. One Kilo novo unit (KNU-S) is the amount of enzyme that can hydrolyze 5.26 g of soluble starch per hour. The glucoamylase used in the saccharification studies was Spirizyme Ultra (Novozymes, North America, stored at 4 °C, density 1.15 g/ml) with optimal temperature of 65 °C and activity of 900 AGU/g protein. One amyloglucosidase unit (AGU) is the amount of enzyme able to hydrolyze 1 μmol of maltose per minute at 37 °C and pH 4.3.

S. cerevisiae Ethanol Red Yeast (Lesaffre Yeast Corp., Milwaukee, WI, optimum growth temperature 35 °C) and *K. marxianus* var *marxianus* NCYC 851 (National Collection of yeast cultures in UK, optimum growth temperature 42 °C) were used for the fermentation studies. *S. cerevisiae* and *K. marxianus* yeast culture freezer stock concentrations (liquid) were on average 1.3 mg dry cells/ml and 1.1 mg dry cells/ml, respectively and cultures were preserved in glycerol (8% salts (KH_2PO_4, $CaCl_2{\cdot}2H_2O$, $(NH_4)_2SO_4$, $MgSO_4{\cdot}7H_2O$), 50% glycerol, and 42% water) at −80 °C. Seed cultures for ISP fermentations were grown in YPD medium (Fisher BioReagents BP-2469, Fisher Scientific, Pittsburgh, PA) under the respective growth conditions until the appropriate cell density was reached for inoculation.

2.3. Experimental design and statistical analysis

2.3.1. Separate hydrolysis and fermentation in white DM02-180 and purple NC-413 ISPs

Use of sugar hydrolysates without prior sterilization can encounter contamination issues by foreign microorganisms and commonly used preservatives during hydrolysis, such as sodium

azide, inhibit subsequent yeast fermentations. In order to determine the effect of heat sterilization on fermentation of sugars from ISPs and examine the benefit of having active enzymes during the subsequent fermentation process (no heat sterilization), experiments with *K. marxianus* and *S. cerevisiae* at 35 °C were studied on hydrolysates that were treated with and without sterilization through autoclaving. The study showed that the sterilization treatments did not result in statistical differences in ethanol production during fermentation ($p > 0.05$, data not shown). As a result, the ISP hydrolysates used during the fermentation part of the SHF experiments were not sterilized by autoclaving.

Hydrolysis of ISPs was completed in sterile 1 l flasks with flour preparations adjusted to a 25% (w/v) substrate loading using sterile deionized water. Moisture inherent to the sweetpotato was accounted for as part of the total liquid volume. One hundred dry grams of flour per 400 ml of total volume was used for purple flour (2.95% moisture content, wet-basis) and white flour (1.59% moisture content, wet-basis) ISPs preparations. Liquozyme SC was added to all preparations under a biological safety cabinet at a level of 0.30% volume of enzyme/g dry swp (4.5 KNU-S/g dry ISP) considering the optimum alpha amylase loading results reported (Bridgers et al., 2010; Duvernay et al., 2013). All flasks were placed in an orbital shaking water bath (80 rpm) and incubated at 85 °C for 2 h. Spirizyme Ultra (2.5 AGU/g dry swp) was added to the flasks (except for controls) and saccharification was completed in an orbital shaking water bath (80 rpm) at 65 °C for a period of 16 h. Hydrolysate (7.8 ml) from the 1 l flasks were transferred to sterile culture tubes (10 ml) and inoculated at 2.5% (v/v) (0.2 ml seed culture) with either *K. marxianus* at 35 °C and 40 °C or with *S. cerevisiae* at 35 °C. The seed cultures had cell densities of 8.7 mg dry cells/ml for *K. marxianus* and 9.8 mg dry cells/ml for *S. cerevisiae* (target cell count of 10^8 cells/ml). Culture tubes were placed in a non-shaking water bath at the corresponding temperature (either 35 °C or 40 °C) for a period of 72 h. Fermented hydrolysate (1 ml) was repeatedly sampled from each container at select time intervals (0, 24, 36, 48 and 72 h) and stored at −80 °C prior to measurement of ethanol, total residual sugars, and residual glucose.

2.3.2. Simultaneous saccharification and fermentation in white DM02-180 ISP

The effects of two Spirizyme Ultra enzyme loading conditions (2.5 and 5 AGU/g dry ISP), two yeast inoculum cell concentrations (8.7 mg dry cells/ml in *K. marxianus* and 9.8 mg dry cells/ml in *S. cerevisiae* ∼inoculum cell count of 10^8 cells/ml; and 3.1 mg dry cells/ml in *K. marxianus* and 3.6 mg dry cells/ml in *S. cerevisiae* ∼inoculum cell count of 10^6 cells/ml), and four incubation times (0, 12, 24, and 48 h) on the conversion of starch into fermentable sugars and fermentation of sugar to ethanol using flour DM02-180 of ISPs during SSF were examined. Treatments were performed with *K. marxianus* (at 35 °C and 40 °C) and with *S. cerevisiae* Ethanol Red Yeast at 35 °C.

All treatment combinations in the repeated measures experimental design were completed in quadruplicates with duplicate saccharification controls (no glucoamylase added during SSF) and duplicate fermentation controls (no yeast added during SSF). Experimental treatments were completed in sterile 50 ml Falcon tubes. Flour white DM02-180 ISP preparations were completed at 25% (w/v) using sterile deionized water and moisture inherent to the sweetpotato and added enzyme stocks were accounted for as part of the total liquid volume. Five dry grams per 20 ml of total volume was used for DM02-180 flour (1.91% moisture content, wet-basis) and the volume of each Falcon tube was adjusted to 25% (w/v) (1:4 ratio) using sterile deionized water (ddH_2O). Liquozyme SC was added to all treatments and controls under a biological safety cabinet at a level of 0.30% volume of enzyme/g dry swp (4.5 KNU-S/g dry ISP) considering the optimum alpha amylase loading results reported (Bridgers et al., 2010; Duvernay et al., 2013). All treatments were placed in an orbital shaking water bath (80 rpm) and incubated at 85 °C for 2 h. Spirizyme Ultra (2.5 and 5 AGU/g dry swp) and yeast [*K. marxianus* (35 or 40 °C) and *S. cerevisiae* (35 °C)] at 2.5% (v/v) were added to the corresponding tubes (except for controls). Treatments were capped with rubber stoppers (with attached check valves (Cat #15-315-33A, Fisher Scientific)) to allow tubes to vent without allowing air in) and placed in an orbital shaking water bath (80 rpm) at the corresponding temperature (either 35 °C or 40 °C) for a period of 48 h. Aliquots (2 ml) of fermentation broth were repeatedly sampled from each tube at selected time intervals (0, 12, 24, and 48 h) and stored at −80 °C prior to measurement of ethanol, total residual sugars, and residual glucose. Analysis of variance for main and interaction effects was evaluated using PROC GLIMMIX in SAS 9.1.3 software (SAS® Inc., Cary, NC) with glucose, total sugar, and ethanol concentration as response variables. In addition, pairwise *t*-test comparisons were made between treatments using maximum ethanol concentration as a response variable. Statistical significance was made at an α value of 0.05.

2.3.3. Simultaneous extraction fermentation in purple NC-413 ISP

The aim of this experiment was to assess the feasibility of extracting anthocyanins through the simultaneous extraction and fermentation (SEF) process of flour purple NC-413 ISPs. The enzyme and yeast parameters selected were based on the optimum conditions obtained from the SSF of DM02-180 ISPs for ethanol production. Furthermore, this study assessed the impact of initial fermentation pH (4.5 or 5.5) in increasing anthocyanin recovery as ethanol was produced. Experimental treatments were completed in sterile 50 ml Falcon tubes. Flour purple NC-413 ISPs preparations were completed at 25% (w/v) using sterile deionized water and moisture inherent to the sweetpotato and added enzyme stocks were accounted for as part of the total liquid volume. Five dry grams per 20 ml of total volume were used for purple NC-413 ISP flour (2.95% moisture content, wet-basis). The volume of each Falcon tube was adjusted to 25% (w/v) (1:4 ratio) using sterile deionized water (ddH_2O). Liquozyme SC was added to all treatments and controls under a biological safety cabinet at a level of 0.30% volume of enzyme/g dry swp (4.5 KNU-S/g dry ISP) considering the optimum alpha amylase loading results reported (Bridgers et al., 2010; Duvernay et al., 2013). All treatments were placed in an orbital shaking water bath (80 rpm) and incubated at 85 °C for 2 h. Spirizyme Ultra (2.5 AGU/g dry swp) and yeast (*K. marxianus* [8.7 mg dry cells/ml ∼10^8 cells/ml in inoculum; 35 or 40 °C], and *S. cerevisiae* [9.8 mg dry cells/ml ∼10^8 cells/ml in inoculum; 35 °C]) at 2.5% (v/v) were added to the corresponding tubes (except for controls). Before enzyme and yeast addition, pH 4.5 treatments were adjusted with dilute HCl while pH 5.5 treatments did not require adjustment. Tubes were capped with rubber stoppers (with attached check valves (Cat #15-315-33A, Fisher Scientific) to allow tubes to vent without allowing air in) and placed in an orbital shaking water bath (80 rpm) at the corresponding temperature (either 35 °C or 40 °C) for a period of 72 h. Sample aliquots (2 ml) were repeatedly sampled from each tube at select time intervals (0, 12, 24, 48, and 72 h) and stored at −80 °C prior to measurement of total monomeric anthocyanin content, ethanol, residual sugars, and residual glucose. Analysis of variance for main and interaction effects were evaluated using PROC GLIMMIX in SAS 9.1.3 software (SAS® Inc., Cary, NC) for maximum ethanol and anthocyanin concentrations and *t*-test pairwise comparisons were made on least square means of treatments. Statistical significance was made at an α value of 0.05.

2.4. Analytical methods

Moisture content of sweetpotatoes was determined using an oven drying method at 105 °C for 24 h and samples were analyzed

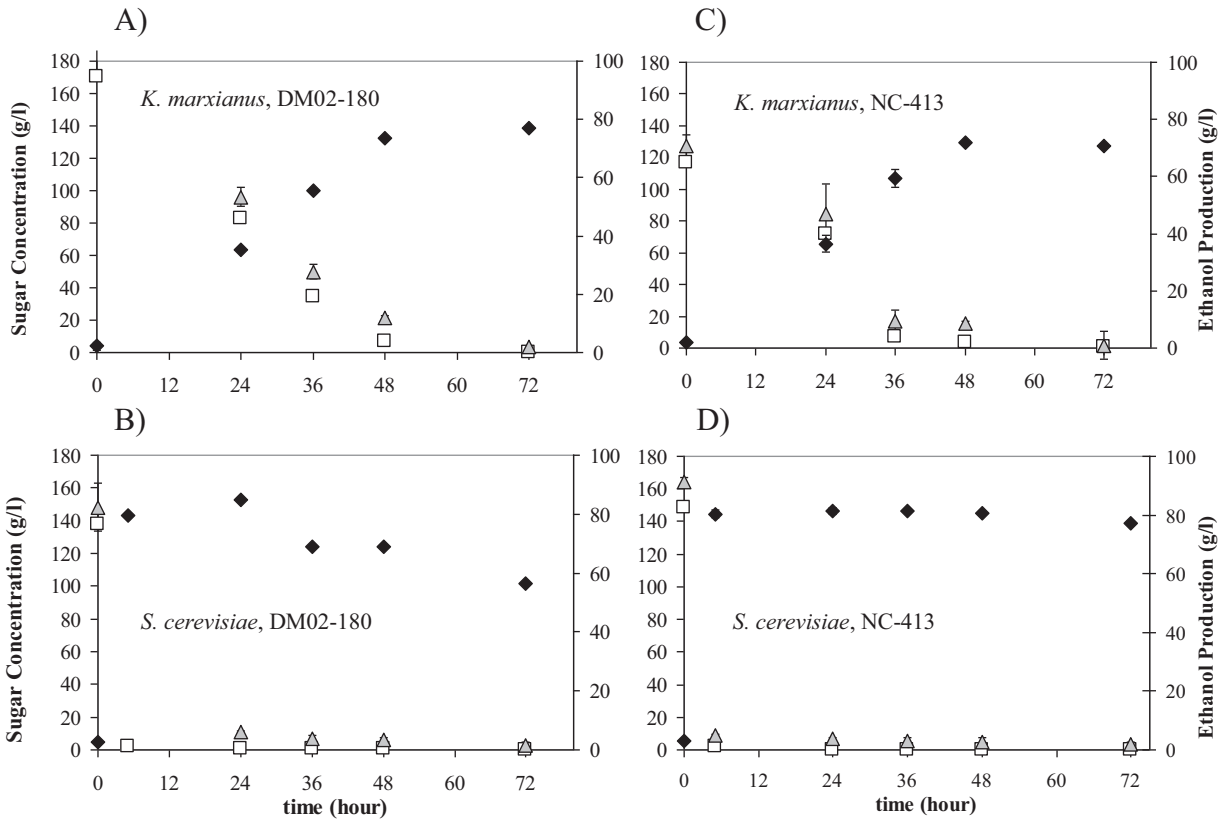

Fig. 1. Glucose (□) total sugar (△), and ethanol concentration (♦) over time with hydrolysate non-autoclaved using *K. marxianus* and *S. cerevisiae* during SHF of flour white DM02180 ISP at 35 °C (A and B, respectively) and during SHF of flour purple NC-413 ISP at 35 °C (C and D, respectively). Total sugars represent maltotriose, maltose, glucose, and fructose.

in triplicate (ASTM E1756-08). Sugar (maltotriose, maltose, glucose, and fructose) and ethanol concentrations during SHF, SSF, and SEF studies were measured by high-performance liquid chromatography (HPLC) using methods described by Bridgers et al. (2010). Total monomeric anthocyanin (TMA) content was determined using a spectrophotometric pH-differential method (Giusti and Wrolstad, 2003). The most representative anthocyanin for this investigation's TMA measurements was cyanidin-3-glucoside with a molar absorptivity (ε) of 26,900 (Jurd and Asen, 1966; Delgado-Vargas and Paredes-Lopez, 2003), therefore results were reported as cyanidin-3-glucoside equivalents (cyd-3-glu-E) per 100 g of fresh weight or dry flour weight (mg cyaniding-3-glucoside/100 g fw or dry flour).

3. Results

3.1. Separate hydrolysis fermentation (SHF) of white DM02-180 and purple NC-413 ISPs

A SHF system was used to establish a baseline for ethanol production and determine differences in the performance of the yeasts *K. marxianus* and *S. cerevisiae* Ethanol Red in fermenting sugar from hydrolysates obtained after hydrolysis of NC-413 and DM02-180 ISP preparations. Fig. 1 shows sugars present and ethanol produced over time in DM02-180 ISPs and NC-413 ISPs at 35 °C using *K. marxianus* and *S. cerevisiae* (10^8 cells/ml) in non-sterile treatments.

Maximum ethanol yield (g ethanol/g glucose used) with DM02-180 ISP hydrolysates using *K. marxianus* was 30% lower than from those hydrolysates using *S. cerevisiae* (0.61 g ethanol/g glucose used, 24 h); however, for NC-413 ISP hydrolysates the yield was 6.6% higher when using *K. marxianus* than when *S. cerevisiae* (0.57 g ethanol/g glucose used, 5 h) was used. Overall, performance in terms of conversion efficiency was similar between ISPs for both yeast. *S. cerevisiae* was capable of fermenting glucose more efficiently than *K. marxianus*, 140 g/l and 143 g/l of glucose almost completely consumed in DM02-180 and NC-413 ISP treatments, respectively after 5 h. More than 15 g/l of total sugars remained unfermented in treatments with *K. marxianus* after 48 h fermentation. Previous studies made on ISPs reported ethanol production rates of 0.7 g/l/h and 0.9 g/l/h in sugar fermentations from flour white-fleshed FTA-94 (~120 g/l) and flour purple-fleshed NC-413 ISPs (~100 g/l) preparations, respectively using similar yeast concentrations of Ethanol Red and without nutrient supplementation (Bridgers et al., 2010; Duvernay et al., 2013). The higher average production rate (28 g/l/h) and ethanol yield (0.61 g ethanol/g glucose used) obtained with *S. cerevisiae* for the ISP lines used in this study could be tied to several factors, including not sterilizing by autoclaving supporting the continued presence of active amylase enzymes and the state of the yeast being hydrated active cells in this work compared to the dry formulations used by the other researchers.

3.2. Simultaneous saccharification fermentation (SSF) of DM02-180 ISPs

Results for glucose, total sugar, and ethanol concentration over time across yeast loading during SSF of DM02-180 preparations under the two yeasts concentrations and the two temperatures used for *K. marxianus* are shown in Fig. 2 ($p < 0.05$). Statistical analysis showed that the effect of enzyme loading on ethanol concentration was not statistically significant ($p > 0.05$, data not shown). SSF using different yeast inoculum concentrations produced significant differences in the ethanol concentrations observed over time and the higher cell concentration significantly improved ethanol production ($p < 0.05$). Increase in yeast inoculum

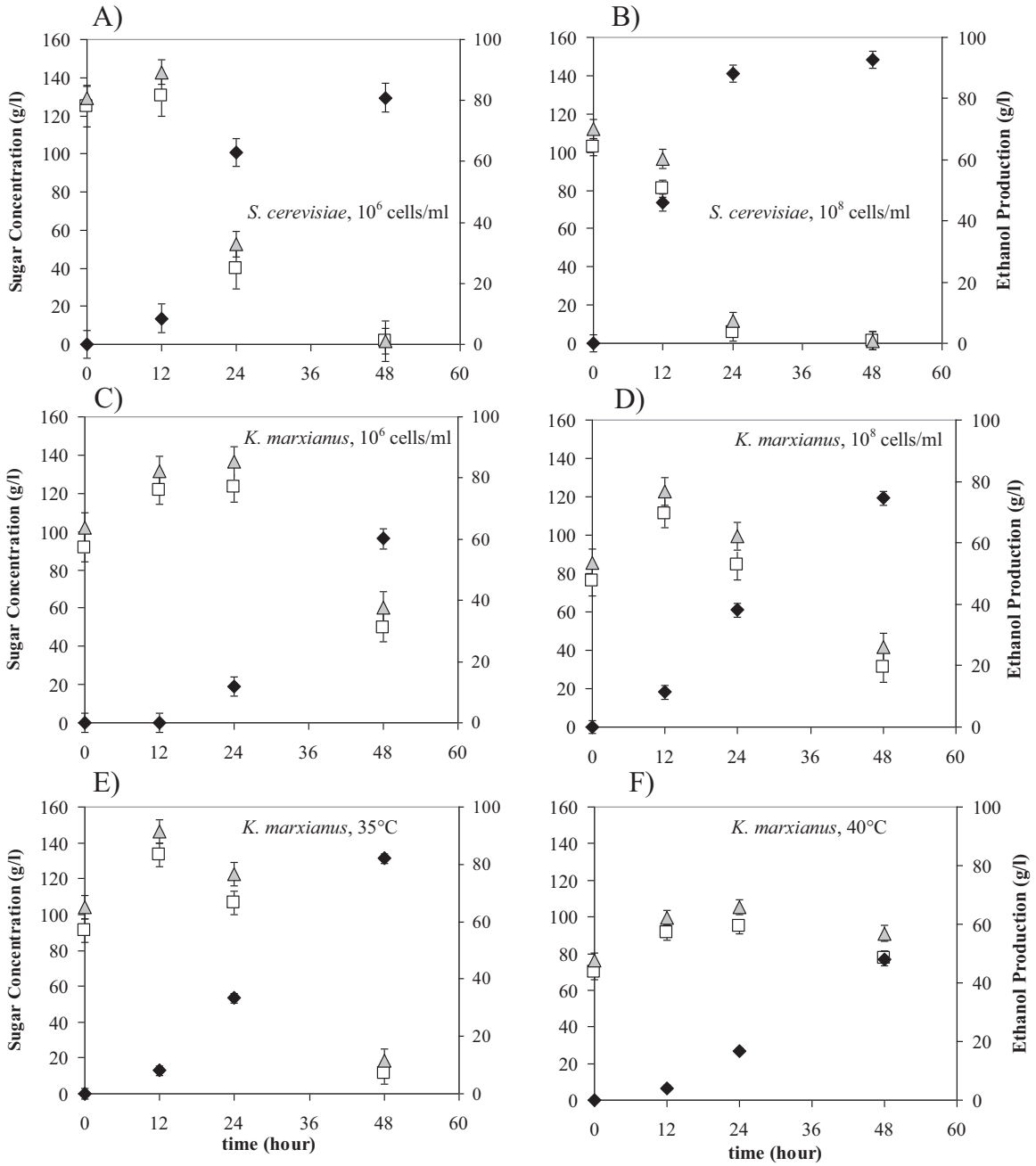

Fig. 2. Glucose (□), total sugar (△), and ethanol concentration (♦) over time in SSF experiments. Total sugars represent maltotriose, maltose, glucose, and fructose. Effect of time across enzyme loading using *S. cerevisiae* at 35 °C (p-value < 0.05), 10^6 cells/ml (A) and 10^8 cells/ml (B). Effect of time across enzyme loading and temperature using *K. marxianus* (p-value < 0.05), 10^6 cells/ml (C) and 10^8 cells/ml (D). Effect of time across enzyme loading and yeast concentration using *K. marxianus* (p-value < 0.05), 35 °C (E) and 40 °C (F).

concentration from10^6 cells/ml to 10^8 cells/ml had a positive effect in increasing the ethanol production rate with 1.2-fold and 1.3-fold increases for *K. marxianus* at 35 °C and *S. cerevisiae*, respectively. In addition, on average the ethanol concentration increased by 31% and 16% for *K. marxianus* and *S. cerevisiae* at 35 °C, respectively. The average ethanol production rate with *S. cerevisiae* (2.39 g/l/h) was at least 28% faster than with *K. marxianus* when using the 10^8 cells/ml inoculum (Fig. 2B and D). The use of *K. marxianus* at 40 °C significantly reduced the ethanol concentration ($p < 0.05$, Fig. 2E–F) with a 29% decrease in ethanol production rate in comparison to the fermentation at 35 °C. Results also revealed that ethanol generated by *S. cerevisiae* had a maximum ethanol content (96.9 g/l) which was 1.07-fold and 1.5-fold greater than that produced by *K. marxianus* at 35 °C and 40 °C, respectively after 48 h SSF.

Table 1 shows residual glucose and the average maximum ethanol concentrations achieved during SSF of flour DM02-180 ISP preparations. Treatments with *S. cerevisiae* were statistically similar for ethanol production and were not statistically different than the concentrations obtained with *K. marxianus* at 35 °C, but statistically higher than ethanol concentrations obtained with *K. marxianus* at 40 °C.

3.3. Simultaneous extraction fermentation (SEF) of purple ISPs

Table 2 shows residual glucose and maximum average ethanol concentration during SEF of NC-413 ISPs. The pH adjustment to 4.5 did not significantly decrease ethanol production ($p > 0.05$). Treatments with *K. marxianus* without pH adjustment produced

Table 1
Residual glucose and maximum average ethanol concentration during SSF of flour DM02-180 ISPs.

Yeast type	Yeast conc. (cells/ml media)	Temp. (°C)	Residual glucose (g/l)	Maximum average ethanol (g/l)
S. cerevisiae	10^8	35	1.9	96.9^{A}
	10^6		41.6	82.5^{A}
K. marxianus	10^8	35	1.8	87.1^{A}
	10^6		90.9	80.9^{AB}
K. marxianus	10^8	40	1.4	64.6^{B}
	10^6		60.0	43^{C}

Statistical comparisons between yeast type, yeast concentration and temperature, values with different letters (A, B and C) are statistically different ($p < 0.05$).

Table 2
Residual sugars and maximum average ethanol concentration during SEF of flour NC-413 ISPs.

Yeast type	pH	Temp. (°C)	Residual glucose (g/l)	Maximum average ethanol (g/l), time (h)
K. marxianus	5.5	35	3.7	85.8 (72 h)^{A}
	4.5		3.8	83.4 (72 h)^{AB}
S. cerevisiae	5.5	35	2.4	80.4 (72 h)^{AB}
	4.5		1.5	77.1 (24 h)^{B}
K. marxianus	5.5	40	17.2	76.9 (72 h)^{B}
	4.5		13.8	76.4 (72 h)^{B}

Statistical comparisons between yeast type, pH, and temperature, values with different letters (A and B) are statistically different ($p < 0.05$).

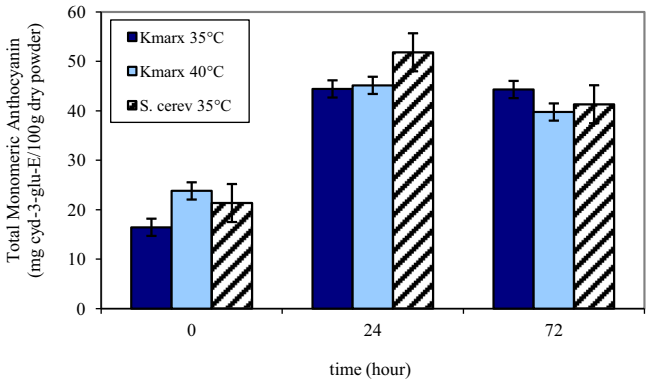

Fig. 3. Effect of time across pH during SEF in purple NC-413 ISP using *S. cerevisiae* at 35 °C and *K. marxianus* at 35 °C and 40 °C ($p < 0.05$).TMA is expressed in mg/100 g dry powder.

significantly higher ethanol concentration compared to all treatments at 40 °C ($p < 0.05$). Unlike SSF on white DM02-180 ISPs, *K. marxianus* at 40 °C and pH 5.5 performed better in terms of ethanol production in treatments with purple NC-413 ISPs. Fig. 3 shows the main effect of time across pH ($p < 0.05$) during SEF of NC-413 ISPs using *S. cerevisiae* and *K. marxianus* for total monomeric anthocyanin concentration (TMA) which describes the color quality of the anthocyanins present. Initial TMA concentration obtained immediately after liquefaction of flour NC-413 ISP was 10.1 mg cyd-3-glu-E/100 g dry flour weight. On average, 24-h incubation with *S. cerevisiae* yeast showed statistically higher anthocyanin concentration (51.8 mg cyd-3-glu/100 g dry flour weight or 17.8 mg cyd-3-glu/100 g fresh weight, with a dry matter content of NC-413 line of 34.0%) than the other extraction times studied. Around 14.8–16.8% more TMA was obtained with *S. cerevisiae* compared to *K. marxianus* ($p < 0.05$). However, over time TMA concentration significantly decreased in treatments with *S. cerevisiae* ($p < 0.05$).

Ethanol produced during the simultaneous fermentation system was effective in extracting anthocyanins. The interaction effect of pH and temperature was statistically significant ($p < 0.05$).

Table 3 shows the average maximum TMA obtained for those different treatment combinations. The pH adjustment to 4.5 significantly increased extraction of TMA when *S. cerevisiae* yeast was used by 33% compared with pH 5.5, 64.4 mg cyd-3-glu/100 g dry flour weight (22.1 mg cyd-3-glu/100 g fresh weight) ($p < 0.05$) and was the highest TMA concentration compared to the treatments with *K. marxianus* ($p < 0.05$).

4. Discussion

4.1. Separate hydrolysis fermentation (SHF) of white DM02-180 and purple NC-413 ISPs

Fermentation of ISP hydrolysates with *K. marxianus* at 40 °C did not improve ethanol concentration in a SHF system. Previous studies have also reported reduced ethanol production and cell viability using *K. marxianus* at high temperatures (>35 °C) (Hacking et al., 1984; Ballesteros et al., 1991). Furthermore, the use of *K. marxianus* at higher temperatures may have required essential nutrients (including K, Mn, Mg, and P) that might not have been available in sufficient quantities in the hydrolysate. Previous studies on SHF using ISPs showed that the storage roots provided enough nutrients to support fermentations using *S. cerevisiae* Ethanol Red yeast (Bridgers et al., 2010; Duvernay et al., 2013). In other studies, supplementation of essential nutrients (especially Mg) to thermotolerant yeasts at 43 °C, resulted in an increase in alcohol

Table 3
Average maximum total monomeric anthocyanin during SEF of flour NC-413 ISPs.

Yeast type	pH	Temp. (°C)	Average max. TMA (mg/100 g fresh weight), (time)	Average max. TMA (mg/100 g dry powder), (time)
S. cerevisiae	4.5	35	22.1 (24 h)	64.4 (24 h)^{A}
	5.5		16.6 (72 h)	48.4 (72 h)^{B}
K. marxianus	4.5	35	15.6 (24 h)	45.6 (24 h)^{B}
	5.5		15.9 (72 h)	46.5 (72 h)^{B}
K. marxianus	4.5	40	15.5 (24 h)	45.3 (24 h)^{B}
	5.5		16.3 (24 h)	47.6 (24 h)^{B}

Statistical comparisons between yeast type, pH, and temperature, values with different letters (A and B) are statistically different ($p < 0.05$). TMA is expressed in mg/100 g fresh weight or mg/100 g dry powder.

production and total glucose utilization compared with treatments without nutrients (Banat et al., 1992; Rocha et al., 2011). The better performance of *K. marxianus* in purple NC-413 ISP may be due to the presence of key nutrients in the purple NC-413 sweetpotatoes compared to the white DM02-180 line (Brinley et al., 2008).

The high amount of sugar present during the SHF with *K. marxianus* may have also inhibited fermentation. Glucose concentrations above 80 g/l have been reported to cause inhibitory effects on the specific growth rate and ethanol productivity in *K. marxianus* strains (Hack and Marchant, 1998). The high glucose concentration (more than 140 g/l) obtained during SHF studies may have altered the microorganism's ability to produce ethanol. Improvement in *K. marxianus* performance was observed in SSF and SEF systems where initial glucose levels were not as high (<92 g/l). Furthermore, in the SHF system the rate of glucose production during hydrolysis was more than 50% higher than the rate of glucose consumption by *K. marxianus* (2.98 g/l/h). The results presented in this study showed that glucose was not used efficiently by the *K. marxianus* strain in alcohol fermentation.

4.2. Simultaneous saccharification fermentation (SSF) of DM02-180 ISPs

Ethanol concentration increased with the increase in yeast concentration for *K. marxianus* and *S. cerevisiae*. In a SSF system, the higher yeast concentration and glucose consumption/ethanol production rates, minimize inhibition effects that can occur due to glucose accumulation, thus improving sugar production and overall potential for more ethanol. The rate of ethanol production using *S. cerevisiae* was more than 1-fold greater than using *K. marxianus*, and had a 20% increase in ethanol concentration compared with the SHF system (data not shown).

Reduction in glucose accumulation observed during SSF resulted in less glucose inhibition for the enzymes, improving starch conversion and thus enhanced ethanol production (Ohgren et al., 2007, 2008). Use of effective thermotolerant organisms in SSF systems can help improve sugar production rates by maintaining temperatures closer the optima for the enzymes used. *K. marxianus* was studied in SSF to evaluate this potential using glucoamylases on ISP lines; however, lower ethanol concentrations were obtained in treatments with *K. marxianus* at 40 °C than with *K. marxianus* at 35 °C. Banat et al. (1992) also reported a decrease in alcohol production in *K. marxianus* IMB strains with an increase in temperature during 42-h incubation. In addition, *K. marxianus* at 40 °C also used glucose as a carbon source to produce glycerol (data not shown). Glycerol (a by-product in yeast fermentation) is accumulated in yeast cells as a protective compound and is synthesized as a response for environmental stresses such as freeze-thaw stress (behaving as a cryoprotectant), heat shock, salt, high concentration of glucose, osmotic stress (osmoprotection), and ethanol stress (Omori et al., 1996; Vriesekoop et al., 2009). This particular system reported seemed to stress *K. marxianus* not only due to temperature but also levels of glucose concentration. The rate of glucose consumption by *K. marxianus* may be too slow relative to the activity of the glucoamylase used and lower activity may show better results.

4.3. Simultaneous extraction fermentation (SEF) of purple ISPs

A better condition for anthocyanin extraction with minimal effect on ethanol concentration was achieved when the initial pH was adjusted to 4.5. Acidified methanol and ethanol solvents have been found to be more efficient in extracting anthocyanins in purple ISPs because their polarity might be similar to anthocyanin characteristics (Bridgers et al., 2010). The high anthocyanin extraction observed in treatments with *S. cerevisiae* at pH 4.5 was likely due to the relative stability of anthocyanins under more acidic conditions and the ethanol polarity which enhanced better extraction. The differences observed in anthocyanin concentration over time in SEF treatments, especially in those using *K. marxianus* can be explained by the ethanol production rates. Higher ethanol production in less time (24 h) was obtained using *S. cerevisiae* yeast compared with *K. marxianus*, thus exposing anthocyanins for less time to potential degradation. The results suggested that longer fermentation time (>70 h) may have thermally degraded extracted anthocyanins (Reyes and Cisneros-Zevallos, 2007; Kechinski et al., 2010). Optimum conditions for extraction of anthocyanins in purple sweetpotatoes reported in the literature are as high as 80 °C, yet the exposure time is limited to 60 min (Fan et al., 2008). The results obtained from the SEF system suggest that the use of the yeast *S. cerevisiae*, which was able to produce ethanol from sugars at high rates and low pH levels, is a potential candidate for anthocyanin extraction in an integrated system.

Extracted anthocyanins from purple-fleshed sweetpotatoes using solvents have been reported in literature ranging from 15 mg cyanidin-3-glu/100 g fw to 186 mg cyanidin-3-glu/100 g fw (Bridgers et al., 2010; Steed and Truong, 2008; Cevallos-Casals and Cisneros-Zevallos, 2003). Teow and coworkers obtained 24.6–43.0 mg/100 g fw using flour (freeze-dried) purple-fleshed sweetpotato lines from the hydrophilic extract (hexane extraction followed by acidified methanol) and measured by the pH-differential method (Teow et al., 2007). Truong and coworkers found anthocyanin concentrations ranging from 0 to 210 g fw from purple-fleshed sweetpotatoes using pressurized-liquid extraction (Truong et al., 2012). Anthocyanin contents obtained in this study were in the range of the ones reported in the literature, yet on the lower end. Higher values may be attained with fresh ISP preparations (not flour) as well as with ISP lines with enhanced anthocyanin content (Bridgers et al., 2010). SEF of flour purple NC-413 ISPs produced 77.1 g/l ethanol after 24 h and simultaneously extracted 22.1 mg cyanidin-3-glu/100 g fw demonstrating the savings in processing costs that is possible to achieve with a SEF system while obtaining two value-added products.

Based on the ethanol yield results and the promising treatment combinations obtained in the SSF and SEF studies, it is projected that about 739 and 588 gallons per acre in a SSF system with NC-413 ISPs and DM02-180 ISPs, respectively can be achieved using Ethanol Red yeast (these values were calculated based on the fermentation data and ISP yields over several growing seasons in NC). In comparison, corn ethanol yields have been reported to be between 300 and 400 gallons/acre (Ziska et al., 2009). The preprocessing methods employed for ISPs can significantly affect ethanol production values attained. The roots used in this work were stored for a period of time from harvest to accommodate the research experiments and it is well known that prolonged storage periods can cause loss in moisture and starch content in sweetpotatoes (Picha, 1987) resulting in lower sugar and ethanol yields. Although all results and data presented here were normalized on a dry matter basis and to their initial starch content at time of use, the values could potentially be higher if reduced storage time is considered. At the same time, we showed decent results and yields from ISP lines that were stored for a 9-month period, supporting their relative stability as a potential renewable biobased product feedstock.

5. Conclusions

ISPs are a promising feedstock for fuel ethanol production. Reduction of energy consumption during processing is a strategy needed to improve its competitiveness for production of valuable products at lower costs. This study showed that using an integrated SSF system is a promising alternative for ethanol production. *S.*

cerevisiae Ethanol Red Yeast showed potential advantages in fermenting sugars in less time with higher glucose consumption and ethanol production rates. It was also observed that glucose was not used efficiently by *K. marxianus* NCYC 851 strain in alcoholic fermentation of the ISPs studied. However, considering the advantage of using a consolidate bioprocess system and the requirement of high temperatures during starch hydrolysis, the use of thermotolerant yeast can be advantageous for large scale chemicals production. Furthermore, the unique SEF approach studied here may find significant application in industrial extraction of natural products from plants.

Acknowledgments

This work was funded in part by the North Carolina Agricultural Foundation, Inc. The authors would like to thank Novozymes North America, Inc. (Franklinton, NC) for providing the enzymes used in these studies, Dr. Craig Yencho and Mr. Ken Pecota of the Sweetpotato Breeding Program of the North Carolina Department of Agriculture and Consumer Services Horticultural Crops Research Station, Clinton, NC for their assistance with the production of the sweetpotatoes used in these investigations. The authors would also like to thank Roger Thompson and Rong Reynods for their assistance with analytical procedures.

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