Appl Biochem Biotechnol (2009) 154:227–237
DOI 10.1007/s12010-008-8399-5

Production of Astaxanthin from Corn Fiber as a Value-Added Co-product of Fuel Ethanol Fermentation

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Received: 15 May 2008 / Accepted: 9 October 2008 /
Published online: 29 October 2008

Abstract Five strains of the yeast *Phaffia rhodozyma*, NRRL Y-17268, NRRL Y-17270, ATCC 96594 (CBS 6938), ATCC 24202 (UCD 67–210), and ATCC 74219 (UBV-AX2) were tested for astaxanthin production using the major sugars derived from corn fiber. The sugars tested included glucose, xylose, and arabinose. All five strains were able to utilize the three sugars for astaxanthin production. Among them, ATCC 74219 was the best astaxanthin producer. Kinetics of sugar utilization of this strain was studied, both with the individual sugars and with their mixtures. Arabinose was found to give the highest astaxanthin yield. It also was observed that glucose at high concentrations suppressed utilization of the other two sugars. Corn fiber hydrolysate obtained by dilute sulfuric acid pretreatment and subsequent enzyme hydrolysis was tested for astaxanthin production by strain ATCC 74219. Dilution of the hydrolysate was necessary to allow growth and astaxanthin production. All the sugars in the hydrolysate diluted with two volumes of water were completely consumed. Astaxanthin yield of 0.82 mg/g total sugars consumed was observed.

Keywords *Phaffia rhodozyma* · Astaxanthin · Fuel ethanol co-products · Lignocellulosic biomass · Corn fiber

Introduction

Fuel ethanol currently is produced by either a dry-grind process or a wet-mill process. In both of these processes, the values added from the co-products are extremely important. Without these co-products, fuel ethanol production is not economically viable [1]. The co-products include corn gluten feed, corn gluten meal, and corn oil in the wet-mill process

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and distillers dried grains with solubles in the dry-grind process. Except corn oil, the other co-products of fuel ethanol are currently sold as animal feeds [2]. There have been strong interests in developing more valuable co-products for fuel ethanol production [3]. Corn fiber, a by-product generated in the wet-mill process or the recently developed enzymatic milling (E-milling) process [4], is of particular interest, since it contains cellulose and hemicellulose, which can be used as starting materials for production of several value-added co-products. For example, the hemicellulose fraction of corn fiber (corn fiber gum) can be extracted and used for food applications [5]. Additionally, both the cellulose and hemicellulose fractions can be hydrolyzed to fermentable sugars, which can be used for production of industrial chemicals and consumer products by microbial fermentation [6].

Astaxanthin is one of the potential co-products of fuel ethanol production [2, 7]. This carotenoid probably is best known for its role in giving the flesh of salmonids, shrimps, lobsters, and crayfish the pinkish-red hue. In the marine environment, astaxanthin is acquired through ingestion of microalgae and phytoplankton, which are natural astaxanthin producers. However, since salmonids are unable to synthesize astaxanthin, the farm-raised fish need to be fed with this carotenoid through their artificial diets [8]. Astaxanthin is a high-value specialty product. The selling price for astaxanthin in 2000 was estimated at ~ $2,500/kg [8] and that of 10% astaxanthin formulas in 2007 was listed at $250/kg [9]. The world market for astaxanthin was predicted to reach over $250 million in 2009 [10]. Recently, astaxanthin was discovered to provide many human health benefits [11, 12]. These discoveries could lead to development of nutraceutical applications and significant expansion of the market for astaxanthin [11].

Currently, astaxanthin is produced commercially by either chemical synthesis or microbial fermentation. The market has been heavily dominated by the synthetic product mainly due to its much lower production cost. However, the growing demand for products from natural sources may open up the market to astaxanthin produced by biological processes. There are only two microbial astaxanthin sources that may be able to compete economically with synthetic astaxanthin. These are the green microalga *Haematococcus pluvialis* and the red yeast *Phaffia rhodozyma* [12]. The yeast *P. rhodozyma* is of particular interest since several strains can utilize various sugars, including glucose, xylose, and arabinose, for growth and astaxanthin synthesis [13]. Thus, *P. rhodozyma* can be used for astaxanthin production using fermentable sugars obtained from hydrolysis of lignocellulosic biomass as carbon sources. The objective of our research program is to develop a process for the production of astaxanthin as a high value-added co-product of corn-based fuel ethanol. We report in this paper the screening of several strains of *P. rhodozyma* for their capability of utilizing fermentable sugars obtained from corn fiber and astaxanthin production by the selected strain.

Materials and Methods

Microbial Strains

P. rhodozyma strains NRRL Y-17268 and NRRL Y-17270 were obtained from the US Department of Agriculture's Agricultural Research Service Culture Collection (Peoria, IL). *P. rhodozyma* strains ATCC 96594 (CBS 6938), ATCC 24202 (UCD 67-210), and ATCC 74219 (UBV-AX2) were obtained from the American Type Culture Collection (Manassas, VA). The characteristics of these strains based on which they were selected for this study are summarized in Table 1.

Table 1 Characteristics of the strains selected for this study.

Strain	Characteristics
NRRL Y-17268	Xylose metabolizing capability [14]
ATCC 24202	Xylose metabolizing capability [14]
NRRL Y-17270	Highly pigmented natural isolate [15]
ATCC 74219	High astaxanthin-producing industrial strain [16]
ATCC 96594	Produced and secreted β-glucanase [17]

β-Glucanase is an important industrial enzyme. It is used to break down the β-glucan in barley to reduce the viscosity of the mash, thus allowing efficient mixing for distribution of yeast and nutrients during ethanol fermentation [18]. This is of particular interest to us since, in our laboratory, we also have a research program on ethanol production from barley

The freeze-dried cultures were reconstituted in 25 mL YM media in 250-mL shake flasks. Following incubation at 22°C and 250 rpm for 1–2 days, two volumes of the broth were mixed with one volume of sterile glycerol, and the stock cultures were stored at −70 °C.

Chemicals, Reagents, and Corn Fiber Feedstock

All chemicals used were of reagent grades. The enzymes Spezyme® EXTRA (α-amylase), Fermenzyme® L-400 (glucoamylase), and GC-220 (cellulase) were provided by Genencor (Rochester, NY), a Division of Danisco (Copenhagen, Denmark). Novozyme 188 (β-glucosidase) was purchased from Sigma-Aldrich (St. Louis, MO). The corn fiber was provided by Archer Daniels Midland (Decatur, IL). The carbohydrate composition of the corn fiber is given in Table 2. The procedure used for the determination of these carbohydrate contents is described in the section on analytical methods below.

Experimental Procedures

The screening of the *P. rhodozyma* strains was performed in 250-mL shake flasks containing 25 mL Difco™ yeast nitrogen base (YNB) medium at regular strength with and without Difco™ yeast extract plus a single sugar. Yeast extract was used at 1 g/L. The sugars were glucose (20 g/L), xylose (10 g/L), and arabinose (10 g/L). The inocula were prepared in YNB medium. Each 250-mL inoculum flask containing 25 mL YNB medium was inoculated with 0.1 mL thawed glycerol culture of the appropriate strains. The inoculum flasks were incubated at 22 °C and 250 rpm for 2 days before 1 mL was used to inoculate the screening flasks. All flasks were sterilized by autoclaving at 121 °C and 20 min and allowed to cool prior to inoculation. The screening flasks were also incubated at 22 °C and 250 rpm. Samples were taken daily, and cell growth was determined by

Table 2 Carbohydrate composition of the corn fiber.

Carbohydrate component	Percent of total mass (dry basis)
Glucan	21.2
Xylan	17.2
Arabinan	12.9
Galactan	4.1
Starch	17.5

measuring optical density (OD) at 600 nm. The screening experiments typically were run for 96 h, but some were stopped at 72 h when no further increase in OD was observed. The final samples were taken for analysis of dry cell mass, astaxanthin, and residual sugars.

To produce fermentable sugars for use in the experiments on astaxanthin production, the corn fiber was pretreated with dilute sulfuric acid and subsequently hydrolyzed with enzymes according to the procedure recommended by Saha and Bothast [19]. The pretreatment conditions were 15% *w*/*v* solid, 0.5% *v*/*v* sulfuric acid, 121 °C, and 1 h. The pH of the slurry then was adjusted to 5 with NaOH. Enzyme hydrolysis was performed at 55 °C for 72 h at enzyme loadings of 30 filter paper units (FPU)/g glucan for GC-220 and 30 cellobiose unit (CBU)/g glucan for Novozyme 188. The residual solids were removed by filtration, and the hydrolysate was used for astaxanthin production. The pH of the filtrate was adjusted to 5, and yeast extract was added to 1 g/L. The hydrolysate media then was sterilized by autoclaving at 121 °C and 20 min prior to inoculation.

All experiments on astaxanthin production were performed in duplicate, and the averages of the results are reported in this paper.

Analytical Methods

The corn fiber was subject to compositional analysis according to the National Renewable Energy Laboratory's standard procedure [20]. Starch content was determined first, then the de-starched corn fiber was subject to carbohydrate composition analysis. Each sample was run in duplicate. Sugars were determined by HPLC. The system was an ISCO model 2350 (Lincoln, NE) using deionized water as solvent at 0.6 mL/min combined with an Aminex® HPX-87P column (Bio-Rad Laboratories, Hercules, CA) operated at 85 °C and an HP 1047A refractive index detector (Hewlett Packard, Palo Alto, CA). The software used for data analysis was Chrom Perfect® Spirit version 4 build 17 (Justice Laboratory Software, Auchtermuchty, Fife, UK).

The extraction and determination of astaxanthin was performed according to the procedure developed by An et al. [21]. One milliliter of sample was placed in a small test tube, and 3 mL of deionized water was added. The test tube was centrifuged for 5 min, and the supernatant was discarded. Glass beads (0.5 mm) were added followed by addition of 1.5 mL acetone. The test tube was vortexed vigorously for 1 min and then sonicated for 5 min. The tube then was centrifuged for 5 min, and the absorbance of the supernatant was read at 480 nm. The concentration of astaxanthin in the sample was determined with a standard curve prepared with pure astaxanthin (Sigma-Aldrich, St. Louis, MO) in acetone.

Dry cell mass was determined by collecting the cells by centrifugation using a 5-mL sample, washing of the cell pellet with deionized water, drying at 135 °C for 2 h, and weighing of the dried pellet.

Results and Discussion

Screening of *P. rhodozyma* Strains

Examples of the growth curves are shown in Fig. 1 for strain ATCC 74219. The growth curves of other strains tested followed similar patterns. The results of the strain screening are summarized in Table 3.

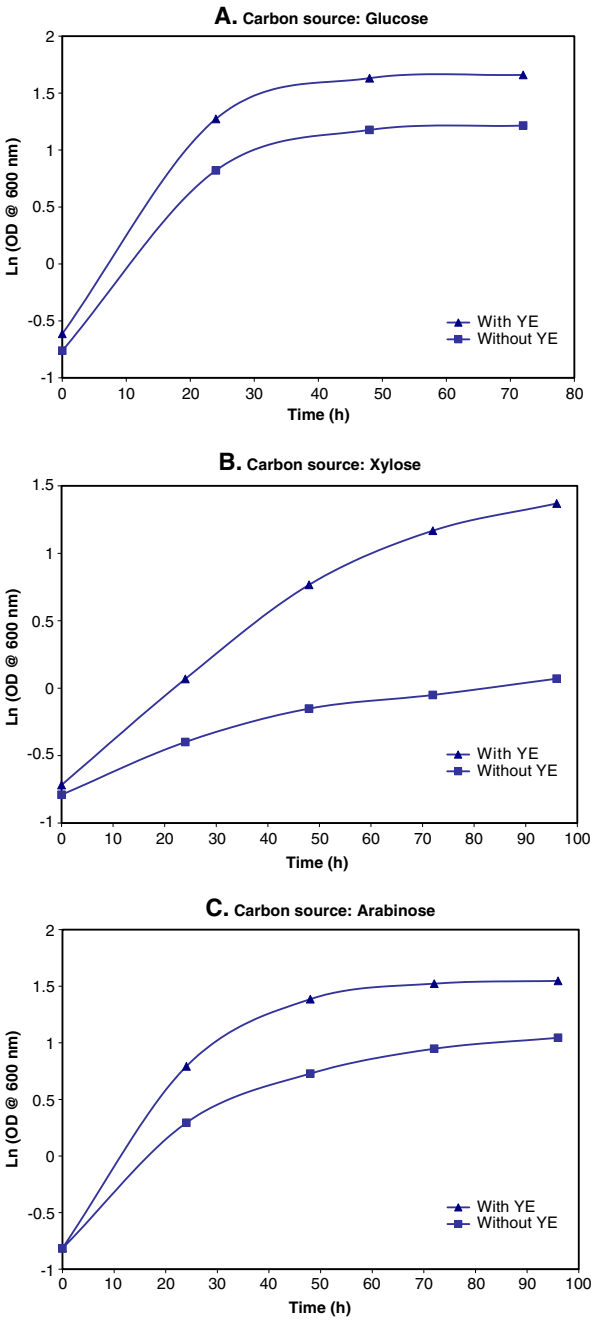

Fig. 1 Growth curves of *P. rhodozyma* strain ATCC 74219

The results from Table 3 indicate that all the strains tested were able to metabolize the three major sugars that could potentially be obtained from corn fiber quite effectively for astaxanthin production. Strain ATCC 74219 was the best astaxanthin producer, irrespective of the sugar substrates. The final astaxanthin production by this strain was several folds

Table 3 Results of screening of *P. rhodozyma* strains for growth and astaxanthin production on glucose, xylose, and arabinose as individual carbon sources.

Strain	Media	X (g/L)	P (mg/L)	ΔS (g/L)	$Y_{P/X}$ (mg/g)	$Y_{P/S}$ (mg/g)	$Y_{X/S}$ (g/g)
ATCC 74219	Glucose, YE	3.67±0.06	17.42±1.37	15.25±0.35	4.73±0.29	1.15±0.06	0.24±0.00
	Glucose	1.59±0.20	10.05±0.48	13.29±0.12	6.34±0.49	0.76±0.04	0.12±0.01
	Xylose, YE	1.92±0.19	10.24±0.29	8.67±0.23	5.34±0.38	1.18±0.00	0.22±0.01
	Xylose	0.41±0.09	4.48±1.61	6.29±0.08	11.25±5.96	0.71±0.25	0.07±0.01
	Arabinose, YE	2.36±0.00	13.46±1.34	8.65±0.12	5.71±0.57	1.55±0.13	0.27±0.01
	Arabinose	1.13±0.14	8.47±1.37	7.04±0.10	7.48±0.28	1.2±0.18	0.16±0.01
ATCC 96594	Glucose, YE	5.59±0.21	2.28±0.37	16.96±0.06	0.41±0.08	0.13±0.02	0.33±0.01
	Glucose	4.76±0.30	2.13±0.06	16.4±0.19	0.45±0.04	0.13±0.00	0.29±0.02
	Xylose, YE	2.68±0.23	0.48±0.04	8.65±0.10	0.18±0.00	0.05±0.01	0.31±0.03
	Xylose	2.28±0.05	0.9±0.02	8.69±0.08	0.39±0.00	0.11±0.01	0.26±0.01
	Arabinose, YE	3.64±0.29	1.17±0.52	8.84±0.48	0.33±0.17	0.13±0.06	0.41±0.01
	Arabinose	1.37±0.94	0.8±0.19	8.89±0.04	0.7±0.34	0.09±0.03	0.16±0.11
ATCC 24202	Glucose, YE	4.86±0.08	1.49±0.09	16.77±0.85	0.31±0.02	0.09±0.01	0.29±0.01
	Glucose	5.71±2.42	1.39±0.03	13.35±0.23	0.26±0.12	0.1±0.00	0.43±0.18
	Xylose, YE	2.61±0.21	0.64±0.15	8.76±0.08	0.24±0.04	0.07±0.02	0.29±0.02
	Xylose	0.92±0.34	0.39±0.03	3.3±0.47	0.44±0.14	0.11±0.01	0.26±0.06
	Arabinose, YE	4.05±0.66	0.71±0.14	8.76±0.14	0.18±0.06	0.08±0.01	0.46±0.08
	Arabinose	3.4±0.03	1.06±0.09	8.97±0.12	0.31±0.03	0.11±0.01	0.37±0.01
NRRL Y-17270	Glucose, YE	4.1±0.14	1.29±0.06	18.24±0.43	0.31±0.00	0.07±0.00	0.22±0.01
	Glucose	3.41±0.24	1.02±0.15	17.21±0.19	0.30±0.02	0.06±0.01	0.2±0.01
	Xylose, YE	2.26±0.01	0.75±0.09	8.69±0.02	0.33±0.04	0.08±0.01	0.26±0.00
	Xylose	1.65±0.04	0.73±0.07	8.93±0.10	0.45±0.05	0.08±0.01	0.18±0.01
	Arabinose, YE	4.02±1.39	1.07±0.00	8.81±0.08	0.28±0.10	0.12±0.00	0.46±0.16
	Arabinose	1.75±0.38	0.65±0.05	4.79±0.21	0.39±0.11	0.14±0.01	0.36±0.06
NRRL Y-17268	Glucose, YE	5.13±0.64	1.19±0.06	16.67±0.20	0.24±0.04	0.07±0.00	0.31±0.04
	Glucose	4.28±0.93	1.22±0.11	17.31±0.55	0.29±0.09	0.07±0.01	0.25±0.05
	Xylose, YE	0.9±0.08	0.39±0.14	9.41±0.35	0.44±0.19	0.04±0.01	0.09±0.01
	Xylose	0.17±0.01	0.08±0.01	3.76±0.28	0.52±0.08	0.02±0.00	0.04±0.01
	Arabinose, YE	3.66±0.14	1.31±0.04	9.01±0.39	0.35±0.00	0.14±0.01	0.43±0.03
	Arabinose	3.99±0.86	1.2±0.29	9.26±0.05	0.3±0.00	0.13±0.03	0.43±0.08

X dry cell mass; *P* astaxanthin production; *ΔS* substrate consumption; $Y_{P/X}$ astaxanthin specific productivity (mg astaxanthin/g dry cell mass); $Y_{P/S}$ astaxanthin yield, mg astaxanthin/g sugar consumed; $Y_{X/S}$ Cell yield, g cells/g sugar consumed

higher than that obtained by the other strains and in several cases, one order of magnitude higher. For this particular strain, the addition of yeast extract clearly helped to improve both cell mass and astaxanthin production. Although the YNB medium already contained the three amino acids histidine, methionine, and tryptophan and various vitamins and co-factors, it was probable that strain ATCC 74219 still benefited from other nutrients in the yeast extract. The beneficial effects of yeast extract toward this strain were most evident when glucose and xylose were used as the main carbon sources. Both astaxanthin yield ($Y_{P/S}$) and cell yield ($Y_{X/S}$) significantly increased when yeast extract was added to the media. The increases of both total astaxanthin production and cell mass production with yeast extract addition, however, resulted in lower astaxanthin specific productivity ($Y_{P/X}$). Thus, if the final product of interest is dry yeast with high astaxanthin contents, the level of yeast extract used in the growth medium must be optimized.

Because strain ATCC 74219 was found to be the best astaxanthin producer, it was selected for further investigation.

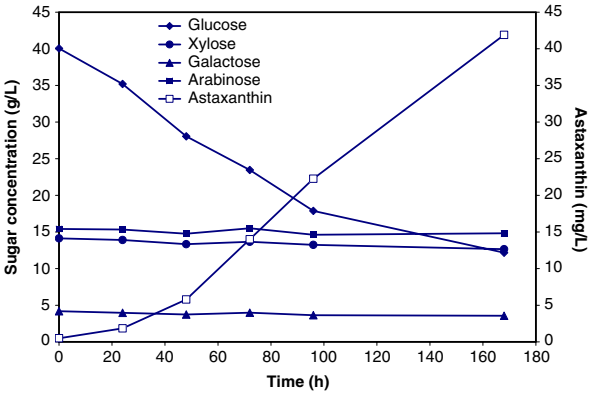

Fig. 2 Astaxanthin production by strain ATCC 74219 in synthetic mixed sugar solution

Astaxanthin Production from Synthetic Mixed Sugars by Strain ATCC 74219

The hydrolysate obtained from enzymatic hydrolysis of lignocellulosic biomass such as corn fiber normally contains several sugars. Thus, a synthetic sugar solution was prepared to simulate the hydrolysate obtained by dilute sulfuric acid pretreatment and subsequent enzyme hydrolysis of corn fiber (see below). The solution was prepared in YNB medium and contained the following sugars: glucose (40 g/L), xylose (14 g/L), galactose (4 g/L), and arabinose (15 g/L). Yeast extract was added at 1 g/L. The synthetic sugar solution was tested for astaxanthin production using strain ATCC 74219. The concentration profiles of astaxanthin and the individual sugars are shown in Fig. 2. The results show that of the four sugars used, only glucose was consumed. The final astaxanthin yield ($Y_{P/S}$) was 1.37 mg astaxanthin/g sugar consumed (Table 4).

For many microorganisms, glucose normally serves as the preferred substrate and tends to repress utilization of other sugars especially when it is available in high concentrations in the medium. To test this hypothesis with the selected strain, astaxanthin production was

Table 4 Production of astaxanthin by strain ATCC 74219 in mixed sugar solutions having high and low initial glucose concentrations and 3× diluted corn fiber hydrolysate.

Media	S_{init}(g/L)	P (mg/L)	ΔS (g/L)	$Y_{P/S}$(mg/g)
Mixed sugars with high glucose[a]	73.77	41.90	30.55	1.37
Glucose+Xylose[b]	18.64	18.26	12.22	1.49
Glucose+arabinose[c]	17.63	28.46	14.62	1.95
Glucose+xylose+arabinose[d]	21.91	27.51	18.52	1.49
Hydrolysate—3× dilution[e]	25.72	20.93	25.68	0.82

S_{init} initial total sugar concentrations, P astaxanthin production, ΔS substrate consumption, $Y_{P/S}$ astaxanthin yield, mg astaxanthin/g sugar consumed

[a] Initial sugar concentrations: glucose, 40.05 g/L; xylose, 14.12 g/L; galactose, 4.18 g/L; and arabinose, 15.42 g/L

[b] Initial sugar concentrations: glucose, 9.19 g/L; xylose, 9.45 g/L

[c] Initial sugar concentrations: glucose, 9.09 g/L; arabinose, 8.54 g/L

[d] Initial sugar concentrations: glucose, 12.39 g/L; xylose, 4.86 g/L; arabinose, 4.66 g/L

[e] Initial sugar concentrations: glucose, 15.27 g/L; xylose, 4.93 g/L; arabinose, 5.52 g/L

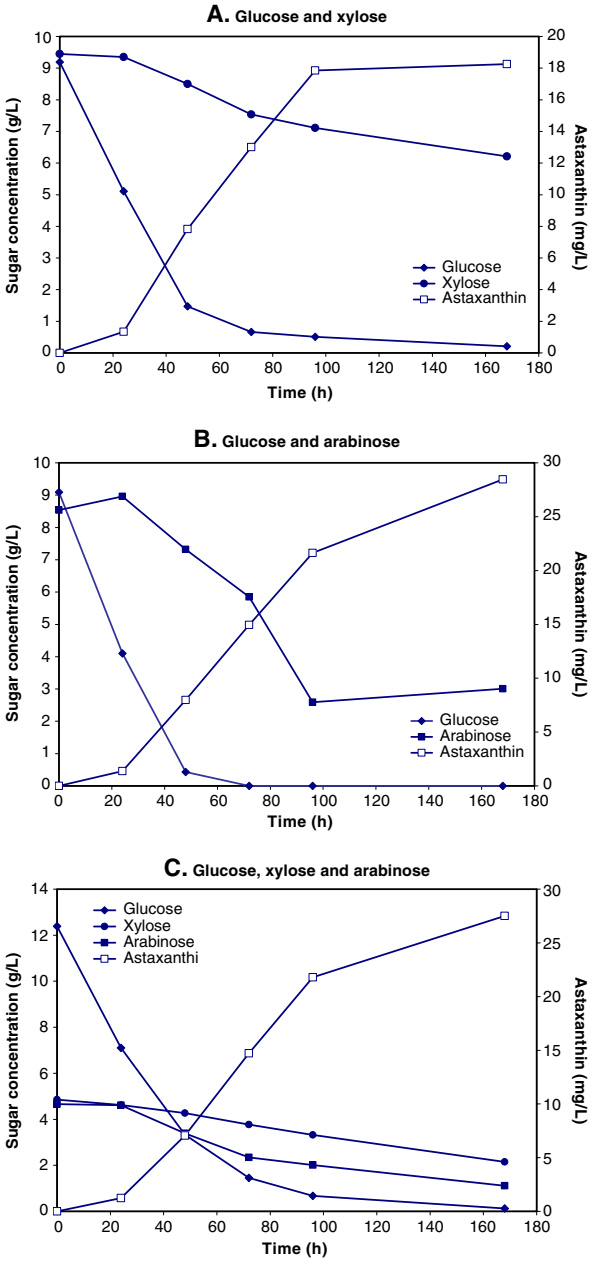

Fig. 3 Sugar consumption and astaxanthin production by strain ATCC 74219 in synthetic mixed sugar solutions with low initial glucose concentrations

studied using several mixed sugar solutions containing lower initial glucose concentrations. All these sugar solutions were prepared in YNB medium and supplemented with 1 g/L yeast extract. Since galactan is only a minor carbohydrate component of corn fiber, the sugar that would be released upon hydrolysis of this polymer, i.e., galactose, was omitted in the low-glucose mixed sugar study. The concentration profiles of astaxanthin and sugars are plotted in Fig. 3, and the results of sugar consumption and astaxanthin production are summarized in Table 4.

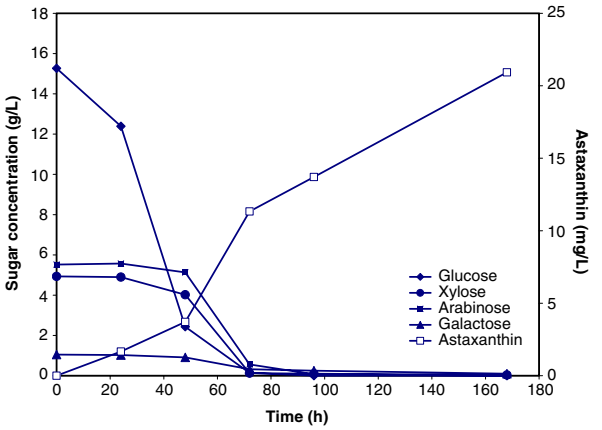

Fig. 4 Astaxanthin production by strain ATCC 74219 using corn fiber hydrolysate with 3× dilution

It can be seen from Fig. 3a and b that consumption of xylose and arabinose did not start until glucose concentration dropped to about 4–5 g/L. The presence of arabinose did not prevent complete glucose consumption. When arabinose was used as the co-substrate in the media, glucose was completely utilized at 72 h. On the other hand, glucose consumption was negatively affected by the presence of xylose. Even at the end of the experiment, i.e., 168 h, there still was some glucose remaining. The results also indicate that arabinose was utilized more effectively than xylose. At the end of the experiments, 65% of the initial arabinose was consumed, whereas for xylose, that value was only 34%. When both xylose and arabinose were used as co-substrates, glucose consumption was incomplete, although the concentrations of both of these sugars were reduced to about 40% of the initial concentration of glucose. The incomplete consumption of glucose in this case probably was more likely caused by the presence of xylose than arabinose. Highest astaxanthin yield was obtained with the glucose/arabinose mixture. When used alone, arabinose also gave highest value of astaxanthin yield (Table 3).

Astaxanthin Production from Corn Fiber Hydrolysate by Strain ATCC 74219

The hydrolysate obtained by dilute sulfuric acid pretreatment and subsequent enzyme hydrolysis of corn fiber was used for astaxanthin production. The hydrolysate contained glucose at 47.0 g/L, xylose at 15.98 g/L, galactose at 3.77 g/L, and arabinose at 16.33 g/L. In the first experiment where the undiluted hydrolysate was used, no sugar consumption was observed. The experiment was terminated after 168 h. The original hydrolysate then was diluted with two volumes of deionized water before it was used for astaxanthin production. When this diluted hydrolysate was used, consumption of all the sugars, including galactose, was observed. Pretreatment of lignocellulosic biomass by dilute sulfuric acid has been known to form compounds toxic to microorganisms [22]. Dilution of the hydrolysate probably reduced the concentrations of the inhibitory compounds to levels sufficiently low to allow sugar utilization by strain ATCC 74219. The concentration profiles of astaxanthin and the individual sugars are shown in Fig. 4. Again, glucose utilization started first. Utilization of xylose and arabinose started only after the initial glucose levels had been reduced. There were insufficient data to determine accurately at what glucose concentration the utilization of the other two sugars began. Unlike the experiment where the synthetic solution containing the three sugars at

approximately equal concentrations were used (Fig. 3c), consumption of all three sugars in the diluted hydrolysate was complete. The astaxanthin yield, however, was lower in the diluted hydrolysate medium (0.82 mg/g sugars consumed) compared to that obtained with the synthetic medium (1.49 mg/g sugars consumed). It is possible that the commercial cellulase enzymes used for hydrolysis of corn fiber also contained proteases, which hydrolyzed the proteins in that material to release free amino acids. The result of this proteolysis was a higher N/C ratio, where N was the available nitrogen, compared to the synthetic sugar medium. Higher N/C ratio tends to be disadvantageous toward synthesis of secondary metabolites [23], such as astaxanthin.

Conclusion

All five *P. rhodozyma* strains tested have shown their capability of utilizing sugars derived from corn fiber for astaxanthin production. Among them, strain ATCC 74219 was the best astaxanthin producer. Kinetics of sugar utilization by this strain was studied, both with individual sugars and with sugar mixtures. All the sugars in the hydrolysate obtained by dilute sulfuric acid pretreatment and subsequent enzyme hydrolysis of corn fiber could be used for astaxanthin production by strain ATCC 74219. However, dilution of the hydrolysate was necessary to alleviate the inhibition of the toxic compounds formed during the dilute acid pretreatment. Investigation of other pretreatment methods that do not generate high levels of inhibitory compounds is recommended.

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