Conversion of soy molasses, soy solubles, and dried soybean carbohydrates into ethanol

Craig C. Long, William R. Gibbons*

(*Biology and Microbiology Department, South Dakota State University, Brookings, SD, 57007 USA*)

Abstract: Soy molasses and soy solubles are byproducts of the conventional soy protein concentrate and soy protein isolate manufacturing processes, respectively. Conversion of the carbohydrates in these byproducts into ethanol was examined. Standardized amounts of commercial cellulase enzymes (Novozyme cellulase, β-glucosidase, and pectinase) were added to soy molasses and soy solubles solutions prepared at various solid loading rates (33%, 50%, 60%, 75%, and 80%) to hydrolyze oligosaccharides, followed by fermentation for 96 h using *Saccharomyces cerevisiae* NRRL Y-2034 and *Scheffersomyces stipitis* NRRL Y-7124. Ethanol-extracted soybean meal (SBM) carbohydrates were also fermented for 96 h without enzymes. *S. cerevisiae* and *S. stipitis* produced about 12.5-45.0 g/L and 6.0-28.0 g/L ethanol, respectively, on molasses and solubles across these solid loading rates. The *S. stipitis* produced about 6.5-17 g/L ethanol and *S. cerevisiae* produced about 6.5-22 g/L ethanol on ethanol-extracted carbohydrates.

Keywords: ethanol production, soy molasses, soy solubles, oligosaccharides

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1 Introduction

Bioethanol production now accounts for approximately 10% of the liquid fuel transportation supply in the USA due to dwindling oil reserves and rising petroleum prices[1,2]. Corn is the most widely used feedstock for bioethanol production[2], but researchers have been developing pretreatment and conversion technologies to use lignocellulosic biomass as a more sustainable feedstock for ethanol production[2-7]. Another class of bioethanol feedstocks has included waste products from various industries (e.g., whey from the cheese industry)[8-11]. These "waste" feedstocks often contain significant levels of fermentable carbohydrates, may not require pretreatment or hydrolysis steps, and are often much cheaper than more conventional substrates[1-7,12-16].

Soy molasses is a by-product of soy protein concentrate (SPC) production (Figure 1). After the oil is removed from the crushed soybean, the defatted flakes (white flakes) or soybean meal (SBM) is washed with 70%-90% aqueous ethanol to remove the carbohydrates and concentrate the protein. The washed solids have a protein content of at least 65% (dry matter basis) and are known as SPC. The liquid fraction is dried to about 50% moisture to recycle as much ethanol as possible, and the resulting syrup (soy molasses) contains a high percentage of carbohydrates, as well as some lipids and protein[1,17,18].

Soy solubles are a by-product of soy protein isolate (SPI) production (Figure 1). Similarly to soy molasses, white flakes or SBM are washed to concentrate protein and remove carbohydrates. White flakes or SBM are washed in an alkaline solution to dissolve the protein, and

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Biography: Craig C. Long, MS, Biology/Microbiology Department, PO Box: 2104A, SDM 215, South Dakota State University, Brookings, SD 57007, Email: cclong@jacks.sdstate.edu.

*__Corresponding author: William R. Gibbons,__ PhD, Professor, Biology/Microbiology Department, PO Box: 2104A, SDM 225A, South Dakota State University, Brookings, SD 57007, Tel: 605-688-5499, Fax: 605-688-6677; Email: william.gibbons@sdstate.edu.

then brought to pH 4.5 to precipitate the protein. The precipitated solids are removed and used to make SPI. The alkaline solution is dried to about 50% moisture and its pH value adjusted to buyer preference. The resulting syrup is called soy solubles[18,19].

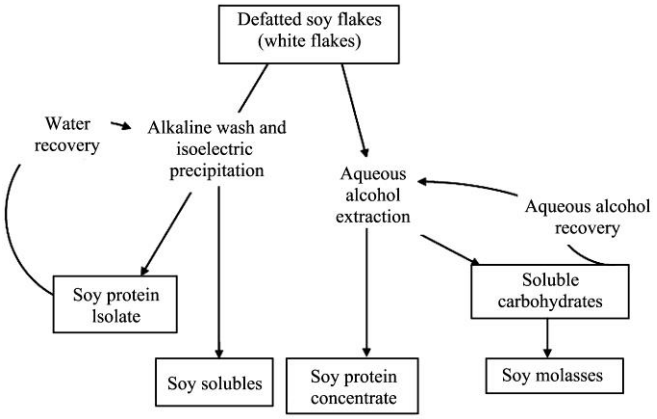

Figure 1 Diagram of SPC and SPI production from soy white flakes

Qureshi et al.[1] used soy molasses as a substrate (80 g/L in water) for producing various solvents (namely acetone, ethanol, and butanol); 10.7 g/L combined solvents were produced after 120 h using *Clostridium beijerinckii* BA101. Siqueira et al.[20] were capable of producing 34.9-63.5 g/L ethanol on soy molasses using *S. cerevisiae* LPB-SC. Montelongo et al.[21] were able to produce 4.0-5.5 g/L of lactic acid using a 2% solution of soy molasses and water, utilizing 85%-89% of the available carbohydrates with *Lactobacillus salivarius* NRRL B-1950. Solaiman et al.[22] produced about 0.6 g/L medium chain-length poly (hydroxyalkanoates) (mcl-PHA) using soy molasses as a medium supplement (5% w/v) for *Pseudomonas corrugata*.

2 Materials and methods

2.1 Substrates

Soy molasses and soy soluble were obtained from Solae, St. Louis, MO. These substrates were subjected to a proximate analysis and carbohydrate quantification by Olson Agricultural Laboratories at South Dakota State University in Brookings, SD. Results are shown in Table 1.

A dried preparation of soluble carbohydrates was also evaluated in this study. This soybean sugar substrate was prepared by a collaborator by mixing ground soybean meal (2 mm Wiley Mill screen) in a 25% (w/v) ratio with 80% (v/v) aqueous ethanol. After heating at 70℃ for 1 h with mixing, the liquid was decanted and replaced with a 50% (w/v) ratio of meal and 80% aqueous ethanol. This slurry was heated at 70℃ for an additional 30 min with periodic mixing. The solids were separated by filtering through a nylon filter and washing with 70℃ sterile deionized water. The combined fluids were evaporated to dryness at 70℃ and subsequently freeze-dried for 72 h using a Virtis shelf dryer.

Table 1 Proximate analysis and carbohydrate quantification of soy molasses and soluble on the basis of dry matter

	Soy molasses	Soy soluble
Dry matter, %	53.1	53.0
Crude protein, combustion, %	11.7	19.1
Crude fat (diethyl ether extract), %	4.91	0.519
Ash, %	21.9	17.0
Fat, Roese Gottlieb, %	4.91	0.519
Crude fiber, crucible method, %	0.565	0.378
Nitrogen free extract, %	61.1	63.0
Sucrose, %	18.5	8.78
Fructose, %	2.96	10.50
Glucose, %	4.67	5.47
Raffinose, %	25.5	12.70
Stachyose, %	34.2	22.3

2.2 Enzymes

Enzymes used in this study were obtained as a gift from Novozymes. NS 50013 (Celluclast 1.5 L) is a cellulase cocktail with an activity of 70 FPU/g. NS50010 (Novozymes 188) is a β-glucosidase with an activity of 250 CBU/g. NS 22016 is a pectinase cocktail with an activity of 3 800 U/mL. Enzymes were stored at 4℃ prior to use.

2.3 Yeast

Saccharomyces cerevisiae NRRL Y-2034 and *Scheffersomyces stipitis* NRRL Y-7124 were obtained from the USDA ARS Culture Collection (Peoria, IL). For short term maintenance, cultures were grown on Potato Dextrose Agar (PDA) plates and slants for 72 h at 35℃, and then stored at 4℃, with subculturing of the organisms every four weeks. Lyophilization in a 20% sucrose solution was used for long term storage.

Inoculum for all trials was prepared by aseptically inoculating sterile 5% glucose, 0.5% yeast extract broth (100 mL in a 250 mL Erlenmeyer flasks) with a 1% (v/v)

aliquot for *S. cerevisiae*, or 5% (v/v) for *S. stipitis*, from broth seed cultures stored at 4℃. Flasks for inoculum were incubated for 24 h at 35℃ in a 250 r/min rotary shaker. Broth seed cultures were grown for 24 h at 250 r/min before refrigeration, and used within 60 days to inoculate flasks for inoculum.

2.4 Buffers and antibiotics

Conversion trials were conducted in a sterile 0.1 M sodium citrate dihydrate buffer with the pH adjusted to 4.8 using concentrated H_2SO_4. A stock solution of 10 mg/mL tetracycline HCl (70% ethanol, filter-sterilized) was prepared and stored at -20℃, from which 0.4 mL/100 mL of total trial volume was used for contamination control.

2.5 Simultaneous Saccharification and Fermentation (SSF) trials of soy molasses and soluble

SSF trials were conducted in 500 mL Erlenmeyer flasks (300 mL working volume), fitted with rubber stoppers that were pierced with 21 gauge syringe needles attached to Whatman 0.2 μm syringe filters to exhaust carbon dioxide while protecting against contamination. Due to the viscosity of the molasses and soluble, it was necessary to dilute them prior to SSF. The substrate loading rate and amounts of enzymes and buffer used in these trials are shown in Tables 2 and 3. The pH of the diluted slurries was initially adjusted to 5.0 using concentrated H_2SO_4 or NaOH. Then 1.2 mL of tetracycline stock solution was added, along with varying amounts of enzymes. Enzyme dosages were based on the assumption that both molasses and soluble contained about 25% glucan or oligosaccharides. Sterile deionized water was added to bring the total volume of each flask to 297 mL, and then 3 mL of a 24 h culture of either *S. cerevisiae* or *S. stipitis* was added. Flasks were incubated for 96 h in a 35℃ reciprocating shaker set at 250 r/min. Control flasks without enzymes were also included to assess ethanol production from the free carbohydrates in the substrates. These controls were prepared in the same manner as described above except that the volumes of enzymes were replaced with sterile buffer.

2.6 Fermentation of dried soybean carbohydrates

Ethanol-extracted, dried soybean carbohydrates were fermented at various substrate loading rates (5%, 10%, and 15%) in 250 mL Erlenmeyer flasks fitted with vented rubber stoppers as described previously. Soybean carbohydrates (3.75, 7.5, or 11.25 g) were mixed with an appropriate volume of tetracycline and 37.5 mL of sterile 0.1 M sodium citrate buffer. The pH value was adjusted to 5.0 using concentrated H_2SO_4 or NaOH, and then sterile deionized water was added to bring the total volume to 74.25 mL. Flasks were then inoculated with 0.75 mL of a 24 h culture of either *S. cerevisiae* or *S. stipitis*, and were incubated for 96 h in a 35℃ reciprocating shaker set at 250 r/min.

Table 2 Components of soy soluble trials with enzymes

Substrate loading/%	Substrate /mL	NS50013 /mL	NS50010 /mL	NS22016 /mL	Buffer /mL	H_2O /mL
33	100	7.00	3.40	3.30	150.00	32.1
50	150	10.50	5.10	4.95	125.25	0.0
75	225	15.75	7.65	7.43	39.97	0.0

Table 3 Components of soy molasses trials with enzymes

Substrate loading/%	Substrate /g	NS50013 /mL	NS50010 /mL	NS22016 /mL	Buffer /mL	H_2O /mL
33	100	7.00	3.40	3.30	150.00	32.1
60	180	12.60	6.12	5.94	95.34	0.0
80	240	16.80	8.16	7.92	25.92	0.0

2.7 Analytical methods

Samples from soy molasses and soy soluble SSF trials (10-15 mL) and dried soybean carbohydrate fermentation trials samples (3 mL) were aseptically collected at 0, 2, 4, 6, 24, 48, 72, and 96 h. Soy molasses and soluble samples were boiled in sealed centrifuge tubes for 5 min to inactivate enzymes. All samples were subsequently centrifuged at 2400 r/min for 10 min to collect the supernatant. After freezing for 24 h at -20℃, supernatant samples were thawed and re-centrifuged at 13 000 r/min for 15 min to remove any precipitates. The supernatant was finally filtered through 0.2 μm syringe filters into HPLC autosampler vials. Ethanol and carbohydrate concentrations were determined using a Waters 717 HPLC with an Aminex HPX-87H column and Waters 2410 refractive index detector (RID). The mobile phase was 0.005 M H_2SO_4 flowing at a rate of 0.6 mL/min, and the column was heated to 65℃.

All trials were run in triplicate. Parameters analyzed included maximum net ethanol concentration, ethanol

productivity, and residual carbohydrates, which were corrected by subtracting enzyme or buffer contaminants that inflated the carbohydrate results on the HPLC. Ethanol Productivity (g/L·h) = (Net Maximum Ethanol Concentration)/Time.

3 Results and discussion

3.1 Fermentation of soy molasses and soy soluble

Figure 2 shows the maximum ethanol titer produced from soy molasses after 96 h SSF or fermentation. The 80% SLR trials evidently were also concentrated, and the low water activity inhibited yeast growth and metabolism. *S. cerevisiae* produced more ethanol than *S. stipitis* on the remaining treatment combinations, with a maximum titer of (12.52±3.13) g/L at 60% SLR with enzymes. The highest ethanol titer for *S. stipitis* was (5.78±5.43) g/L at 33% SLR without enzymes, indicating that it was more sensitive to low water activity. Addition of enzymes actually had a negative impact on ethanol titers.

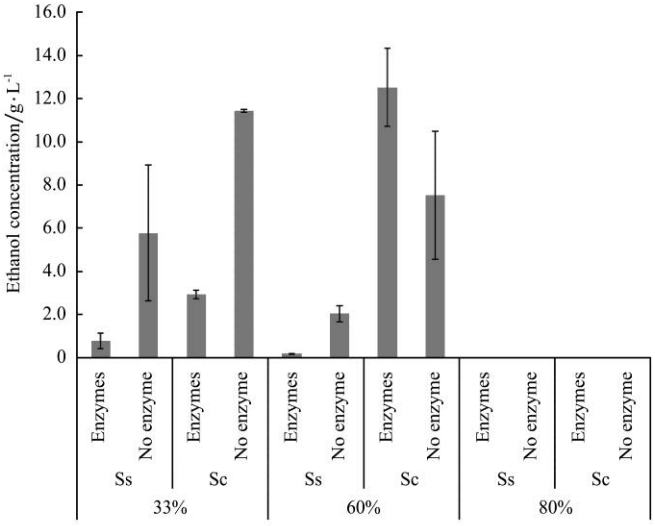

Figure 2 Maximum ethanol titer after 96 h from soy molasses. Error bars represent one standard error of the mean

Figure 3 shows the ethanol productivity at the time of maximum ethanol concentration from the soy molasses during 96 h SSF or fermentation. Productivity trends were similar to the ethanol titer data, with *S. cerevisiae* outperforming *S. stipitis* in each treatment combination. Trials lacking enzymes again showed higher productivity rates than trials with enzymes. The maximum ethanol productivities for both yeasts were in the 33% SLR trials lacking enzymes, with (0.16±0.001) g/L/h for *S. cerevisiae*, and (0.06±0.06) g/L/h for *S. stipitis*.

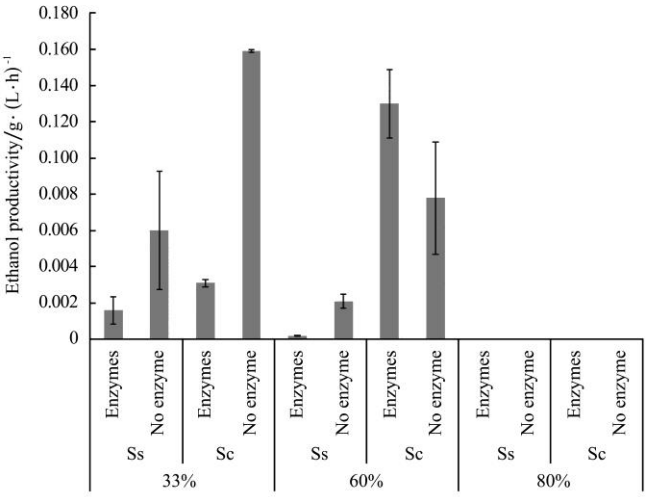

Figure 3 Maximum ethanol productivity from soy molasses. Error bars represent one standard error of the mean

Figure 4 shows the residual carbohydrates from the soy molasses after 96 h SSF or fermentation. As expected, for each combination of yeast and enzyme, residual carbohydrate levels increased at higher SLRs due to the water activity inhibition of yeast. Trials with enzymes resulted in higher residual carbohydrates compared to trials without enzymes, but there was little difference between comparable trials using *S. cerevisiae* versus *S. stipitis*. *S. cerevisiae* treatment should have resulted in lower carbohydrate levels than *S. stipitis*, since *S. cerevisiae* is capable of using the invertase enzyme to hydrolyze the fructose residue from stachyose and raffinose[23,24].

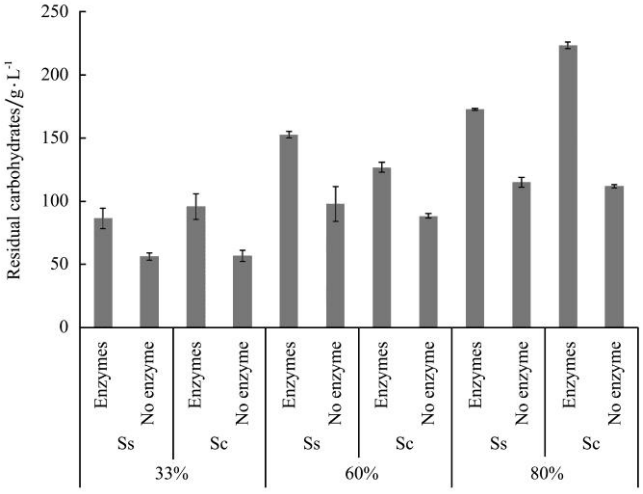

Figure 4 Residual carbohydrates after 96 h from soy molasses. Error bars represent one standard deviation

Figure 5 shows the maximum ethanol produced from the soy solubles after 96 h SSF or fermentation. Both yeasts were again inhibited at the highest SLR due to the low water activity. The highest ethanol titers occurred in the 50% SLR trials lacking enzymes, (39.66±0.94) g/L and (28.24±1.68) g/L for *S. cerevisiae* and *S. stipitis*, respectively. The yeast had substantially less variability on the solubles than on the molasses. *S. cerevisiae* again produced more ethanol than *S. stipitis* in comparable trials.

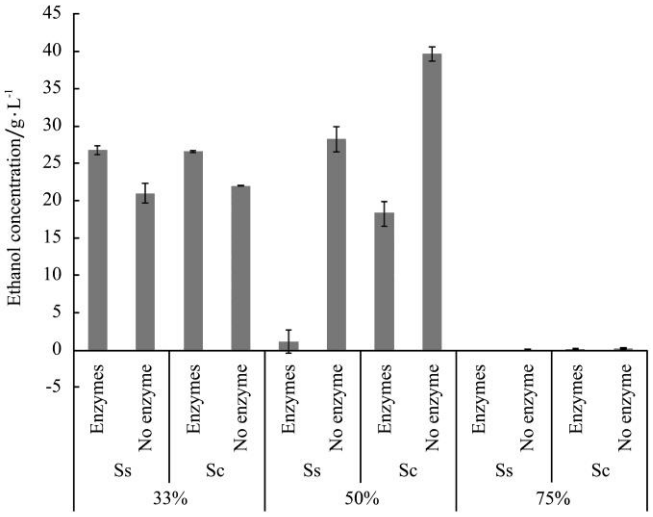

Figure 5 Maximum ethanol titer after 96 h from soy soluble. Error bars represent one standard deviation

Figure 6 shows the maximum ethanol productivity from the soy solubles during 96 h SSF or fermentation. Productivity showed the same trends as observed with ethanol titers. There were very similar productivities in comparable trials at the 33% SLR, higher productivities in 50% SLR's without enzymes, and lower rates at 50% SLR with enzymes than at 33% SLR. Productivity was minimal at the 75% SLR. The maximum productivity rates were (0.41±0.01) g/(L h) (50% SLR enzyme-free) and (0.29±0.017) g/(L h) (50% SLR enzyme-free) for *S. cerevisiae* and *S. stipitis*, respectively.

Figure 7 shows the residual sugars from soy solubles after 96 h of SSF or fermentation. As expected, the residual carbohydrates increased as the SLR increased. At the 33% and 50% SLR's, *S. cerevisiae* had lower levels of residual carbohydrates compared to *S. stipitis*. This was expected, since *S. cerevisiae* is more capable of using soy oligosaccharides, and therefore produced more ethanol than *S. stipitis*.

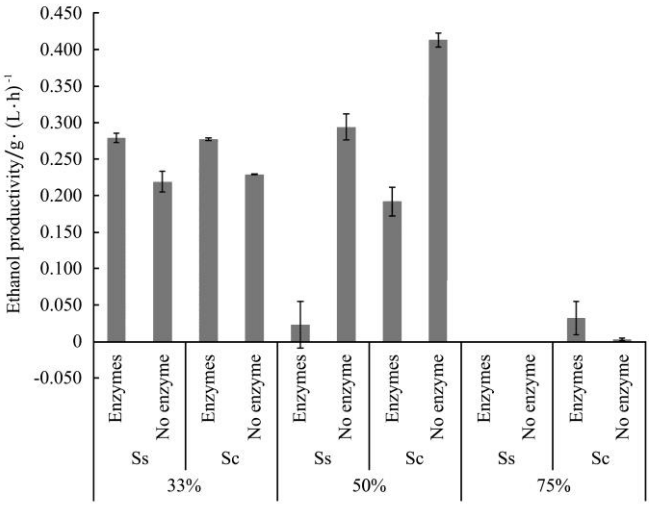

Figure 6 Maximum ethanol productivity from soy soluble. Error bars represent one standard deviation

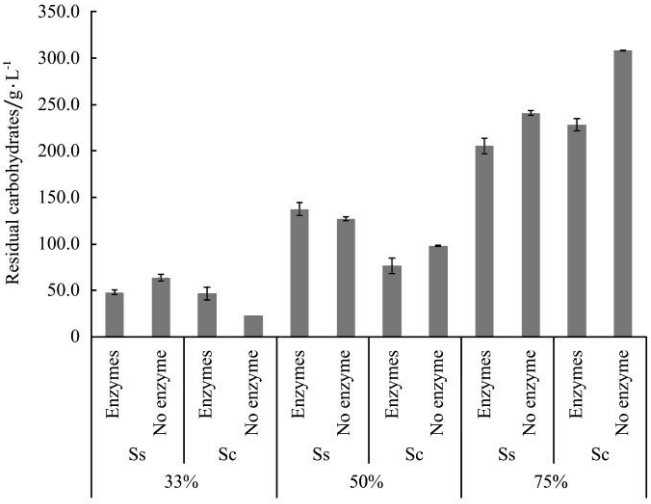

Figure 7 Residual carbohydrates after 96 h from soy soluble. Error bars represent one standard deviation

3.2 Fermentation of dried soybean carbohydrates

Aqueous ethanol-extracted soybean meal (SBM) carbohydrates were subjected to fermentation by either *S. cerevisiae* or *S. stipitis* at three substrate concentrations (5%, 10%, or 15% SLR). Figure 8 shows the maximum ethanol titers during 96 h fermentation. *S. cerevisiae* produced more ethanol than *S. stipitis* at all SLR's, although the differences were not statistically significant. Ethanol titers increased as SLR increased, but the increase from 10% to 15% was not proportional to the increased ethanol titer observed between SLR's of 5% and 10%. The maximum ethanol titers were produced at 15% SLR, being (21.70±4.44) g/L for *S. cerevisiae* and

(17.30±4.42) g/L for *S. stipitis*.

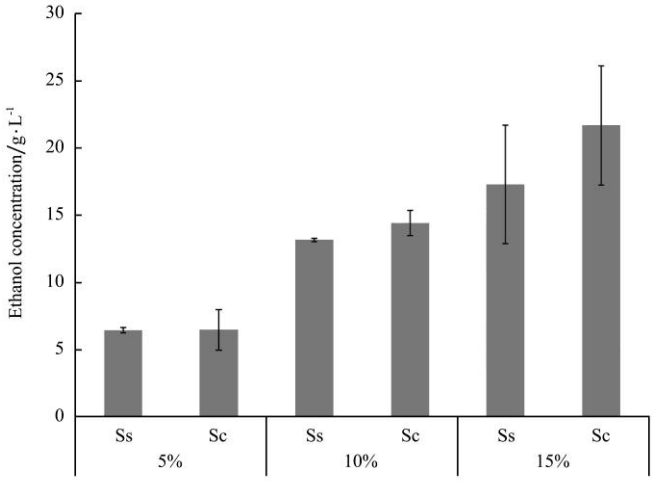

Figure 8 Maximum ethanol titer from dried soybean carbohydrates after 96 h fermentation.
Error bars represent one standard deviation

Figure 9 shows the ethanol productivities calculated at the maximum ethanol concentration during the 96 h fermentation. Ethanol productivities were relatively uniform at the 10% and 15% SLR levels, but were much lower at the 5% SLR. Aside from the 15% trials, *S. stipitis* had higher productivities than *S. cerevisiae*. The maximum productivities for both yeasts were (0.23±0.05) g/(L ħ) for *S. cerevisiae* at 15% SLR, and (0.27±0.01) g/(L ħ) for *S. stipitis* at 10% SLR.

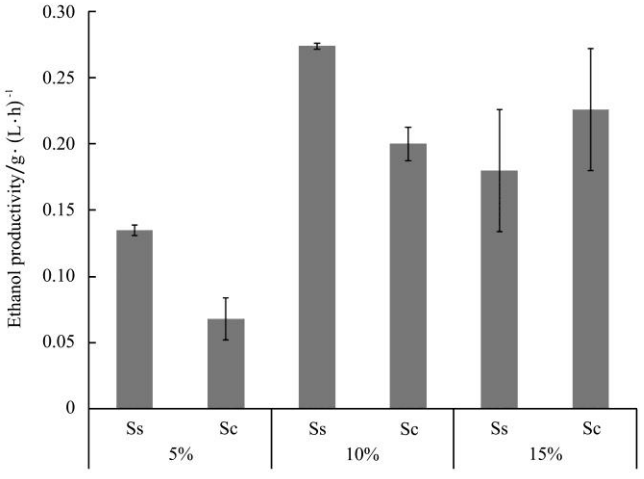

Figure 9 Ethanol productivity from dried soybean carbohydrates after 96 h fermentation.
Error bars represent one standard deviation

Figure 10 shows that the residual carbohydrate levels increased with SLR for the dried soybean carbohyhdrate fermentations. As with ethanol titers and productivity, the differences in residual carbohydrates between strains was not significant at SLRs of 10% and 15%. The maximum level of carbohydrates in solution for each yeast was (14.11±1.13) g/L for *S. cerevisiae*, and (16.88±5.76) g/L for *S. stipitis*, both at the 15% SLR.

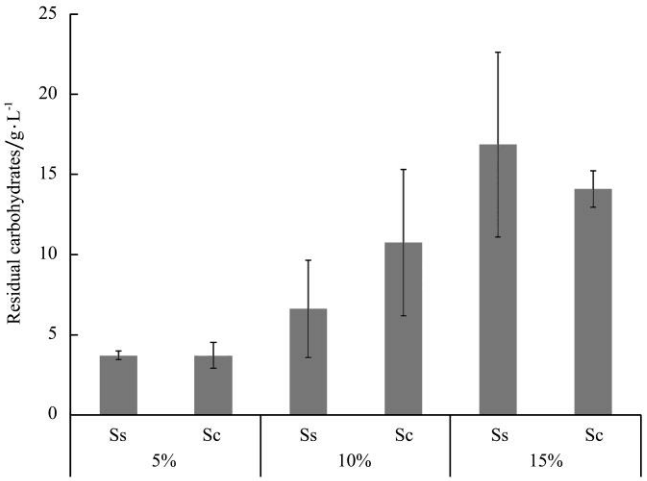

Figure 10 Residual carbohydrates from dried soybean carbohydrates after 96 h fermentation.
Error bars represent one standard deviation

4 Summary and conclusions

The yeasts were able to produce ethanol and reduce the carbohydrate concentration, but not greatly so. Fermentation of the molasses resulted in a maximum ethanol titer of about 12.5 g/L using 60% molasses as the substrate; however, about 125 g/L of carbohydrates were left in the medium. There were about 40 g/L of ethanol produced using 50% soy solubles as the medium, but this still left nearly 100 g/L of unfermented carbohydrates. In both cases, the oligosaccharides raffinose and stachyose (or partially hydrolyzed oligosaccharides) were a large component of the remaining carbohydrates. The enzymes seemed to be more detrimental to fermentation than helpful, possibly due to lower water activity in the medium. Substantial reductions in carbohydrate concentrations were seen during dried carbohydrate fermentation (with decreases to roughly 10% of starting SLR), but also showed low efficiency. The substrate likely contained some components that were not fermentable carbohydrates, and with the yeast being unable to fully ferment the oligosaccharides, the fermentation efficiency would be lower than calculated.

Future experimentation should involve lower SLR's and optimization of enzyme volumes to increase water

activity of the medium. Using a different organism that is more capable of utilizing the oligosaccharides fully (or employing additional pretreatment to hydrolyze the α1-6 galactosidic bonds) would increase the ethanol titer as well.

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