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Xylose-Enriched Ethanol Fermentation Stillage from Sweet Sorghum for Xylitol and Astaxanthin Production

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Received: 26 August 2019; Accepted: 17 September 2019; Published: 23 September 2019

Abstract: Developing integrated biorefineries requires the generation of high-value co-products produced alongside cellulosic ethanol. Most industrial yeast strains produce ethanol at high titers, but the small profit margins for generating ethanol require that additional high-value chemicals be generated to improve revenue. The aim of this research was to boost xylose utilization and conversion to high-value co-products that can be generated in an integrated biorefinery. Pretreated sweet sorghum bagasse (SSB) was hydrolyzed in sweet sorghum juice (SSJ) followed by ethanol fermentation. Ethanol was removed from the fermentation broth by evaporation to generate a stillage media enriched in xylose. *Candida mogii* NRRL Y-17032 could easily grow in non-detoxified stillage media, but a high xylitol yield of 0.55 g xylitol/g xylose consumed was achieved after recovered cells were resuspended in synthetic media containing supplemented xylose. *Phaffia rhodozyma* ATCC 74219 could be cultivated in non-detoxified stillage media, but astaxanthin generation was increased 4-fold (from 17.5 to 71.7 mg/L) in detoxified media. Future processing strategies to boost product output should focus on a two-step process where the stillage media is used as the growth stage, and a synthetic media for the production stage utilizing xylose generated from SSB through selective hemicellulase enzymes.

Keywords: sweet sorghum; xylose; *Candida mogii*; *Phaffia rhodozyma*; xylitol; astaxanthin

1. Introduction

As greater emphasis is placed on developing a sustainable bioeconomy, integrated biorefineries producing both biofuels and high-value co-products are essential to limit and reduce petroleum dependence. Although manufacturers of electric vehicles are improving battery performance and vehicle output due to market demands [1], liquid transportation fuel will continue to represent up to 50% of overall transportation energy in 2075 [2]. While biofuels from agricultural feedstocks can contribute to the overall liquid transportation fuel market, challenges remain for the development of integrated biorefineries due to economic constraints. In the United States, biorefineries producing corn ethanol have been successfully developed with favorable economics due to generating additional co-products from corn such as corn oil, corn gluten meal, and distiller's dried grains with solubles (DDGS) [3]. Alternatively, biorefineries for cellulosic ethanol production have yet to be widely commercialized due to wide-ranging challenges including feedstock supply [4], the handling of solids during pretreatment and enzymatic hydrolysis [5], and overall capital investment [6]. Like the corn ethanol industry, the generation of high-value co-products during cellulosic ethanol production can improve overall profitability of integrated biorefineries. The challenge is to identify the feedstock fraction for conversion and the type of co-product to generate.

The purpose of most integrated biorefineries is to biochemically convert fermentable sugars to ethanol from pretreated agricultural feedstocks. These sugars can be obtained by deconstructing the plant cell wall polysaccharides of cellulose and hemicellulose by enzymatic hydrolysis following chemical pretreatment. Common pretreatment chemistries utilized to make plant cell walls amenable to deconstruction include dilute acid [7], alkali ranging from NaOH [8] to ammonia [9], or organic solvents with an added acid catalyst [10]. The liquid hydrolysates recovered after enzymatic hydrolysis contain high concentrations of fermentable sugars, mostly composed of glucose and possessing over 90% conversion yields of cellulose after 72 h hydrolysis [11]. Other sugars present at lower concentrations in the hydrolysate include five-carbon sugars xylose and arabinose that originate from the hemicellulose fraction of the plant cell wall [12]. The fermentable sugars will serve as the substrate for ethanol producing yeast strains, such as *Saccharomyces cerevisiae*. It is well known that wild-type *S. cerevisiae* lacks the ability to ferment xylose, but more recent efforts have focused on genetically modifying the strain to improve ethanol titers by co-fermenting glucose and xylose simultaneously [13,14]. While genetic improvements have been made on industrial yeast strains, additional high-value co-product generation is necessary to improve revenue and the small profit margins associated with ethanol production [15].

Other types of yeast strains can be employed by integrated biorefineries to simultaneously generate high-value co-products. The *Candida* family of yeast are well known to ferment xylose and produce xylitol. As xylose is fermented by *Candida* strains, the sugar enters the pentose phosphate pathway by way of a two-step process that utilizes xylose reductase to generate xylitol followed by conversion to xylulose through xylitol dehydrogenase [16]. Xylose is typically utilized by these yeast strains for biomass growth and overall cell maintenance, but xylitol remains in the fermentation broth as a byproduct due to a co-factor imbalance mechanism existing between xylose reductase and xylitol dehydrogenase, resulting in excess xylitol generation [16,17]. Once recovered, this sugar alcohol is regarded as one of the most important value-added chemicals that can be obtained from agricultural feedstocks [18] owing to its use as a natural sweetener while possessing a low glycemic index [17]. Aside from use as a food additive, xylitol can also function as an intermediate for polymer synthesis [19]. A second yeast strain that can metabolize xylose for high-value co-product generation is *Phaffia rhodozyma*. This yeast strain is a well-known astaxanthin producer with an affinity for xylose metabolism without the presence of glucose [20,21] and generates a relatively pure form of astaxanthin that has high market value in aquaculture feed [22]. As both yeast strains producing xylitol or astaxanthin do not require genetic modification, they provide an attractive utilization in a biorefinery setting.

This research took the approach of assessing the biochemical conversion of xylose after ethanol fermentation to generate higher-value chemicals. Sweet sorghum was utilized as the feedstock as it can provide both nonstructural sugars (i.e., sucrose, glucose, and fructose) in the form of juice, and structural sugars (i.e., glucose and xylose) from the lignocellulosic bagasse remaining after juice extraction [23]. Sweet sorghum bagasse was pretreated using the low-moisture anhydrous ammonia (LMAA) process followed by enzymatic hydrolysis of the pretreated bagasse in sweet sorghum juice. The hydrolysate underwent ethanol fermentation to ferment hexose sugars followed by broth recovery. The broth was treated to remove ethanol and produce a stillage media enriched with xylose. Two separate yeast strains were utilized for xylose fermentation to assess chemical production. *Candida mogii* NRRL Y-17032 was cultivated for xylitol production, and *Phaffia rhodozyma* ATCC 74219 was grown for astaxanthin production. The enriched xylose stillage media was shown to be an adequate media for xylose conversion to xylitol, but additional detoxification is necessary for astaxanthin yield improvement.

2. Materials and Methods

2.1. Chemicals and Yeast Strains

All chemicals utilized for this work were suitable for cell and microbiological cultures. Cellic CTec2 and HTec2 were obtained from Novozymes (Franklinton, NC, USA). The xylitol-producing strain *Candida mogii* NRRL Y-17032 was obtained from the U.S. Department of Agriculture's National Center for Agricultural Utilization Research (Peoria, IL, USA). The freeze-dried culture was reconstituted in 25 mL of yeast malt (YM) media in a 250 mL flask. Following incubation at 30 °C and 250 rpm for 16 h, 20 mL of the culture was mixed with 10 mL glycerol, which was presterilized by autoclaving at 121 °C for 20 min and dispensed into 1.5 mL cryogenic vials. The stock culture vials then were stored in a freezer maintained at –80 °C. Active dry ethanol red yeast culture (*Saccharomyces cerevisiae*) was provided by Lesaffre Yeast Corporation (Milwaukee, WI, USA). The yeast culture in powder form was kept refrigerated. *Phaffia rhodozyma* ATCC 74219 (UBV-AX2) stock cultures were reactivated following storage at –80 °C as described previously [24].

2.2. Feedstock

Sweet sorghum juice (SSJ) and sweet sorghum bagasse (SSB) were obtained from Delta BioRenewables (Memphis, TN, USA). SSJ was stored at −20 °C and thawed before utilization in enzymatic hydrolysis experiments. SSB was processed according to previous research [25]. In brief, the SSB was ground through a Wiley mill (Arthur H. Thomas Company, Philadelphia, PA, USA), and the fraction that passed a 1 mm screen was collected.

2.3. Pretreatment

SSB was pretreated by the low-moisture anhydrous ammonia (LMAA) process as described previously [25,26]. A simplified schematic is presented in Figure 1 outlining the experimental procedures from pretreatment to fermentation.

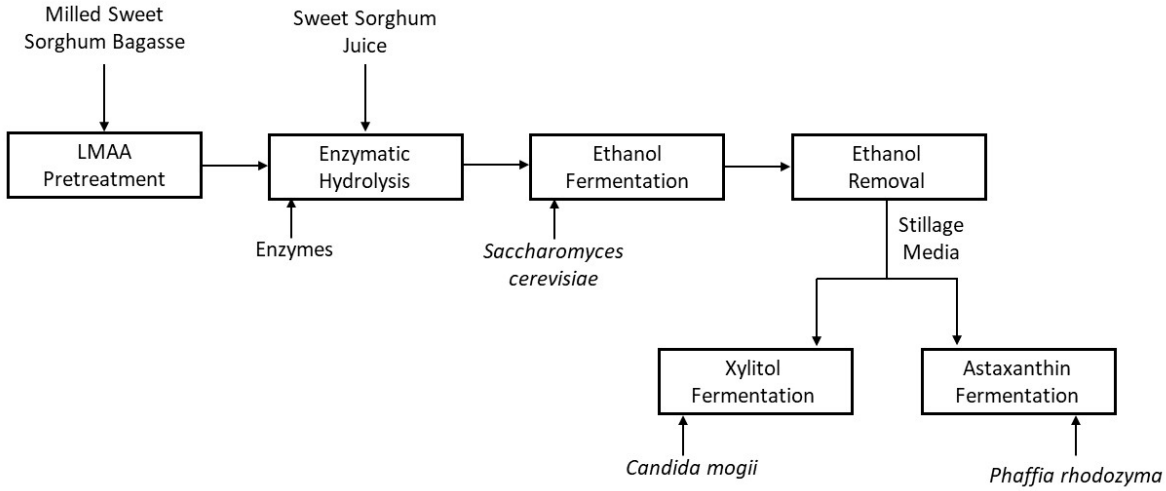

Figure 1. Simplified experimental progress from sweet sorghum bagasse (SSB) pretreatment to stillage media fermentation.

After pretreatment, the SSB was washed with deionized (DI) water to remove potential inhibitory compounds that were formed in the pretreatment, e.g., water-soluble lignin. Several 250 mL plastic bottles were used for washing. An amount of pretreated SSB equivalent to 20.0 g dry solids was placed in each bottle, and DI water was added to give a solid content of 10 wt%. The bottles were capped and shaken in an incubator at 250 rpm and room temperature. Upon removal from the incubator, the bottles were centrifuged at 4000 rpm for 20 min. The supernatants were decanted, and 100 mL of DI

water was added to each bottle. The bottles were shaken as previously described then centrifuged, and the supernatants were decanted. The washing step using 100 mL DI water was repeated one more time. The washed SSB was spread out on an aluminum pan for drying and placed in a 55 °C oven for 20 h.

2.4. Enzymatic Hydrolysis and Ethanol Fermentation

Prior to ethanol fermentation, the pretreated, washed, and partially dried SSB was hydrolyzed in SSJ. An amount equivalent to 25.0 g dry SSB was placed in a 500 mL flask. SSJ was added to give a solid content of 10 wt%. CTec2 and HTec2 then were added at a dosage of 0.020 mL/g dry solids for each enzyme. The flask was tightly capped with a rubber stopper and incubated at 55 °C and 250 rpm for 72 h. After removal from the incubator, the flask was cooled to room temperature and urea was added to 0.4 g/L. An inoculum was prepared for ethanol fermentation by suspending 0.7 g *S. cerevisiae* in 14 mL DI water and stirred for about 30 min. A volume of 13.2 mL of the yeast suspension was then added to the flask. The flask was tightly capped with a rubber stopper punctured by a hypodermic needle and incubated at 32 °C and 200 rpm. The flask was weighed three times per day, and the weight loss due to CO_2 production was plotted vs. time. When the weight loss curve levelled off at 50 h, which indicated the end of the fermentation, the flask was removed from the incubator. Samples were taken immediately after inoculation and at the completion of the fermentation. The samples were centrifuged on a microcentrifuge, and the supernatants were filtered through a 0.2-micron filter then stored in a freezer until HPLC analysis. The fermentation broth was stored in a freezer until further processing.

2.5. Ethanol Removal and Stillage Detoxification

Ethanol was removed before the fermentation broth was used as media for xylitol production. The frozen ethanol fermentation broth was thawed and gently boiled on a hot plate. Agitation of the broth was provided by a magnetic stir bar. Samples were taken intermittently and run on the HPLC to determine the residual ethanol concentration. When the ethanol concentration dropped to 2 g/L, the heating was stopped. The resultant stillage then was used to prepare the media for xylitol fermentation experiments.

Xylitol fermentation experiments were performed with the stillage obtained as described previously with and without detoxification. Detoxification was performed in 250 mL flasks. About 80 g stillage was placed in a flask with 8 g (i.e., 10 wt%) of activated charcoal (AC). The flask was tightly capped with a rubber stopper and incubated at 50 °C and 250 rpm for one hour. The AC and other solids were removed by centrifugation. The supernatant then was used to prepare the media for xylitol fermentation.

2.6. Xylitol Fermentation

The inoculum for xylitol fermentation was prepared by inoculating 0.2 mL stock culture into 25 mL YM media supplemented with 5 g/L xylose in a 250 mL flask, which was previously sterilized by autoclaving at 121 °C for 20 min and allowed to cool to room temperature. The flask then was incubated at 30 °C and 250 rpm for about 16 h.

2.6.1. Xylitol Production in Synthetic Media

Media 1 contained 22.2 g/L xylose and 18.75 g/L corn steep liquor (CSL). Media 2 contained 44.4 g/L xylose and 18.75 g/L CSL. Media 3 contained 88.8 g/L xylose and 18.75 g/L CSL. The suspended solids in the CSL were removed by centrifugation prior to use for preparation of the fermentation media. All media were adjusted to pH 5.5. In each experiment, 20 mL media was placed in a 250 mL flask, stoppered with a foam plug, and autoclaved at 121 °C for 20 min. The flasks were cooled, inoculated with 5 mL *C. mogii* inoculum, and incubated at 30 °C and 250 rpm. Samples were removed at intervals, centrifuged on a microcentrifuge, and filtered through a 0.2-micron syringe filters. The supernatants were used for HPLC analysis of xylose and xylitol. Each set of experiments was performed in triplicate.

2.6.2. Xylitol Production in Stillage Media without Detoxification

The stillage obtained after ethanol removal was concentrated to one half of the original volume by gentle boiling on a hot plate. The concentrated stillage was cooled to room temperature, and CSL was added to 18.75 g/L. The pH of the media was adjusted to 5.5, and 20 mL was placed in a 250 mL flask. The flask was stoppered with a foam plug and autoclaved at 121 °C and 20 min. After being allowed to cool to room temperature, the flask was inoculated with 5 mL inoculum and incubated at 30 °C and 250 rpm. In a parallel experiment, the stillage was used without concentration. Xylose was added to the stillage to about 2X the original concentration, i.e., to be approximately equal to the xylose concentration in the concentrated stillage. CSL then was added to 18.75 g/L. Following pH adjustment to 5.5, 20 mL media was placed in a 250 mL flask, which then was treated in the same manner as described for the flask containing the concentrated stillage media. A separate experiment using the nonconcentrated stillage media was also performed. After 72 h of incubation, the cells in this experiment were recovered by centrifugation and resuspended in 25 mL of a synthetic media containing 40 g/L xylose and 20 g/L CSL at pH 5.5 in a 250 mL flask. The flask then was stoppered with a foam plug and incubated at 30 °C and 250 rpm. All experiments were performed in duplicate, and samples were removed at intervals for HPLC analysis.

2.6.3. Xylitol Production in Stillage Media with Detoxification

In the first experiment, CSL was added to the detoxified stillage at 18.75 g/L. Following pH adjustment to 5.5, 20 mL media was placed in a 250 mL flask, autoclaved, inoculated, and incubated as described previously. In the second experiment, the detoxified stillage was concentrated by 33% and used for xylitol fermentation in the same manner as described for the nonconcentrated detoxified stillage media. A separate experiment was performed using a synthetic media containing 18.75 g/L CSL plus xylose and glycerol at concentrations approximately equal to those in the second experiment. All experiments were performed in duplicate, and samples were removed at intervals for HPLC analysis.

2.7. Astaxanthin Fermentation

Sterilized YM media at 25 mL was inoculated with a single colony of *P. rhodozyma* from an agar plate and allowed to cultivate for four days at 22 °C in a shaking incubator at 200 rpm. Flasks were sealed with a foam rubber stopper. After four days, the culture obtained a very light pink coloration indicating that astaxanthin production had begun and samples could be inoculated. Samples were supplemented with a minimum of 2.0 g/L yeast extract, 0.4 g/L urea, and several minerals according to prior research [24]. The pH of each sample was adjusted to 5.5 followed by filter sterilization through a 0.2-micron filter cup. A media sample volume of 30 mL was utilized for all experiments with a 1.0 mL inoculum. *P. rhodozyma* cultivation experiments on stillage were conducted in duplicate. HPLC samples were collected at 24 h time intervals to determine sugar consumption. All samples were centrifuged on a microcentrifuge followed by filtering through a 0.2-micron syringe filter. Biomass growth and astaxanthin production were only determined after fermentation completion.

2.7.1. Astaxanthin Production in Stillage Media with and without Detoxification

Stillage media was gently boiled to remove ethanol. The media was concentrated by 63.5%, at which point no ethanol remained in the stillage media as confirmed by HPLC. About 60% of the water evaporated was added back to the stillage media in the form of DI water. Half the volume of the stillage media underwent AC detoxification as described in Section 2.5, while the other half was utilized for experiments without detoxification.

2.7.2. Astaxanthin Production in Stillage Media with Increased Nitrogen and Nutrient Supplementation

A second fraction of stillage media was gently boiled to remove ethanol but concentrated at two different levels. The first stillage media fraction was only concentrated by 36% to allow some ethanol to remain in the media. The second fraction was concentrated by 60% to remove all ethanol. Both fractions were then diluted back to their initial volume by adding back 100% of water evaporated in the form of DI water. The concentrated media samples contained the same loading of urea and minerals as stated in Section 2.7, but each concentrated sample was also supplemented with either a higher yeast extract loading at 6.0 g/L or a similar yeast extract loading of 2.0 g/L coupled with an increase in nitrogen with a 1.0 g/L ammonium sulfate loading

2.8. Analytical Methods

An Agilent (Santa Clara, CA, USA) 1260 series HPLC equipped with a refractive index detector was utilized to quantify sugar, glycerol, organic acid, and ethanol concentration in the samples. The samples were separated on a Bio-Rad (Hercules, CA, USA) Aminex HPX-87H column equipped with a cation H^+ guard column. The operation conditions of the HPLC have been previously reported [24,25]. The astaxanthin was extracted from the cells using acetone according to previous methods [24]. A 1 mL aliquot of fermentation broth was mixed with 3 mL of water in a glass test tube. The sample was centrifuged, and the supernatant was removed by aspiration. Glass beads with a diameter of 0.5 mm were added to the test tube with 1.5 mL acetone and vortexed for 1 min followed by sonication for 5 min. The sample was centrifuged again, and the acetone phase containing astaxanthin was transferred to a quartz cuvette. The absorbance of the acetone phase was read at 480 nm on a Shimadzu (Somerset, NJ, USA) UV-1800 UV/vis spectrophotometer. A blank measurement was made with acetone. Biomass growth for *P. rhodozyma* was determined gravimetrically according to previous methods [27,28]. After fermentation was terminated, a 5 mL aliquot of broth was transferred to a conical centrifuge tube. The samples were centrifuged, and the supernatant was discarded. The biomass pellet was washed with DI water and centrifuged a second time. The washed biomass was transferred to preweighed aluminum pans and placed in an oven for drying at 135 °C for 2 h. Samples were cooled in a desiccator before weight determination. The biomass weight was corrected for the presence of astaxanthin. The composition of the SSB was determined according to the procedure developed by the National Renewable Energy Laboratory (NREL) [29].

3. Results and Discussion

3.1. Ethanol Production from Sweet Sorghum Juice (SSJ) and LMAA-treated Sweet Sorghum Bagasse (SSB)

The composition of the LMAA-treated SSB are presented in Table 1. Losses in polysaccharide content are not expected during pretreatment as the LMAA process does not extract plant cell wall fractions. Minor sugar losses might occur after washing the pretreated feedstock. However, Koo et al. [30] reported similar sugar and lignin fractions to those displayed in Table 1 for washed and untreated SSB.

Table 1. Composition of low-moisture anhydrous ammonia (LMAA)-treated SSB (% dry basis).

Glucan	Xylan	Arabinan	AI Lignin	AS Lignin	Total Lignin	Ash
38.3 ± 2.3	22.4 ± 1.5	3.0 ± 0.3	13.4 ± 3.9	3.1 ± 1.4	16.6 ± 4.7	0.2 ± 0.0

Note: AI: Acid insoluble, AS: Acid soluble.

The ethanol fermentation results obtained in sweet sorghum juice (SSJ) only and in SSJ combined with LMAA-treated SSB fermented together are summarized in Table 2. In the latter case, the results obtained after most of the ethanol in the fermentation broth was removed by boiling are also included.

Table 2. Ethanol fermentation results for sweet sorghum juice (SSJ) and SSJ combined with LMAA-treated SSB.

	SSJ Only	SSJ + SSB	SSJ + SSB (Ethanol Removed)
Xylose (g/L)	0	18.1 ± 0.1	23.4 ± 1.4
Glycerol (g/L)	6.4 ± 0.1	7.1 ± 0.2	9.5 ± 1.1
Ethanol (g/L)	60.9 ± 0.4	69.6 ± 0.1	2.3 ± 0.1

The results indicated that significant additional ethanol was produced when the LMAA-treated SSB was hydrolyzed in the SSJ prior to the start of ethanol fermentation. It was calculated that 13.0 g additional ethanol was produced per liter of juice when the SSB and SSJ were used together for ethanol fermentation. The efficiency of ethanol production from the SSB-derived glucose was 60.0% of the theoretical value. A similar conversion of SSB-derived glucose to ethanol was reported to be 63% for bagasse material that was subjected to dilute acid pretreatment followed by partial delignification in alkali prior to simultaneous saccharification and fermentation with *S. cerevisae* [31]. Since the yeast could not utilize xylose, this sugar that was generated by xylan hydrolysis accumulated in the fermentation broth. The theoretical xylose yield from the xylan in the SSB was calculated to be 53.3%. The calculations for additional ethanol production, ethanol efficiency, and xylose yield can be found in supplementary information.

3.2. Xylitol Production in Post-Ethanol Media

3.2.1. Synthetic Media

Xylitol production was first studied in synthetic media containing xylose as the sole carbon source at three different initial concentrations. The concentration profiles of xylose and xylitol in these experiments are plotted in Figure 2. It can be seen from these plots that as the initial concentrations of xylose increased, its consumption rates decreased, which was atypical of substrate inhibition.

Moreover, the production rates of xylitol were directly proportional to the consumption rates of xylose, i.e., lower initial xylose concentrations resulted in higher production rates of xylitol. However, in the first experiment, where the lowest initial xylose concentration was used, xylitol concentration started to decrease when xylose concentration dropped to 6 g/L. The result indicated that the cells consumed xylose and produced xylitol, but when xylose concentration was low, they turned to xylitol and used it as the second carbon source. This mechanism in *C. mogii* has been observed previously and is influenced by low xylose concentrations that direct the organism to consume xylitol for sustaining biomass growth [32]. This observation will have an implication on the design of a fermentation process for xylitol production from xylose. In such a process, the recovery of xylitol should be started before the onset of its consumption rather than waiting for the exhaustion of the substrate (i.e., xylose) as is common practice.

3.2.2. Stillage Media with Detoxification

The concentration profiles of xylose, glycerol, and xylitol in the experiment using the detoxified stillage media are plotted in Figure 3. Xylose and glycerol were utilized simultaneously by the cells. Xylitol concentration increased and then decreased, i.e., similar to the pattern observed in the experiment using synthetic media with an initial xylose concentration at 22 g/L.

Because of the high solid contents in the stillage media, a sample volume of at least 1 mL was required to provide enough supernatant for HPLC analysis. Thus, it was decided to sample the flasks only daily to avoid losing too much liquid. The subsequent small number of samples did not allow determination of the exact time when xylitol consumption by the cells started. The detoxified stillage was concentrated 33% by gentle boiling to remove water by evaporation then used for xylitol production in the next experiment. The concentration profiles of xylose, glycerol, and xylitol are plotted in Figure 3B, which again show simultaneous utilization of xylose and glycerol. Like the observation in

Figure 3A for the nonconcentrated detoxified stillage media, xylitol was first produced then consumed by the cells.

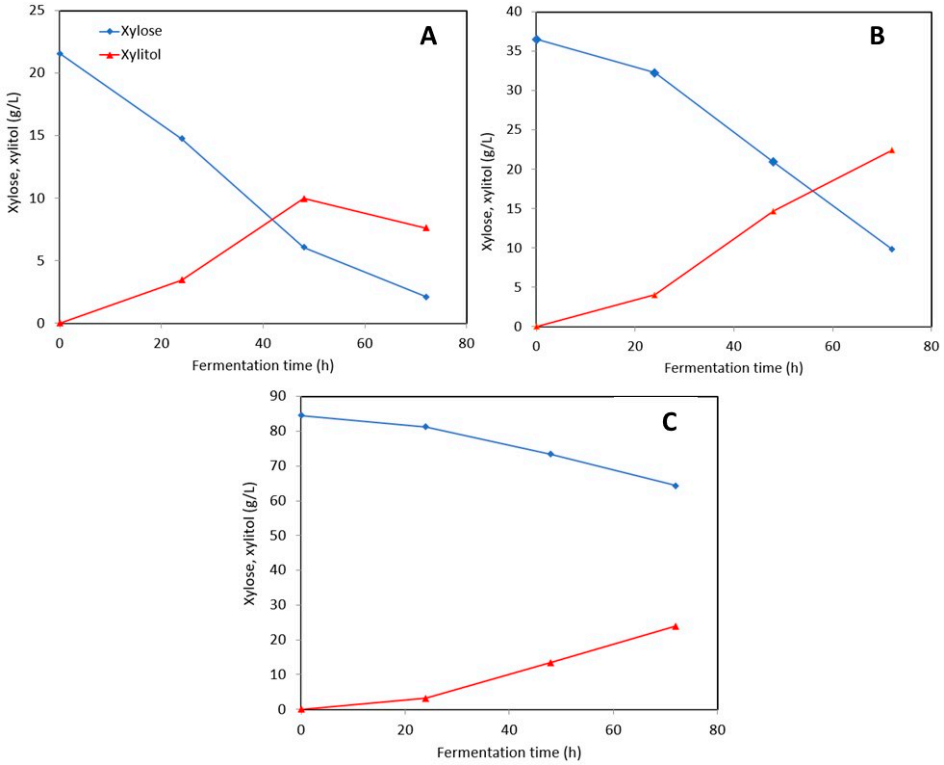

Figure 2. Concentration profiles of xylose (♦) consumption and xylitol (▲) generation by *C. mogii* cultivated in synthetic media with xylose as the sole carbon source at initial concentrations of (**A**) 22.2 g/L, (**B**) 44.4 g/L, and (**C**) 88.8 g/L.

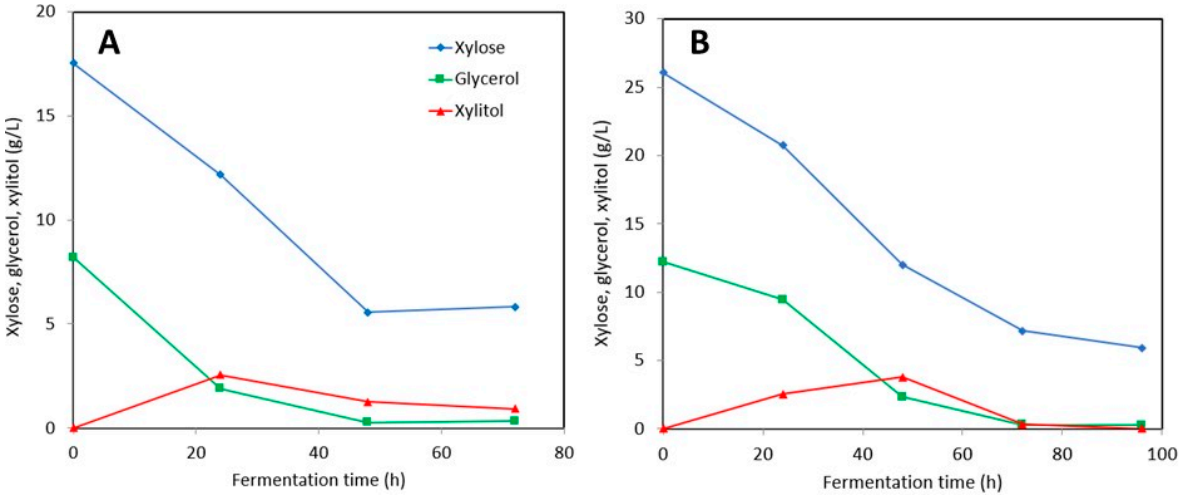

Figure 3. Concentration profiles of xylose (♦) and glycerol (■) consumption, and xylitol (▲) generation by *C. mogii* cultivated in (**A**) detoxified stillage media, and (**B**) stillage media that was detoxified and concentrated by 33%.

The results obtained in the experiment using the detoxified and concentrated stillage media are compared with those obtained in the experiment using synthetic media containing equal initial concentrations of xylose and glycerol. The concentration profiles of xylose, glycerol, and xylitol in

this experiment are plotted in Figure 4. The consumption rates of xylose and glycerol in the synthetic media were both significantly faster than those observed in the stillage media.

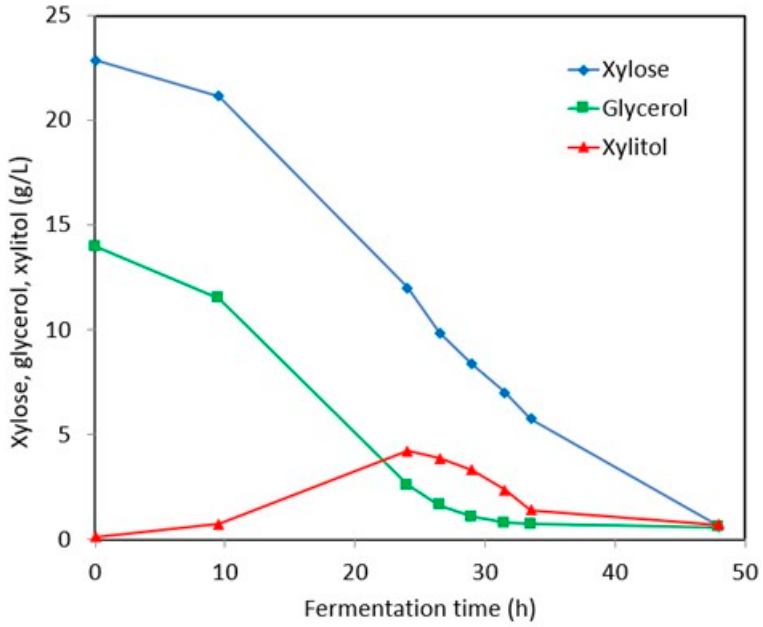

Figure 4. Concentration profiles of xylose (♦) glycerol (■) consumption, and xylitol (▲) generation by *C. mogii* cultivated in synthetic media containing xylose and glycerol at initial concentrations equal to those in detoxified and concentrated stillage media.

Xylitol also was consumed by the cells in the synthetic media when the concentration of xylose dropped to about 12 g/L. The yield at the highest xylitol concentration was 0.39 g xylitol/g xylose consumed. Similar xylitol yields at this level have been reported for *C. mogii* when cultivated on hydrolysate obtained from big bluestem grass [33] and corn stover hydrolysate after detoxification [34]. Other xylitol fermentation research utilizing *Candida guilliermondii* cultivated on hemicellulosic hydrolysates from forage sorghum generated a xylitol yield around 0.35 g xylitol/g xylose consumed which also compares well with the yield calculated from Figure 4 [35]. Using *C. guilliermondii* on sorghum straw hydrolysate produced a yield of 0.44 g xylitol/g xylose consumed, which slightly outperforms the yield obtained here [36]. The results indicated that although the stillage media could support xylitol production, it might contain inhibitory compounds, which negatively affect the consumption rates of the two substrates, i.e., xylose and glycerol, especially when they accumulated to high levels due to concentration.

3.2.3. Stillage Media without Detoxification

Xylitol production was also studied in stillage media without detoxification. After removal of ethanol, the stillage was concentrated to one half its original volume by evaporation of water and used for xylitol production. The initial xylose and glycerol concentrations were 42.8 g/L and 10.8 g/L, respectively. The results are plotted in Figure 5A, which clearly shows there was no microbial activity.

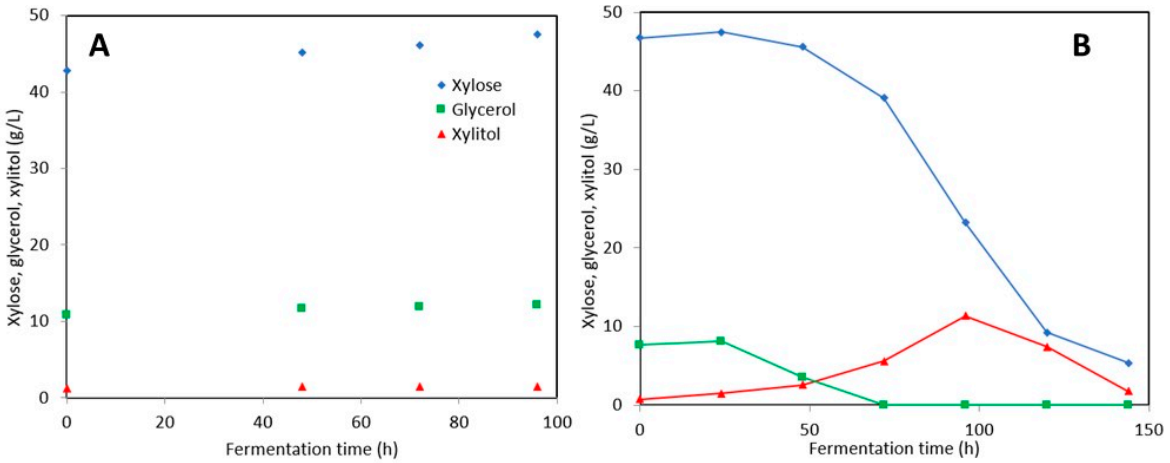

Figure 5. Concentration profiles of xylose (♦) glycerol (■) consumption, and xylitol (▲) generation by *C. mogii* cultivated in (**A**) non-detoxified stillage media concentrated to one half original volume by water evaporation, and (**B**) non-detoxified stillage media supplemented with xylose to 46.5 g/L.

In a separate experiment, xylose and glycerol were added to the original stillage to bring their initial concentrations to the same levels as those in the non-detoxified and concentrated stillage media. The stillage media supplemented with xylose and glycerol then was used for xylitol production. The results are plotted in Figure 5B, which show utilization of both xylose and glycerol and production of xylitol. The yield at the highest xylitol concentration before it started to decrease was 0.48 g xylitol/g xylose consumed. This higher xylitol yield obtained corresponds favorably to *C. mogii* cultivation on overlimed and detoxified rice straw hemicellulosic hydrolysate [37]. At the end of this experiment, the cells were recovered by centrifugation and resuspended in a synthetic media containing 40 g/L xylose as described previously in the Materials and Methods section. The concentration profiles of xylose and xylitol obtained in this synthetic media are plotted in Figure 6. The results show high rates of xylose consumption and xylitol production.

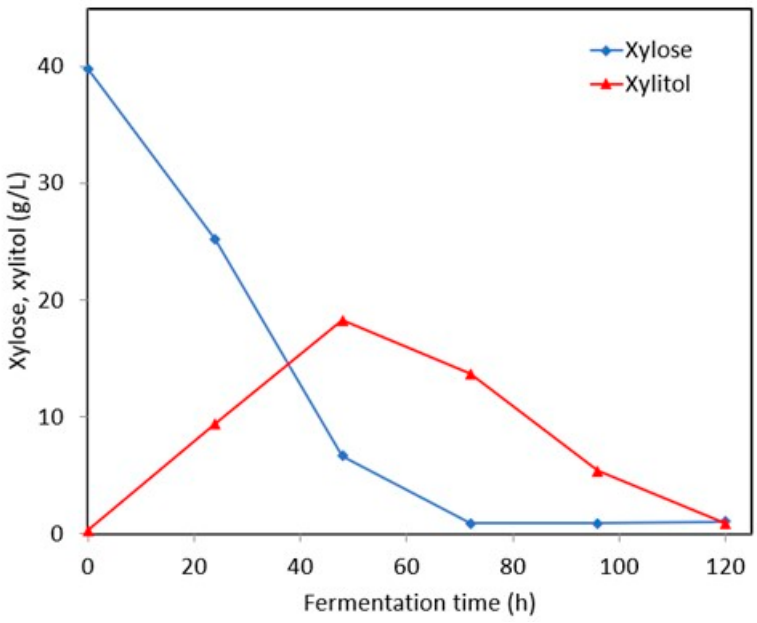

Figure 6. Xylose (♦) consumption and xylitol (▲) generation by *C. mogii* cells recovered from non-detoxified stillage media with xylose supplementation and resuspension in synthetic media.

The highest xylitol concentration achieved before it started to decrease was 18.3 g/L and the yield was 0.55 g xylitol/g xylose consumed. This xylitol yield reported is higher than previously reported results for partially neutralized and detoxified corn fiber hydrolysate cultivated with *Candida tropicalis* [38] and *Debaryomyces hansenii* grown on non-detoxified and detoxified brewery spent grain hydrolysate [39]. These results indicate that the non-detoxified stillage media could be used for xylitol production but only if it was not overconcentrated, for example, to one half its original volume, which would result in high concentrations of the potential inhibitory compounds. In addition, the cells grown on the non-detoxified and nonconcentrated stillage media could be used for xylitol production in a synthetic media with xylose as the substrate. This may lead to the development of a two-stage process, which consists of a growth stage using the stillage media and a production stage using a synthetic media. The xylose in the synthetic media may be generated from selectively hydrolyzing pretreated SSB with commercially available hemicellulase cocktails.

3.3. Astaxanthin Production in Post-Ethanol Media

3.3.1. Stillage Media with and without Detoxification

The stillage media after ethanol removal was next utilized as a substrate to cultivate *P. rhodozyma* for astaxanthin generation. Prior research on *P. rhodozyma* has shown that yeast has an affinity for xylose metabolism without the presence of glucose [25,27]. Figure 7 displays the consumption of xylose and glycerol by *P. rhodozyma* in non-detoxified and detoxified stillage media. It can be identified that the rate of glycerol consumption is relatively steady in both samples, but the rate of xylose consumption is accelerated in detoxified media.

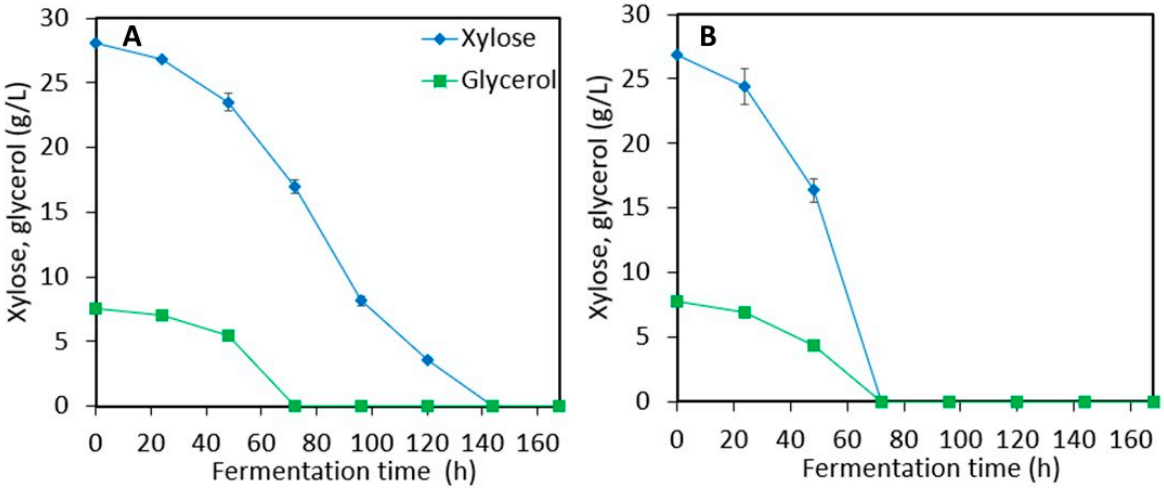

Figure 7. Xylose (♦) and glycerol (■) consumption by *P. rhodozyma* in (**A**) non-detoxified stillage media and (**B**) detoxified stillage media.

When cultivated in a mixture of fermentable sugars, *P. rhodozyma* has been documented to metabolize glucose first before switching to other sugars, i.e., xylose [28]. Glycerol does not appear to have the same effect on *P. rhodozyma* as both glycerol and xylose consumption occur simultaneously and, as shown in Figure 7, are consumed completely within 72 h in the detoxified stillage media. The fermentations progressed for a total of one week since longer fermentation times produce greater astaxanthin output by the organism [40]. Table 3 displays final biomass and astaxanthin product yields along with astaxanthin productivity.

Table 3. Biomass and astaxanthin output following *P. rhodozyma* cultivation in non-detoxified and detoxified stillage media after ethanol removal.

	X (g/L)	P (mg/L)	$Y_{P/S}$ (mg/g)	$Yx_{/S}$ (g/g)	$Y_{P/X}$ (mg/g)	Q_P (mg/L/h)
Non-Detoxified	17.5 ± 0.9	17.5 ± 1.8	0.47 ± 0.05	0.47± 0.02	1.0 ± 0.1	0.10 ± 0.01
Detoxified	18.2 ± 1.3	71.7 ± 3.3	1.9 ± 0.1	0.5 ± 0.03	3.9 ± 0.3	0.43 ± 0.02

Note: X is dry cell mass concentration; P is astaxanthin concentration; $Y_{P/S}$ is astaxanthin yield (mg astaxanthin/g carbon consumed); $Y_{X/S}$ is dry cell mass yield (g dry cell mass/g carbon consumed); $Y_{P/X}$ is cell astaxanthin content (mg astaxanthin/g dry cell mass); and Q_P is astaxanthin volumetric productivity (mg astaxanthin/L/h).

As exhibited in Table 3, biomass growth by *P. rhodozyma* after one week of fermentation is essentially the same in both the non-detoxified and detoxified stillage media. While the non-detoxified hydrolysate still allowed for biomass growth by the organism, the astaxanthin production was severely inhibited in this media. *P. rhodozyma* cultivated in detoxified stillage media could produce 4 times more astaxanthin in terms of both final titer and cell astaxanthin content. A previous hydrolysate generated from pretreated SSB that was not subjected to ethanol fermentation and therefore contained a mixture of glucose, xylose, and arabinose exhibited a similar increase in astaxanthin output after detoxification [25]. It is assumed that soluble aromatic compounds originating from lignin are the primary cause for product inhibitory effects during yeast fermentation [41,42]. However, the inhibition exhibited by *P. rhodozyma* is slightly different as the astaxanthin output by the yeast strain was affected and not biomass growth. Apart from inhibition, other factors, such as nitrogen and nutrient supplementation, also contribute to the ability of the organism to generate astaxanthin [43,44].

3.3.2. Stillage Media with Increased Nitrogen and Nutrient Supplementation

Non-detoxified stillage media was supplemented with higher loadings of nitrogen and nutrients to determine if astaxanthin generation could be improved without detoxification. Two types of concentrated stillage media were utilized in this experiment. The first stillage media was concentrated to 36%, while the second stillage media was concentrated to 60%. Both samples were either supplemented with a higher nutrient loading of yeast extract at 6.0 g/L, or an increase in nitrogen content with 1.0 g/L ammonium sulfate addition while holding the yeast extract loading at the same level (2.0 g/L) utilized in Section 3.3.1. Figures 8 and 9 display the xylose and glycerol consumption profile for each supplementation strategy. As shown in Figure 8A,B, a small amount of ethanol remained in the stillage media concentrated by 36% but was readily consumed within 96 h of fermentation.

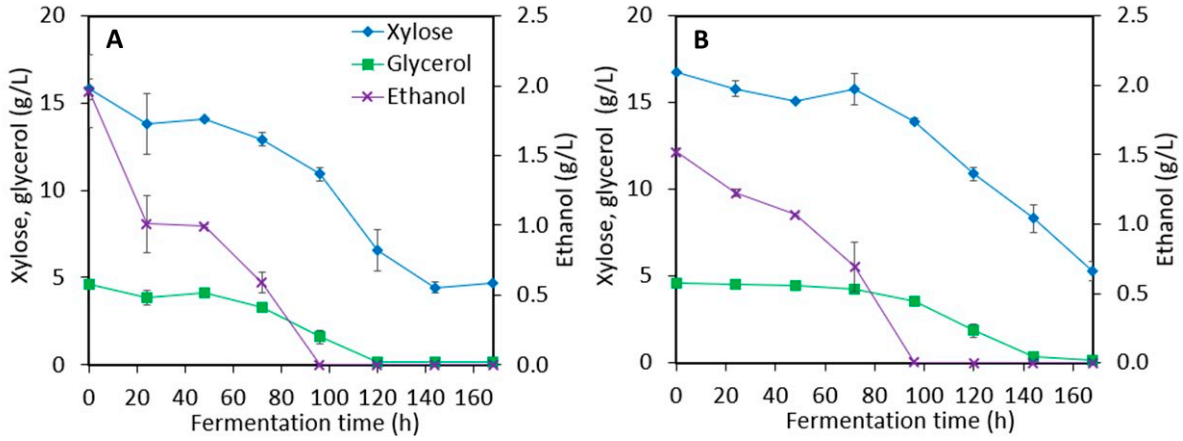

Figure 8. Xylose (♦), glycerol (■), and ethanol (x) consumption by *P. rhodozyma* in (**A**) 36% concentrated stillage media with 6.0 g/L yeast extract and (**B**) 36% concentrated stillage media with 1.0 g/L ammonium sulfate.

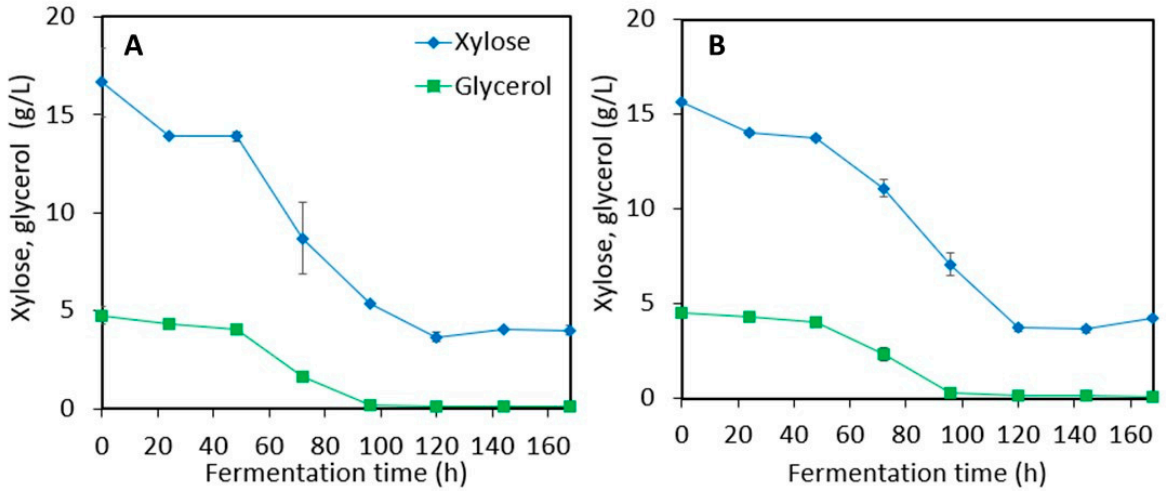

Figure 9. Xylose (♦) and glycerol (■) consumption by *P. rhodozyma* in (**A**) 60% concentrated stillage media with 6.0 g/L yeast extract and (**B**) 60% concentrated stillage media with 1.0 g/L ammonium sulfate.

The consumption profiles presented in Figures 8 and 9 display several notable trends. First, around 5 g/L of xylose remained unconsumed by *P. rhodozyma* after 168 h of fermentation. This result contrasts non-detoxified stillage media in Figure 7A as xylose was completely consumed. Secondly, although glycerol was completely consumed, Figures 8 and 9 show a much slower glycerol consumption rate when compared to Figure 7. The inability to completely metabolize xylose coupled with slower glycerol utilization indicated that increased nitrogen and nutrient supplementation in non-detoxified stillage media failed to produce adequate growing conditions for *P. rhodozyma*. This aspect is exhibited more definitively in Table 4 which shows final fermentation performance metrics for biomass growth and astaxanthin generation.

Table 4. Biomass and astaxanthin output following *P.* cultivation in non-detoxified stillage media with increased nitrogen or nutrient loadings.

		X (g/L)	P (mg/L)	$Y_{P/S}$ (mg/g)	$Y_{X/S}$ (g/g)	$Y_{P/X}$ (mg/g)	Q_P (mg/L/h)
36% Concentrated	**6.0 g/L YE**	8.5 ± 0.3	17.3 ± 1.0	0.87 ± 0.05	0.43 ± 0.01	2.0 ± 0.1	0.10 ± 0.01
	1.0 g/L AS	6.0 ± 0.2	14.2 ± 1.0	0.72 ± 0.05	0.31 ± 0.01	2.4 ± 0.1	0.08 ± 0.01
60% Concentrated	**6.0 g/L YE**	8.6 ± 0.2	18.1 ± 1.8	0.98 ± 0.1	0.46 ± 0.01	2.1 ± 0.3	0.11 ± 0.01
	1.0 g/L AS	7.9 ± 0.5	15.3 ± 1.6	0.87 ± 0.1	0.45 ± 0.03	1.9 ± 0.2	0.09 ± 0.01

Note: YE is yeast extract; AS is ammonium sulfate; X is dry cell mass concentration; P is astaxanthin concentration; $Y_{P/S}$ is astaxanthin yield (mg astaxanthin/g carbon consumed); $Y_{X/S}$ is dry cell mass yield (g dry cell mass/g carbon consumed); $Y_{P/X}$ is cell astaxanthin content (mg astaxanthin/g dry cell mass); and Q_P is astaxanthin volumetric productivity (mg astaxanthin/L/h).

The results in Table 4 show that all fermentation performance metrics for biomass development and astaxanthin product generation were inferior to the detoxified stillage media results listed in Table 3. The astaxanthin concentration remained relatively constant between the non-detoxified stillage media, but the biomass growth was reduced by half when compared to the non-detoxified stillage media sample in Table 3. The balance of carbon-to-nitrogen loading in *P. rhodozyma* cultivation is of critical importance in terms of both yeast growth and carotenogenesis. The underlying influence of carbon-to-nitrogen ratio during fermentation is still poorly understood, and recent research efforts have identified that while cell growth and astaxanthin formation improve with an increasing carbon-to-nitrogen ratio, the astaxanthin composition per cell of yeast decreases as this ratio increases [45]. Moreover, the presence of residual ethanol remaining in the 36% concentrated non-detoxified stillage media did not boost astaxanthin production as documented in previous studies [46,47]. Even with increased supplementation, the non-detoxified stillage media was not a superior media compared to detoxified

stillage media. The cell astaxanthin content of 3.9 mg astaxanthin/g dry cell was higher than what has been demonstrated for *P. rhodozyma* cultivated on date juice [48], but overall biomass growth at 18.2 g/L still lagged behind fermentations performed on molasses [49] and an optimized media and fed-batch process that could produce 114 g per kg broth [50]. The formation of carotenoids by pigmented yeast strains such as *P. rhodozyma* can also be inhibited by the presence of amine compounds. Diphenylamine at low concentrations has been shown to irreversibly inhibit β-carotene formation in the carotenoid pathway to astaxanthin [51]. Due to the type of pretreatment utilized on the SSB, it is probable that some residual lignin was transformed into nitrogenous phenolic compounds that can be solubilized during hydrolysis and inhibit downstream fermentation [52]. While LMAA-pretreated SSB hydrolysate provides an adequate substrate for cell growth by *P. rhodozyma*, it is apparent that hydrolysate detoxification is a requirement for astaxanthin carotenogenesis by the organism.

4. Conclusions

SSB pretreated by the LMAA process could be subjected to enzymatic hydrolysis in SSJ followed by ethanol fermentation. The resulting ethanol was removed to prepare a post-ethanol stillage media enriched in xylose. This media provided adequate conditions for the cultivation of *C. mogii* and *P. rhodozyma*, two separate yeast strains that have a natural xylose metabolism and can generate high-value co-products xylitol and astaxanthin, respectively. A high xylitol yield of 0.55 g xylitol/g xylose consumed could be achieved after *C. mogii* was cultivated for growth in non-detoxified stillage media with additional xylose supplementation followed by cell recovery and resuspension in synthetic media. A substantial astaxanthin concentration of 71.7 mg/L and cell astaxanthin content of 3.9 mg astaxanthin/g dry cell mass was achieved in detoxified stillage media. Increasing the nitrogen and nutrient loadings in non-detoxified stillage media did not improve astaxanthin output and hampered overall biomass growth. Although xylose-enriched post-ethanol fermentation stillage media proved to be a useful media for xylose conversion by the yeast strains, a dual process might be necessary for efficient xylose utilization and product generation. The stillage media could be utilized for biomass production by the organisms followed by cell recovery and transfer to a second production stage fermentation for product generation. A synthetic xylose media could be utilized for this second stage fermentation that would be generated from pretreated SSB subjected to hydrolysis with specific hemicelluase enzymes.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/2311-5637/5/4/84/s1.

Author Contributions: N.P.N. conceptualized the research; R.J.S. and N.P.N. supervised the research; R.J.S., N.P.N., and R.J.L. developed experimental procedures and plans; N.P.N. analyzed the xylitol fermentation data and wrote the Materials and Methods and Results section for xylitol fermentation; R.J.L. analyzed astaxanthin fermentation data and wrote the Materials and Methods for astaxanthin fermentation; R.J.S. analyzed astaxanthin fermentation data, wrote the Introduction, Results, and Discussion for astaxanthin fermentation, Discussion for xylitol fermentation, and the Conclusion. All authors reviewed, edited, and approved the final manuscript.

Acknowledgments: The authors graciously thank Delta Biorenewables for the supply of sweet sorghum juice and bagasse utilized in this research. Additionally, the authors thank Matthew Toht for his assistance in pretreating the sweet sorghum bagasse and setting up enzymatic hydrolysis, ethanol fermentation, and xylitol fermentation experiments.

Conflicts of Interest: The authors declare no conflicts of interest. Mention of trade names or commercial products in this publication is solely for providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. ARS is an equal opportunity provider and employer.

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