Bioconversion of Meat and Bone Meal and Starch into Astaxanthin

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Abstract

Astaxanthin is a valuable superior antioxidant and has been extensively used in cosmetics, pharmaceuticals, food applications, and animal feed. In present studies, valorization of rendering by-products (meat and bone meal-MBM) and corn/wheat starches into astaxanthin through bio-transformation with *Paffia rhodozyma* was investigated. First, the effect of carbon (glucose) to nitrogen (yeast extract+peptone) (C/N) ratio in the *P. rhodozyma* fermentation media on astaxanthin production was studied, and the optimal C/N ratio of 5 was achieved to obtain maximum astaxanthin content of 3.6 mg/g dry cell biomass. Secondly, MBM was utilized as a nitrogen source for astaxanthin production. MBM was hydrolyzed by enzymes (proteinase) and alkaline (i.e., carbon hydroxide] to convert protein into soluble amino acids to supply nitrogen source for *P. rhodozyma* fermentation. With 40 g/L glucose and alkaline MBM hydroysate, *P. rhodozyma* fermentation achieved a maximal astaxanthin content of 1.02 mg/g dry cell biomass. At last, corn and wheat starches were studied as carbon sources for astaxanthin production by *P. rhodozyma*. Starches were hydrolyzed by amylase and glucoamylase into glucose, starches hydrolysates were diluted by distilled water for further fermented by *P. rhodozyma* for astaxanthin production. The corn and wheat starches achieved maximum astaxanthin contents of 2.15 mg/g dry cell biomass and 3.62 mg/g dry cell biomass, respectively. Future work is needed to further enhance the astaxanthin yield to produce astaxanthin yield of industrial interest.

Key word: Astaxanthin; *Phaffia rhodozyma*; Meat and bone meal; Starch; Hydrolysis; Fermentation

Table of Contents

List of Figures....vi
List of Tables....vii
Acknowledgements....viii
Chapter 1 - Introduction....1
1.1 Astaxanthin....1
1.2 Astaxanthin production....2
1.3 Studies on *Phaffia rhodozyma* for astaxanthin production....3
1.4 Economic assessment and review....6
Chapter 2 - Studies of the effects of carbon and nitrogen source loadings on astaxanthin production by *Phaffia rhodozyma*....8
2.1. Introduction....8
2.1.1 Astaxanthin biosynthesis by *P. rhodozyma*....8
2.1.2 *P. rhodozyma* fermentation....11
2.2. Materials and Methods....11
2.2.1 Culture preparation....11
2.2.2. Effect of glucose loading on AX production....12
2.2.3. Effect of nitrogen loading on AX production....13
2.3. Analytical methods....14
2.3.1 Optical density and cell mass concentration measurements....14
2.3.2 Glucose quantification by HPLC....14
2.3.3 Astaxanthin quantification by HPLC....15
2.4. Results and Discussion....16
2.4.1. Effect of glucose concentration on AX production....16
2.4.2. Effect of nitrogen loading on AX production....19
2.5. Conclusions....22
Chapter 3 - Biotransformation of meat and bone meal (MBM) into astaxanthin....23
3.1 Introduction....23
3.1.1 Astaxanthin as a feed additive....23
3.1.2 Meat and bone meal for microbial fermentation....24

3.2 Materials and Methods 24
3.2.1 MBM defatting 24
3.2.2 Astaxanthin production from enzymatic/alkaline hydrolysate of meat and bone meal 25
3.2.3 Total nitrogen content measurement 26
3.2.4 Meat and bone meal hydrolysates (MBMHs) fermentation 26
3.2.5 Yeast nitrogen-based medium (YNB) 27
3.3 Results and Discussion 27
3.3.1 Total nitrogen content of MBMHs 27
3.3.2 MBMHs fermentation 29
3.4 Conclusions 33
Chapter 4 - Astaxanthin production from corn and wheat starches by *Phaffia rhodozyma* 34
4.1 Introduction 34
4.2 Materials and Methods 35
4.2.1 Test of the extracellular amylolytic activity of *P. rhodozyma* 35
4.2.2 Hydrolysis of corn/wheat starches 35
4.2.3 Corn/Wheat starch hydrolysate fermentation 36
4.3 Results and discussion 37
4.3.1 Detection of extracellular amylolytic activity of *P. rhodozyma* 37
4.3.2 Corn and Wheat starch hydrolysis and fermentation 37
4.4 Conclusions 42
Chapter 5 - Summary 43
References 45

List of Figures

Figure 1. Comparison between different AX production method (A) price and (B) cost distribution 7
Figure 2. Astaxanthin configurational isomers (a-c) and geometric *cis* isomer (Higuera-Ciapara et al., 2006; Bjerkeng et al., 1997) 9
Figure 3. Astaxanthin biosynthesis pathway in *P. rhodozyma* (Ducrey Sanpietro & Kula, 1998) 10
Figure 4. Effect of glucose concentration on: (A) cell growth; (B) glucose consumption; and (C) AX content 19
Figure 5. Effect of nitrogen concentration on: (A) cell growth; (B) glucose consumption; and (C) AX content 21
Figure 6. Total nitrogen content: (A) Total nitrogen content in freeze-dried MBMHs; (B) Total nitrogen mass in MBMHs 28
Figure 7. MBMHs fermentation cell growth: (A) AM4, AM8, FM4, and FM8; (B) YAM4, YAM8, YFM4, and YFM8; (C) Pk4, Pk8, Pb4, and Pb8; (D) YPk4, YPk8, YPb4, and YPb8; (E) CM4, CM8, and CM16; (F) YCM4, YCM8, and YCM16. 31
Figure 9. MBMHs fermentation: percentage of glucose consumption 33
Figure 10. Test of extracellular amylolytic activity of *P. rhodozyma* (A) SC plate at 0 h; and (B) SC plate at 120 h 37
Figure 11. CHS fermentation: (A) Cell growth; (B) Glucose consumption; and (C) Final AX content 40
Figure 12. WHS fermentation: (A) Cell growth; (B) Glucose consumption; and (C) Final AX content 42

List of Tables

Table 1. Agriculture waste fermented by *P. rhodozyma* 5
Table 2. Nitrogen and glucose loadings 13
Table 3. Medium profile for the nitrogen loading study 13
Table 4. Mobile phase profile variation for AX analysis on HPLC 15
Table 5. Hydrolysis conditions of MBM 25

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Chapter 1 - Introduction

1.1 Astaxanthin

Astaxanthin (AX) has been widely used in pharmaceutical applications as a functional compound in skin health protection, cardiovascular disease prevention, eye protection, inflammation reduction, liver function enhancement and detoxification, immune function improvement, and so on. On the other hand, the customer desired color of salmon and trout comes from AX which is used as a red pigment in aquaculture feed and, thus AX is massively demanded in the aquaculture industry. Therefore, the skyrocketing market demand has stimulated the research and production of AX.

Carotenoids can be classified by the oxygenation degree into carotenes and xanthophylls. Carotenes include β-carotene and α-carotene while xanthophylls includes lutein, zeaxanthin, canthaxanthin, and astaxanthin (Schmidt et al., 2011). AX is considered as the most powerful natural antioxidant (Comhaire, Garem, Mahmoud, Eertmans, & Schoonjans, 2005), and its free radical scavenger activity is several-fold higher than that of vitamin E and β-carotene (Guerin, Huntley, & Olaizola, 2003). AX could be produced through three ways, such as chemical synthesis, microbial fermentation, and crustacean waste extraction (Spinelli, Lehman, & Wieg, 1974). Biological method can provide natural AX which is more preferable over the chemical synthesized. Microalgae [e.g., *Chlorella* sp., *Chlorococcum* sp. (Maddipati, Atiyeh, Bellmer, & Huhnke, 2011), *Scenedesmus* sp. (Qin, Liu, & Hu, 2008), and *Haematococcus pluvialis* (Del Campo et al., 2004; Maddipati et al., 2011; Qin et al., 2008; Lorenz and Cysewski, 2000)] and yeasts (e.g., *Rhodotorula* sp. and *Phaffia rhodozyma*) (Stark, Kleinschmidt, & Probst, 1963) have received extensive research for production of natural AX. Among all the species, the red yeast *P.*

rhodozyma and green microalga *H. pluvialis* have been commercially used to produce AX for animal feed and human consumption, respectively. Compared to *H. pluvialis*, *P. rhodozyma* is easier to be cultivated and achieves higher AX yield at faster rate even though its AX has been used for lower-end uses due to product purity and quality, such as animal feed for aquaculture and poultry. *P. rhodozyma* is able to metabolize a broad range of substrates (e.g., sugars, glycerol and glucuronic acid), and able to produce astaxanthin without light under both aerobic and anaerobic conditions (Schmidt et al., 2011), which could reduce the cost contribution of substrate for AX production. Since *P. rhodozyma* possesses superior astaxanthin productivity, researchers have done mutant strain screening, fermentation condition optimization and genetic studies to enhance natural AX production.

1.2 Astaxanthin production

Synthetic astaxanthin is still the major source of astaxanthin in the market. During the chemical synthetic process, different stereoisomers were produced, consists of (3S, 3'S), (3R, 3'S), (3S, 3'R), and (3R, 3'R), with a ratio of 1:2:2:1 (Higuera-Ciapara, Félix-Valenzuela, & Goycoolea, 2006; Nguyen, 2013), whereas naturally produced AX is (3S, 3'S) structure. Natural astaxanthin has relatively higher price and can only be produced at smaller scale than the synthesized astaxanthin. Therefore, the productivity of natural AX is limited. To enhance natural AX production by *H. pluvialis* and *P. rhodozyma*, extensive studies have focused on process optimization on fermentation conditions, such as pH, temperature, rotating speed, medium optimization, extraction, carotenoid purification, and process design.

Natural AX can be extracted from underutilized crustacean byproducts, such as crustacean shell. Astaxanthin is soluble in organic solvent and oil so that it can be extracted by using organic solvents (e.g., hexane and ethanol) and edible oils [sunflower oil (Razi Parjikolaei et al., 2017) and flaxseed oil (Pu, Bechtel, & Sathivel, 2010)]. Using edible oil can significantly lower astaxanthin oxidation rate, and is environmentally friendly and sustainable (Pu et al., 2010). The AX extraction efficiency of edible oil reached 3.7-8.4 mg AX/100 g wet feedstock, but the extraction efficiency may vary from different feedstocks (Razi Parjikolaei et al., 2017).

1.3 Studies on *Phaffia rhodozyma* for astaxanthin production

P. rhodozyma has recently become an important microorganism in food and pharmaceutical industry, and it also became the most promising pathway to produce natural astaxanthin. Fermentation condition optimization has been widely studied, such as pH (Chan & Ho, 1999), temperature (An, 1997), carbon source (Vázquez, Santos, & Parajó, 1997), nitrogen source (Ni et al., 2007), and supplementary nutrients (Wang, Ling, Zheng, Xiong, & Lu, 2011), and so on. *P. rhodozyma* is capable of utilizing a wide spectrum of carbon and nitrogen sources, making it great candidate for large-scale AX production for animal feed applications, especially the utilization as a pigment for coloring salmon and trout (Storebakken, Sørensen, Bjerkeng, & Hiu, 2004). Among available *P. rhodozyma* strains, ATCC 74219 has been commercialized because it is superior to other strains in AX productivity, cell density and cell growth rate (Johnson, 2003). Recent study of *P. rhodozyma* ATCC 74219 could be summarized into three aspects: (1) Metabolic engineering to improve astaxanthin production; (2) Fermentation condition and process optimization; and (3) Alternative low-cost substrates.

Generally used nitrogen source for AX production by *P. rhodozyma* are yeast extract (YE) and peptone (P). However, industrial price of spray-dried yeast extract is as high as \$9.2 kg^{-1} (Maddipati et al., 2011) and peptone is about \$3 kg^{-1}(Xiao, Liu, Qin, & Xu, 2007). Hence, cheaper nitrogen source is desired to produce affordable AX to meet the fast-growing market demand. Rendering animal meals (e.g., meat and bone meals, MBM) are rich in animal protein, which has been mainly used for animal feed as nutrient additives. As the current market of animal feed started to saturate, the rendering industry by-products has been recently exploring new markets and alternative high-value non-feed applications of rendering animal protein is preferred. Using rendering animal protein to replace high-cost yeast extract and peptone for AX production by *P. rhodozyma* could be a potential high-value outlet for rendering animal protein, which would solve respective issues in both biological AX production with lower cost and novel way to utilize rendering industry waste and explore a new market outlet.

As for carbon source for *P. rhodozyma* fermentation, a wide spectrum of carbon sources can be used AX fermentation, such as glucose, maltose, xylose, and arabinose. However, the low-cost alternative is desired to replace regular carbon source, such as agriculture waste rich in saccharides (**Table 1**). In addition to agricultural waste, starch is also a preferable carbon source for *P. rhodozyma* fermentation. Starch is a long chain polysaccharide, which fundamentally consists of glucose units that are connected by glycosidic bonds. Starch is potentially a preferable carbon source to support *P. rhodozyma* cell growth to accumulate AX because it can be converted into glucose and maltose which are preferable sugars for *P. rhodozyma* fermentation (Montanti, Nghiem, & Johnston, 2011a). Starch (e.g., corn and sorghum starch) has been used for ethanol production and exported as corn/sorghum grains for long history. The U.S.

is the largest starch producer in the world and most starches have been used for ethanol production. However, the corn market has many concerns about downward pressure on ethanol price because the average earnings before interest, tax, depreciation, and amortization are down about 4 cents per production gallon over the last 4 years. Identifying alternative high-value markets for crop starches would greatly benefit the U.S. agriculture (Bai, Anderson, & Moo-Young, 2008). The utilization of starch for *P. rhodozyma* fermentation to produce AX will demonstrate a new high-value outlet for crop starches. Also, the new outlet for crop starch will increase the domestic starchy crop use and reduce export pressure. For AX production, starch as a carbon source for *P. rhodozyma* fermentation can further reduce the cost of AX.

Table 1. Agriculture waste fermented by *P. rhodozyma*

Carbon sources	Treatments	Astaxanthin concentration (mg/L)	References
Barley straw	Enzymatic hydrolysis: xylanase	3.0	(Montanti, Nghiem, & Johnston, 2011b)
Sugarcane bagasses	Enzymatic hydrolysis: xylanase	2.6	(Montanti et al., 2011b)
Raw coconut milk	Waste of coconut pulp	1.9	(Domínguez-Bocanegra & Torres-Muñoz, 2004)

Grape juice	-	2.1	(Meyer & du Preez, 1994)
Yucca fillifera juice	-	6.2	(Ramirez, Nunez, & Valdivia, 2000)
Sweet sorghum juice	-	6.5	(Stoklosa, Johnston, & Nghiem, 2018)

Therefore, MBM and starch are potential low-cost alternative nitrogen and carbon sources for *P. rhodozyma* to produce affordable AX for animal feed applications. The overarching goal of this project is to study the feasibility and characteristics of the utilization of MBM and starch for AX production with *P. rhodozyma*. The objectives to achieve this goal included: (1) Optimization of C/N ratio of YEP (yeast extract and peptone) medium for *P. rhodozyma* fermentation; (2) Utilization of MBM as a feasible nitrogen source for *P. rhodozyma* fermentation; (3) Study of corn and wheat starches as inexpensive carbon source for *P. rhodozyma* fermentation.

1.4 Economic assessment and review

So far, only *H. pluvialisi* and *P. rhodozyma* have been used to produced natural AX of commerce. Because of the limitation of biosynthesis, natural AX has relatively higher price.

Based on the BASF report (Baker & Saling, 2003), the synthetic astaxanthin costs $2,000/kg, while the red yeast and microalga astaxanthin cost $2,500/kg and >$7,000/kg, respectively (Figure 1A). Based on the cost distribution, biosynthesis has higher costs for material, energy input and ORAC (Oxygen radical absorbance capacity) value (Chew & Park, 2004). Compared to *H. pluvialisi*, *P. rhodozyma* is more cost-competitive to produce natural AX because of its' rapid cell growth and high cell density (Nguyen, 2013). Another advantage is its capability of utilizing various of carbon and nitrogen sources. *P. rhodozyma* shown a strong vitality in various of carbon and nitrogen source, such as agriculture wastes and animal protein.

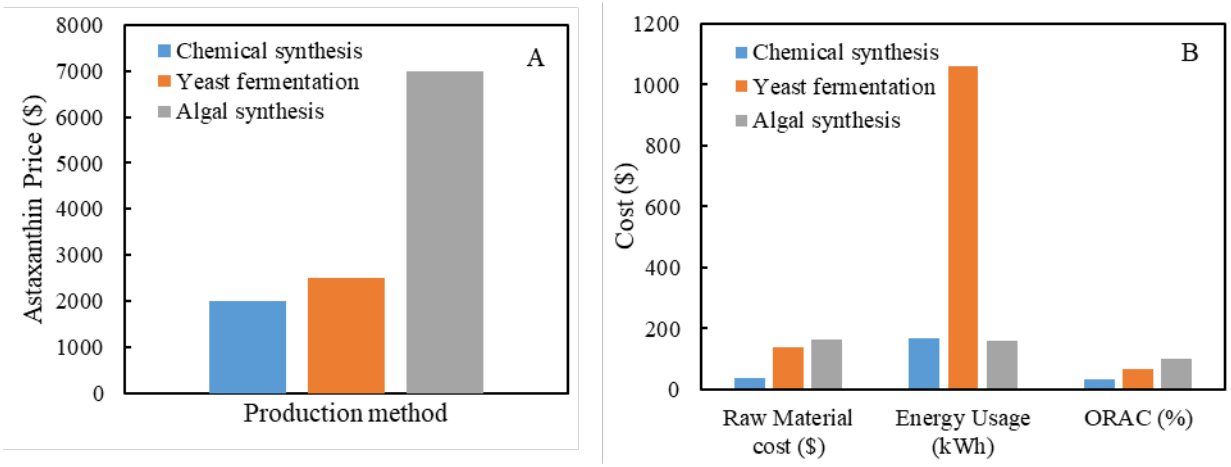


Figure 1. Comparison between different AX production method (A) price and (B) cost distribution

Chapter 2 - Studies of the effects of carbon and nitrogen source loadings on astaxanthin production by *Phaffia rhodozyma*

2.1. Introduction

Studies have showed that *P. rhodozyma* fermentation is one of the most promising methods to produce natural AX (Johnson & An, 1991; Johnson, 2003). The optimization of fermentation conditions is an important approach to improve the production of natural AX by this yeast. Nitrogen source is one of the most critical components in fermentation media, and different type of nitrogen sources can greatly affect the yield of astaxanthin (Du, Zhu, Wu, & Liang, 2005). On the other hand, carbon source is also an important component of fermentation media, and the type of carbon source can result in different AX yield (Vázquez et al., 1997). Glucose has been identified the optimum carbon substrate for *P. rhodozyma* fermentation. In this study, glucose and different nitrogen sources were used in the fermentation of *P. rhodozyma*, and the optimal combination of glucose and nitrogen source and the effect of C/N ratio were determined to maximize the AX yield.

2.1.1 Astaxanthin biosynthesis by *P. rhodozyma*

AX is a pigment that is in the same family of β-carotene. In nature, AX has three isomers such as (3R, 3'R), (3R, 3'S) and (3S, 3'S) with a proportion of 1:2:1. In *P. rhodozyma,* the majority of the produced AX is (3R, 3'R), as shown in **Figure 2**. In the synthesis pathway, AX shares the main metabolic pathway with β-carotene, lutein and zeaxanthin (Hussein, Sankawa, Goto, Matsumoto, & Watanabe, 2006). In 1979, Johnson and Lewis reported the biosynthesis pathway of *P. rhodozyma*, as schematically shown in **Figure 2**. Two probable pathways were postulated for *P. rhodozyma*. As γ-carotene is formed, one pathway goes to β-carotene and finally forms

trans-astaxanthin, and the other goes to torulene that is finally converted to cis-astaxanthin (Johnson & Lewis, 1979).

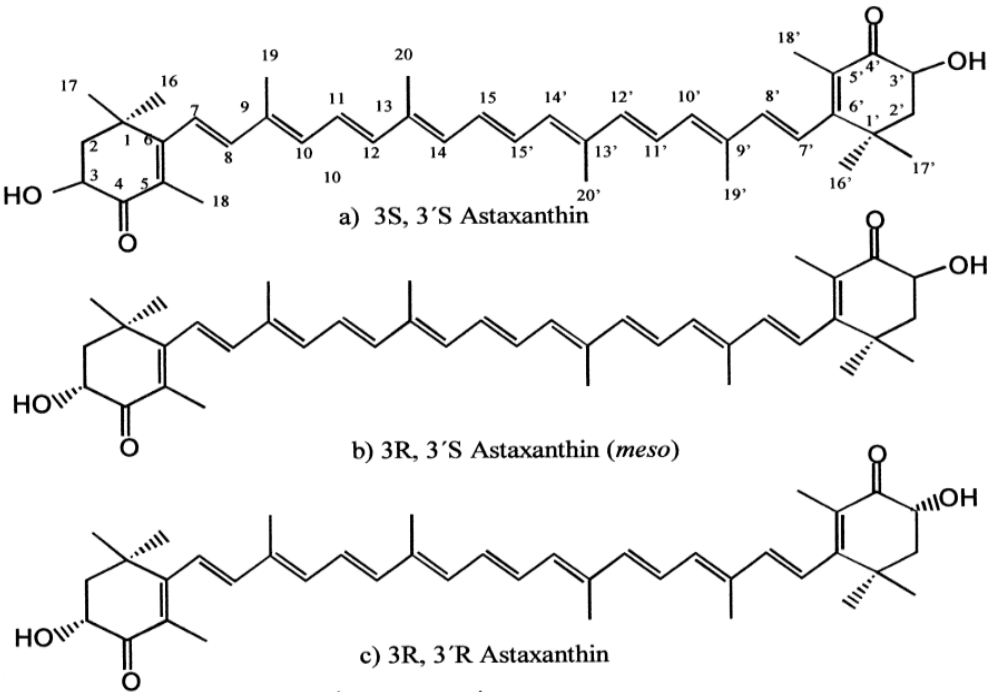


Figure 2. Astaxanthin configurational isomers (a-c) and geometric *cis* isomer (Higuera-Ciapara et al., 2006; Bjerkeng et al., 1997)

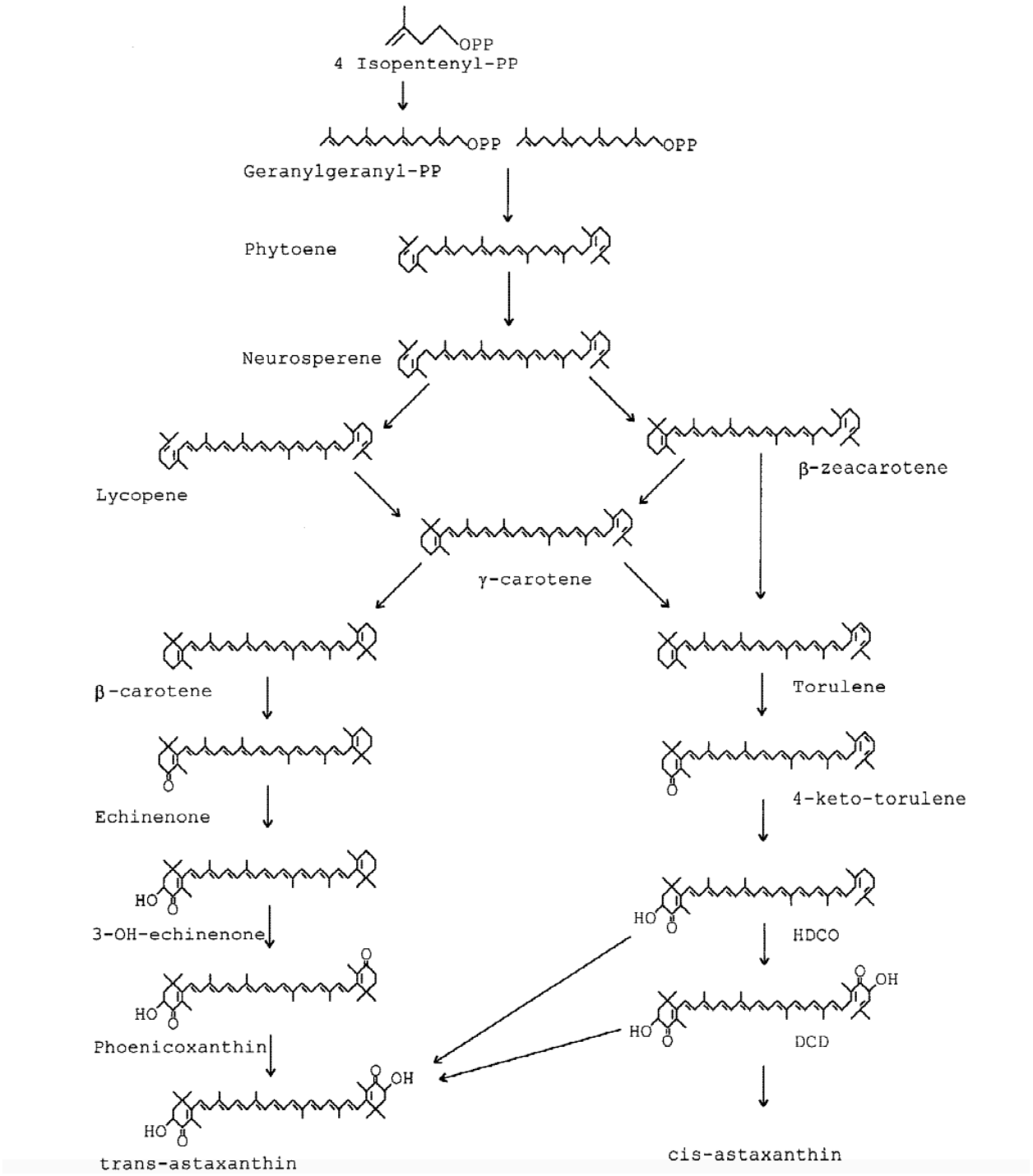


Figure 3. Astaxanthin biosynthesis pathway in *P. rhodozyma* (Ducrey Sanpietro & Kula, 1998)

2.1.2 *P. rhodozyma* fermentation

Carbon and nitrogen sources are the most important ingredient in fermentation broth. Carbon source provides the energy for microbial cell growth, and nitrogen source provides the necessary ingredient for structure formulation (e.g., proteins and enzymes). The difference in chemical ingredients and formulas could affect the final yield of astaxanthin in *P. rhodozyma*.

P. rhodozyma could utilizing various carbon sources, including saccharides (e.g., glucose, sucrose, arabinose, xylose, maltose, cellobiose, and etc.), sugar alcohols (e.g., mannitol), and acids (e.g., acetic acid, D-malic acid, oxalacetic acid, and etc.). Among these, most of the compounds could be utilized by *P. rhodozyma*, but the yield may be different. Different carbon source could affect the pigmentation of the yeast differently, i.e., trehalose could affect pigmentation, while acetic acid and succinic acid could inhibit pigment accumulation (Palágyi, Ferenczy, & Vágvölgyi, 2001). During *P. rhodozyma* fermentation, nitrogen sources, such as yeast extract, peptone, and ammonia sulfate have been used, and the yeast extract and peptone (YEP) were found to be the best nitrogen sources. The common medium for *P. rhodozyma* is YPD, including yeast extract, peptone and glucose. However, little research was done on optimization of glucose and nitrogen (yeast extract and peptone) loading to achieve maximum cell growth and AX production.

2.2. Materials and Methods

2.2.1 Culture preparation

P. rhodozyma ATCC 74219 used for AX production in this study was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultivated in

the yeast malt broth medium (YMB) recommended by the ATCC. YMB medium includes (per liter): 3 g of yeast extract, 3 g of malt extract, 10 g of dextrose, and 5 g of peptone. When making YMB agar plates, 20 g of agar was added into YMB medium. The medium was autoclaved at 121 °C for 18 min prior to use. The initial pH of the YMB medium was around 5.2 and was not controlled during fermentation. To prepare seed culture for AX fermentation, 500 μL stock culture was inoculated into sterilized YMB medium with the inoculation size of 1% (v/v). The seed culture was incubated at 22 °C and 200 rpm for 48 h in a New Brunswick (Edison, NJ, USA) Innova 4300 incubator. The seed culture was then ready for use as an inoculum for fermentation. AX standard was purchased from Sigma Aldrich (St. Louis, MO, USA), and other chemicals were all bought from Thermo Fisher Scientific (Pittsburgh, PA).

2.2.2. Effect of glucose loading on AX production

In YMB medium, glucose was the only carbon source and a series of glucose concentrations in the range of 10-100 g/L were tested to determine the optimum glucose concentration with the constant nitrogen loading (3 g/L yeast extract+5 g/L peptone), the medium profile is listed in **Table 2**. The seed culture prepared in Section 2.2.1 was inoculated (with inoculation size of 1%, v/v) in the media in 1-L flasks with 200 mL working volume to initiate fermentation. Flasks were incubated at 22 °C and 200 rpm. During fermentation, aliquots of 5 mL uniform fermentation broth were taken at 0, 24, 48, 72, 96, and 120 h for analyses of cell mass and optical density (OD) for calculating cell concentration. Also, glucose concentration and AX production were measured by HPLC. All experiments were duplicated unless specified, otherwise.

Table 2. **Nitrogen and glucose loadings**

Glucose (g/L)	20	40	60	80	100
Peptone (g/L)			5.00		
Yeast extract (g/L)			3.00		

2.2.3. Effect of nitrogen loading on AX production

In Section, 2.2.2, glucose concentration of 40 g/L was determined to be the optimum value to achieve the highest AX content (mg/g dry cell mass). As such, it was selected and used in this section to determine the optimum nitrogen loading (i.e., yeast extract and peptone, g/L). The nitrogen source was formed by adding both yeast extract and peptone, and the ratio of yeast extract/peptone was kept at constant of 3:5 (g/g) (**Table 3).** As such, the carbon source/nitrogen source ratios (i.e., "C/N" = glucose / (yeast extract + peptone), g/g) were 5, 10, 15, 20, and 25. The inoculation and sample analysis procedures were the same as Section 2.2.2. All experiments were duplicated unless specified, otherwise.

Table 3. **Medium profile for the nitrogen loading study**

Glucose (g/L)	40	40	40	40	40
Peptone (g/L)	5.00	2.50	1.67	1.25	1.00
Yeast extract (g/L)	3.00	1.50	1.00	0.75	0.60
Total nitrogen loading (P+YE) (g/L)	8.00	4.00	2.67	2.00	1.60
"C/N"	5	10	15	20	25

2.3. Analytical methods

2.3.1 Optical density and cell mass concentration measurements

Sampling was done every 24 h, where 5 mL fermentation broth was collected, and the cell growth was monitored by measuring OD of the culture at λ=600 nm (OD_{600}) using a BioTek (Winooski, VT, USA) Epoch 2 microplate spectrophotometer, while cell mass was also measured in dry weight basis (Nghiem, Montanti, & Johnston, 2009).The linear correlation between OD_{600} and cell mass was established and was used to calculate cell mass by just measuring OD_{600} in subsequent experiments. After OD measurement, the rest of 4 mL of fermentation broth was centrifuged at 4,000 rpm for 5min in a Sorvall Instruments (Newtown, CT, USA) Super T21 refrigerated centrifuge, and the pellet was washed by using distilled water to remove residual medium components in the fermentation broth. This washing step was repeated twice. Afterwards, pellets were washed by filtration through a pre-weighted 0.45-μm Whatman (GE Healthcare Life Sciences, Marlborough, MA, USA) filter paper, and filter paper with retained cells was dried in an oven at 105 °C for 2 h. The dried filter paper and cells was weighed, and the cell dry mass was then calculated by correcting the weight of filter paper.

2.3.2 Glucose quantification by HPLC

Glucose quantification was performed on a Shimazu LC-20AB LC system (Shimadzu Scientific Instruments, Somerset, NJ, USA) HPLC equipped with a reflective index detector (RID-10A). The analytical column was Rezex ROA organic acid H^+ column (Phenomenex Inc., Torrance, CA). The retention time for glucose analysis was 30 mins. The mobile phase was 0.005 N sulfuric acid solution and the flow rate was set at 0.6 mL/min for analysis. The separation temperature was set at 60 °C. Prior to HPLC analysis, fermentation broth samples were

centrifuged at 20,000 rpm for 2 min and supernatants were collected and filtered through 0.45-μm filters into HPLC vials.

2.3.3 Astaxanthin quantification by HPLC

At the end of fermentation, the cells were collected by centrifugation of fermentation broth at 4,000 rpm centrifuged for 30 min. The supernatant was discarded and the cell pellet was freeze-dried and stored at -20 °C until used. To measure AX content, 20 mg of the freeze-dried cell samples were mixed with 5 mL DMSO and vortexed for 30 s for AX extraction in the presence of glass beads for aiding cell disruption. The mixture was heated at 55 °C for 20 min and then centrifuged to collect the supernatant. This extraction cycle was repeated until cell biomass became colorless. All DMSO supernatant were combined and subject to AX analysis on an Agilent (Santa Clara, CA, USA) 1100 Series HPLC equipped with a diode array detector (DAD). A ZORBAX Eclipse Plus C18 column (Agilent, Santa Clara, CA, USA) was used for AX separation at 25 °C. The mobile phase was the mixture of acetonitrile, methanol, dichloromethane, and 1% formic acid, and the mobile phase profile was shown in **Table 4**. The mobile phase flow rate was adjusted to 1.5 mL/min. The signals were recorded at wavelength of 470 nm. The AX production was reported as AX content (mg/g dry cell mass) and concentration (mg/L).

Table 4. **Mobile phase profile variation for AX analysis on HPLC**

Time (min)	A (vol%)	B (vol%)	C (vol%)	D (vol%)
0.00	0.0	50.0	15.0	35.0
2.00	0.0	50.0	15.0	35.0
5.00	5.0	50.0	10.0	35.0

12.00	40.0	30.0	10.0	20.0
16.00	40.0	30.0	10.0	20.0
18.00	0.0	50.0	15.0	35.0

Note: A-dichloromethane; B-acetonitrile; C-formic acid; D-methanol.

2.4. Results and Discussion

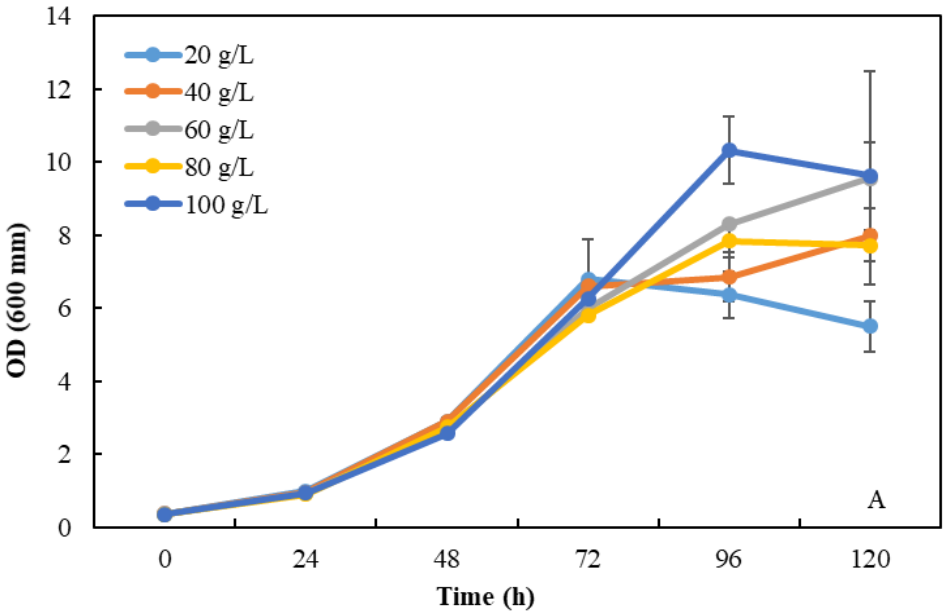
2.4.1. Effect of glucose concentration on AX production

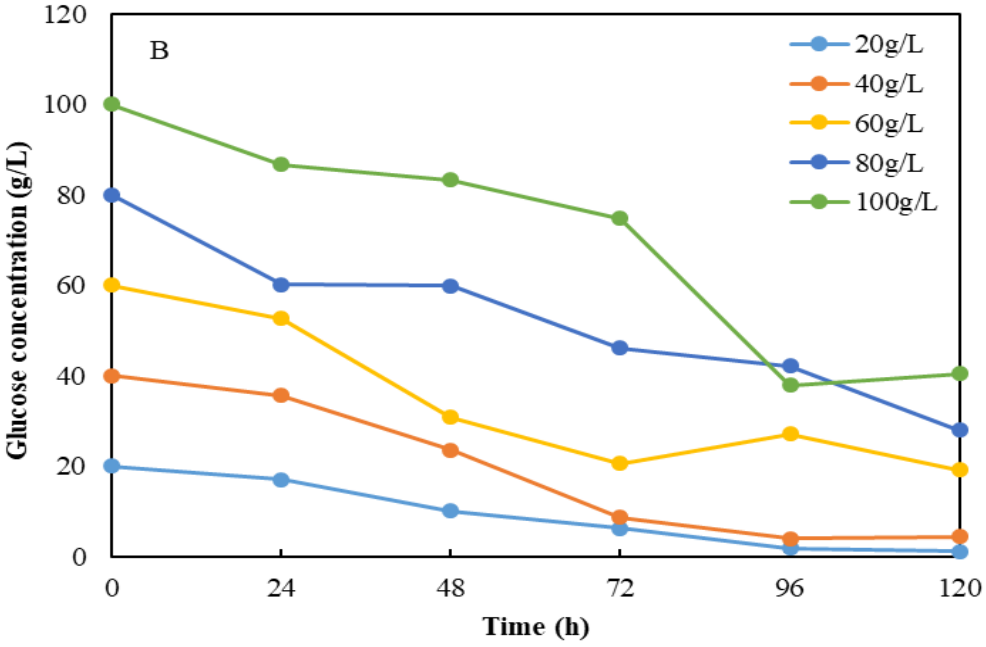
As shown in **Figure 4A**, the growth curves for all glucose concentrations did not clearly display typical four-phase cell growth progress. Lag phase seemed to be in the first 24 h, and 24-96 h appeared to be the exponential growth phase except 20 g/L glucose concentration whose exponential growth occurred between 24-72 h, while cells entered decay phase at the 72 h in the presence of 80 and 100 g/L of glucose. No evident stationary phase was observed. The highest ODs for all glucose concentrations occurred at 96 h, and the maximum OD at 96 h increased from 9.56 to 9.632 with the increase of glucose concentration from 60 and 100 g/L, while the maximum ODs of 5.49 and 7.98 were achieved with the glucose concentrations being 20 and 40 g/L, respectively.

In monitoring glucose consumption, it was found that glucose in the medium was continuously consumed. With the initial glucose concentrations of 20 and 40 g/L, almost all glucose was consumed in about 96 h, while 35-40% of initial glucose still left after 120 h of fermentation and glucose consumption rate became very slight after 96 h at the initial glucose concentrations of 60, 80 and 100 g/L (**Figure 4B**). This result was consistent with that in **Figure 3A** that cells started decaying after 96 h so that slight glucose consumption was used for cell maintenance.

The nitrogen limitation could be one of reasons to inhibit cell growth even though there was till decent amount of carbon source (i.e., glucose), given that nitrogen source is indispensable for protein synthesis for cell formation while carbon source provides carbon and energy for cell growth. The relative carbon and nitrogen contents (usually expressed in C/N ratio) should be balanced to achieve maximum cell growth. As such, carbon source may be excessive when glucose concentration exceeded 40 g/L.

Carotenoid family compounds like AX have an obvious orange or red color, thus the color change can indicate carotenoid accumulation in the cells. With the progress of fermentation, the fermentation broth appeared a distinct color change, from light yellow to orange, and finally ended with a reddish color. At the end of fermentation, initial glucose concentration of 40 g/L achieved the highest AX content of 3.4 mg/g dry cell mass (**Figure 4C**). Compared **Figure 4A** with **Figure 4B**, it is seen that high glucose concentration is not necessary to achieve both high cell mass and AX content, which again indicates that C/N loading ratio should be adjusted to maximize AX production, and cell growth can't be used as a direct indicator for AX accumulation. As such, 40 g/L of glucose was used in the following study on the effect of nitrogen loading.


20 g/L
40 g/L
60 g/L
80 g/L
100 g/L
OD (600 mm)
Time (h)
A


B
20g/L
40g/L
60g/L
80g/L
100g/L
Glucose concentration (g/L)
Time (h)

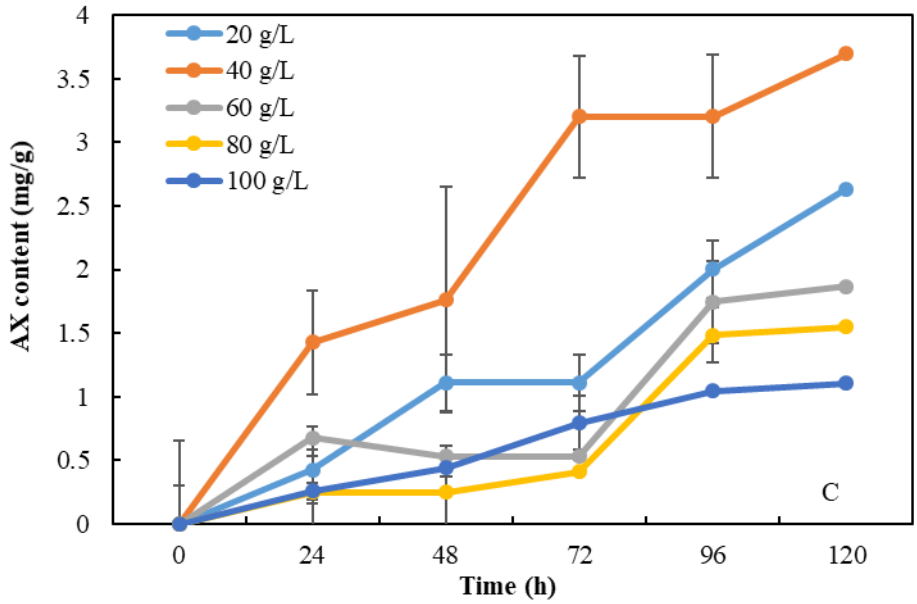
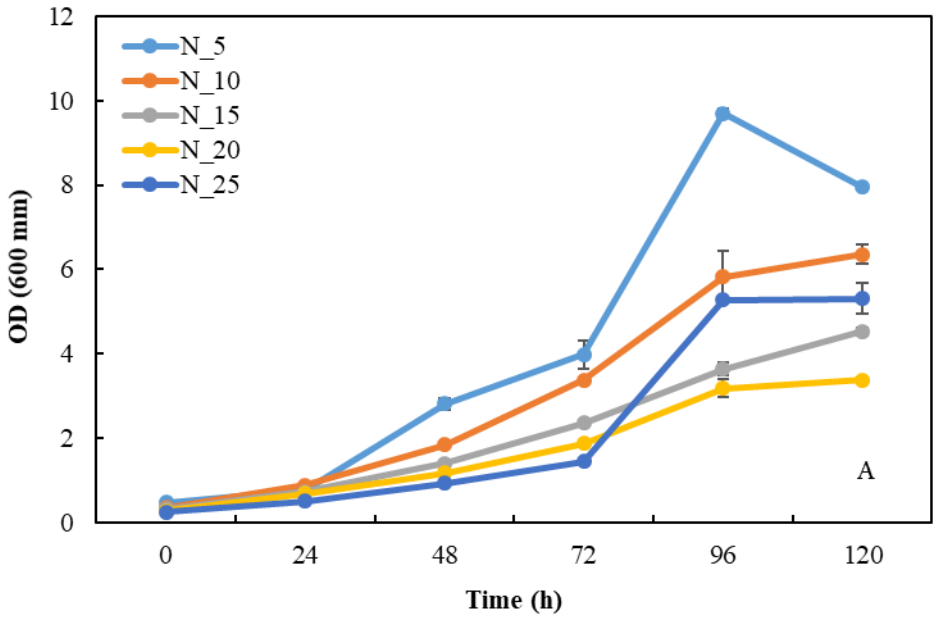


Figure 4. Effect of glucose concentration on: (A) cell growth; (B) glucose consumption; and (C) AX content

2.4.2. Effect of nitrogen loading on AX production

Similar to the results for glucose concentration study, the cell growth curves for all nitrogen loadings did not show typical four-phase cell growth progress (**Figure 5A**). The first 24 h was lag phase, and 24-96 h appeared to be the exponential growth phase except 1.6 and 8 g/L nitrogen loadings. The cells growth entered stationary phase right after 96 h for all nitrogen loadings except 1.6 g/L. In addition, no decay phase was observed for all nitrogen loadings except 1.6 g/L. The maximum OD at 96 h increased from 7.22 to 9.82 with the decrease of nitrogen loading from 8 to 2.67 g/L, while further reduction of nitrogen loading to 2.00 and 1.60 g/L caused the decrease of OD to 7.22 and 6.86, respectively. Therefore, excessive C/N ratio is unfavorable for cell growth.

The glucose concentration decreased rapidly for all nitrogen loadings in the first 48 h, and glucose concentration decrease slowed down for 8 and 4 g/L of nitrogen loadings until 120 h, while little glucose decrease was found with the nitrogen loading exceeding 4 g/L (**Figure 5B**). This result could reflect that insufficient nitrogen supply impaired carbon (i.e., glucose) consumption. The AX content showed a different trend from cell mass, i.e., the AX content increased with the decrease of nitrogen loading. The maximum AX content was 2.6 mg/g cell mass at nitrogen loading of 1.6 g/L. Therefore, high C/N ratio benefited AX accumulation although unfavorable for cell growth, which means excessive carbon could be used for AX production instead of cell growth due to lack of enough nitrogen source.



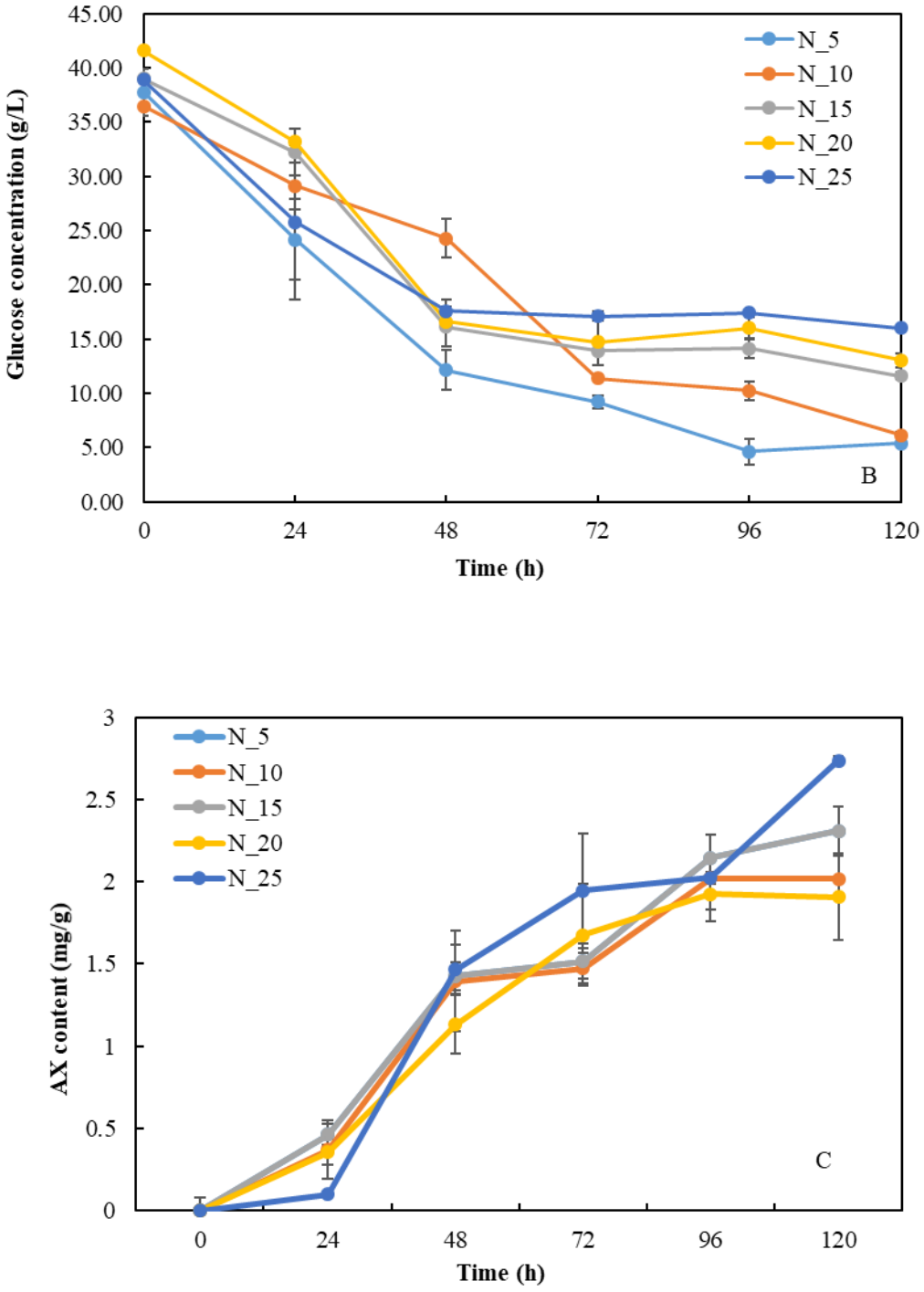


Figure 5. Effect of nitrogen concentration on: (A) cell growth; (B) glucose consumption; and (C) AX content

2.5. Conclusions

In terms of AX production (in AX content of cells), 40 g/L glucose and 8 g/L nitrogen loadings (3 g/L yeast extract+5 g/L peptone) were determined to be the best conditions. The C/N ratio is a key factor affecting both cell growth and AX accumulation, i.e., low C/N ratio is favorable for cell growth, while high C/N ratio benefits AX accumulation with the carbon source loading below the inhibition level (i.e., excessive glucose loading can inhibit cell growth leading to low AX production). Therefore, C/N balance should be well controlled to achieve the either maximum cell growth or AX production.

Chapter 3 - Biotransformation of meat and bone meal (MBM) into astaxanthin

3.1 Introduction

Meat and bone meal (MBM) is an important by-product of the rendering industry. It generally includes organs, fat/lard, skin, feet, abdominal/ intestinal contents, bone, and blood (Jayathilakan, Sultana, Radhakrishna, & Bawa, 2012). It is costly to treat and dispose these by-products ecologically (Toldrá, Mora, & Reig, 2016). Since MBM is rich in protein, it has been used as animal feed ingredients (Piazza & Garcia, 2014). However, the feed industry market is saturated so that new market needs to be explored. Given the high protein content of MBM, MBM could be used as a nitrogen source to support microbial fermentation. Using inexpensive nitrogen source can reduce fermentation cost (Rodrigues, Teixeira, & Oliveira, 2006). On the other hand, the utilization of rendering by-products to produce value-added products would make additional profit to the rendering industry, given the fact that the yield of animal by-product is about 50-60% of the animal total weight (Irshad, 2015). In this study, MBM was studied as a nitrogen source to replace expensive yeast extract and peptone for *P. rhodozyma* fermentation to produce astaxanthin.

3.1.1 Astaxanthin as a feed additive

Astaxanthin is not only used as pharmaceutical products, but also widely used in animal feed. *P. rhodozyma* is usually added into animal feed as an additive for aquaculture, poultry and swine industries Astaxanthin is used in aquaculture feed due to its function to bring customer preferable color to the fish flesh. For poultry, the purpose of using astaxanthin in the feed is to obtain

deeper color egg yolk and increase the fertility. Astaxanthin additive in swine feed is able to help swine gain weight (Swick, 1996).

3.1.2 Meat and bone meal for microbial fermentation

Most of the proteins in MBM are soluble but undigestible due to their long polypeptide chains. In order to make animal protein more digestible for microorganisms, they need to be converted into short-chain polypeptides and/or amino acids. Enzymatic and alkaline hydrolysis methods have been widely used to effectively to degrade animal protein. Garcia et al. (2011) used alkaline and enzyme to hydrolyze MBM into nitrogen rich hydrolysate which served as a nitrogen source to support the growth of *Escherichia coli, Pythum irregular*, and *Schizochytriu limacinum* to produce long-chain polyunsaturated fatty acid, such as docosahexaenoic acid (DHA). But no research has been done one the MBM utilization for AX production.

3.2 Materials and Methods

3.2.1 MBM defatting

MBM was provided by the Animal Co-Products Research & Education Center, Clemson University. Prior to MBM hydrolysis, MBM was defatted to prevent the soap formation during alkaline hydrolysis. Soxhlet extraction was used for defatting with hexane as a solvent. Each extraction thimble contained 25 g of dry MBM and the used hexane was in amount of 15:1 (hexane/MBM, v/w). Each extraction had 6 cycles per hour and lasted for 6 h. The weight difference before and after extraction was calculated as the total fat content of MBM. Each extraction was conducted in three replicates. The fat content was determined to be 20.4% (dry weight basis).

3.2.2 Astaxanthin production from enzymatic/alkaline hydrolysate of meat and bone meal

MBM hydrolysis conditions were listed in **Table 5**. At the end of alkaline hydrolysis, CO_2 was sparged into MBM hydrolysate till pH dropped to 9, and sulfuric acid was then added to further reduce pH to 7. In enzymatic hydrolysis, the reaction was terminated by heating the hydrolysate to 90 ℃ to deactivate the enzymes. Both hydrolysates were centrifuged, and supernatants containing hydrolyzed MBM proteins named MBM hydrolysates (MBMHs) were collected and stored at 4℃ until used for fermentation.

Table 5. **Hydrolysis conditions of MBM**

Hydrolysis Type	Reagent	Hydrolysis conditions	Code*	Reference
Enzymatic hydrolysis	Alcalase 2.4L	Alcalase 2.4L, 0.4 U/g substrate, 50 °C, pH 8.5, 4 h	AM4	(Garcia, Pyle, Piazza, & Wen, 2011)
		Alcalase 2.4L,0.4 U/g substrate, pH 8.5, 50 °C, 8 h	AM8	
	Flavourzyme	Flavourzyme, 50 LAPU/g substrate, pH 7.0, 4 h	FM4	
		Flavourzyme, 50 LAPU/g substrate, pH 7.0, 8 h	FM8	
	Proteinase K	1 mg/g substrate, pH 7.5, 37 ℃, 4 h	PK4	
		1 mg/g substrate, pH 7.5, 37 ℃, 8 h	PK8	
		1 mg/g substrate, pH 7.5, 37 ℃, 4 h	PB4	

	Proteinase, bacterial	1 mg/g substrate, pH 7.5, 37 ℃, 8 h	PB8
Alkaline hydrolysis	Calcium hydroxide	0.1 g/g MBM, 4 h	CM4
		0.1 g/g MBM, 8 h	CM8
		0.1 g/g MBM, 16 h	CM16

* Code example: In AM4, A =Alacalase 2.4L; M=MBM; and 4=hydrolysis for 4 h.

3.2.3 Total nitrogen content measurement

All MBMHs were freeze-dried and measured for the total nitrogen content with a 2400 Series II CHNS/O Elemental Analyzer (PerjubElmer Inc. Waltham, MA). Samples were all grounded into particles, and small quantity of samples were loaded into capsules and then pressed into pellets. The combustion temperature of the oven was set at 975 ℃ and the capsule was loaded into the oven by using an auto-sampler. The inflow were helium, oxygen, and nitrogen.

3.2.4 Meat and bone meal hydrolysates (MBMHs) fermentation

Glucose was the only carbon source for fermentation, and its concentration of 40 g/L was used with MBMHs during fermentation. Glucose was directly mixed in MBMHs, and the pH was adjusted to 5.25. The mixture of glucose and MBMHs was added into 250-mL shaking flasks with working volume of 50 mL. Fermentation broth was autoclaved for 20 min at 121 ℃. Each experiment was duplicated.

3.2.5 Yeast nitrogen-based medium (YNB)

YNB was used as a supplementary nutrient which was expected to improve cell growth and astaxanthin production. It consisted of vitamins (biotin, thiamine hydrochloride, etc.), trace elements (boric acid, copper sulfate, etc.), and minerals (sodium chloride, magnesium sulfate, etc.). In the YNB medium, no extra nitrogen or carbon source included. Therefore, glucose, yeast extract and peptone were the only carbon and nitrogen source in this study. Mineral and vitamins were sensitive to temperature change, thus this supplement nutrient solution was sterilized via filtration. The preparation process is as follows: (1) 1.7 g of ×10 YNB was dissolved in a 100-mL sterilized water in a 250-mL beaker; (2) The solution was filtered through 0.2-μm membrane for sterilization; (3) The sterilized YNB stock was refrigerated under 4 ℃. A 10 times YNB stock was prepared as followed the instructions on the manual and YNB stock was diluted to required concentration and volume before added to fermentation broth.

3.3 Results and Discussion

3.3.1 Total nitrogen content of MBMHs

As shown in **Figure 6A**, the total nitrogen contents of yeast extract and peptone were measured as references, and they were 11.4% and 14.0%, respectively. All MBMHs has a higher total nitrogen content than yeast extract. Among enzymatic hydrolyzation groups, Pb4, Pb8, and Pk8 exceed peptone, and they were 14.35%, 15.29%, and 15.59%. Among alkaline hydrolyzation groups, no group exceed peptone.

Elemental analyzer measured the total nitrogen content in solid freeze-dried MBMHs, but during MBMHs were directly fermented without freeze drying, thus the crude nitrogen content was

quantified by calculation. In **Figure 6B**, all of the MBMHs contained 2.91-3.82 g of nitrogen, yeast extract (3 g/L) and peptone (5 g/L) contained total of 5.21g nitrogen.

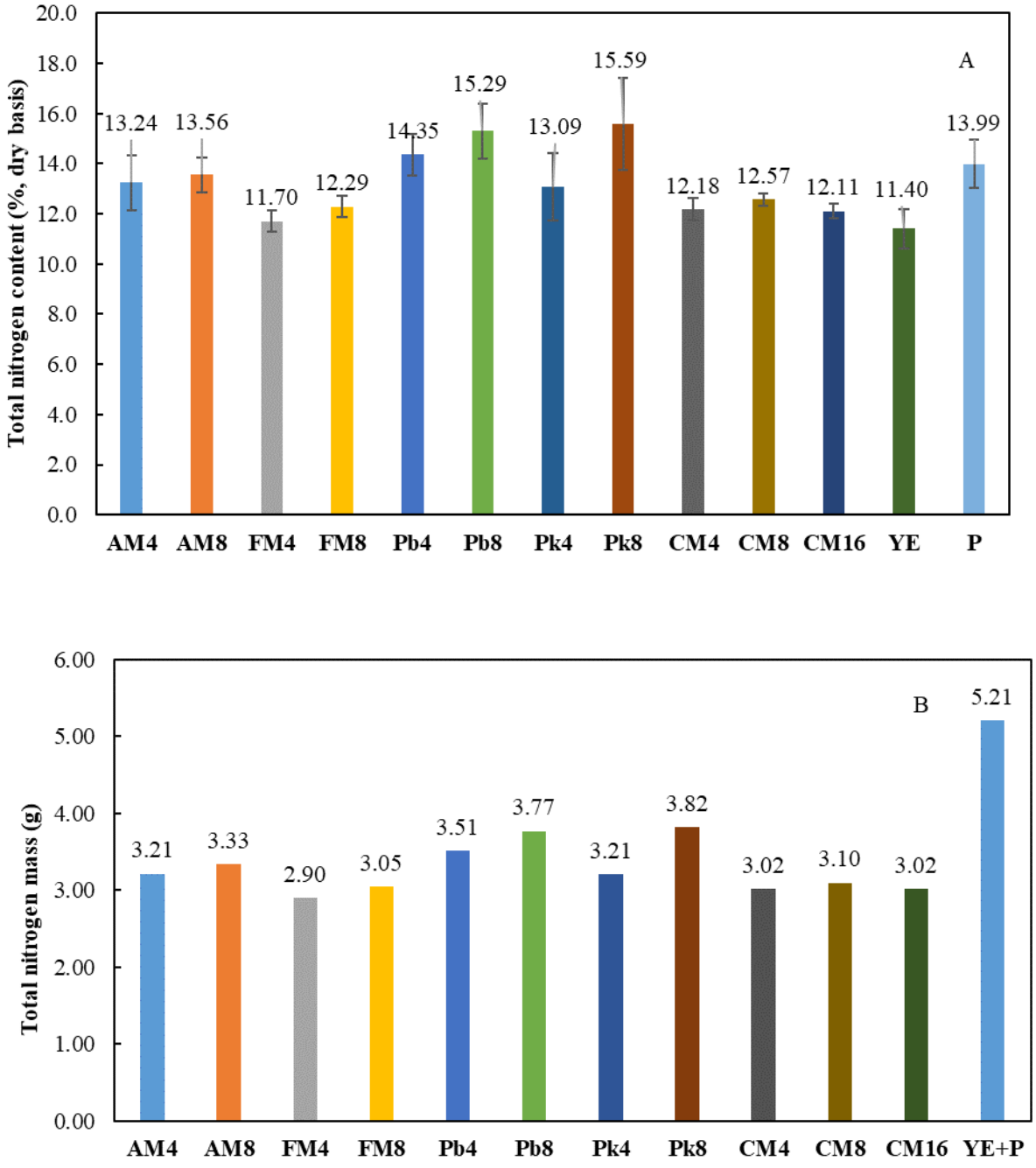


Figure 6. Total nitrogen content: (A) Total nitrogen content in freeze-dried MBMHs; (B) Total nitrogen mass in MBMHs

In this study, the effect of hydrolysis time was also investigated. Longer time achieved higher total nitrogen content in the hydrolysate during enzymatic hydrolysis, while time did not result in much difference in total nitrogen content of MBMHs from alkaline hydrolysis.

Compared with yeast extract, all MBMHs had higher total nitrogen content, while only Pb4, Pb8 and Pk8 achieved higher total nitrogen content than peptone. In addition, enzymatic hydrolysis was found to be more efficient than alkaline hydrolysis because it achieved higher total nitrogen content in the hydrolysate. Also, among enzymatic hydrolysis groups, protease from bacterial (Pb) and protease K (Pk) resulted in higher total nitrogen content than Alcalase 2.4L and Flavourzyme. The Pk hydrolysis for 8 h resulted in the maximal total nitrogen content (15.6%) among all experiment groups, its total nitrogen content was even higher than that of peptone (14.0%).

3.3.2 MBMHs fermentation

Among enzymatic hydrolyzation groups, liquid enzyme Alcalase 2.4L and Flavoryzyme were used for MBM hydrolyzation, the cell growth curve was shown in **Figures 7A** and **7B**. MBM hydrolyzed by Alcalase 2.4L (AM4 and AM8) did not have any cell growth, even though YNB supplement was applied (YAM4 and YAM8). For MBM hydrolyzed by Flavourzyme (FM4 and FM8), only FM8 and YFM8 were observed to have cell growth, and achieved OD of 1.30 and 1.85, respectively. As shown in **Figures 7C** and **7D** on experimental groups using protease K (Pk4 and Pk8) and protease from bacterial (Pb4 and Pb8), Pk8 and Pb8 were the only two enzymes to result in OD of 0.782 and 1.247, respectively. The addition of YNB improved the OD of to about 2.2 (YPk8 and YPb8).

For alkaline hydrolyzation groups (CM4, CM8 and CM16), calcium hydroxide was applied as a protein degradation agent, and cell growth curve is shown in **Figures 7E** and **7F**. In **Figure 7F**, cells in YCM4 and YCM8 grew constantly from 24 to 72 h, and cells in YCM16 entered exponential phase and had a faster cell growth rate than YCM4 and YCM8. YCM16 entered stationary phase from 72 h to 96 h, and cell growth started to decrease afterwards. In **Figure 7E**, the cell growth tendency was uniformed as in **Figure 7F**, but the cell growth rate were all enhanced approximately two times.

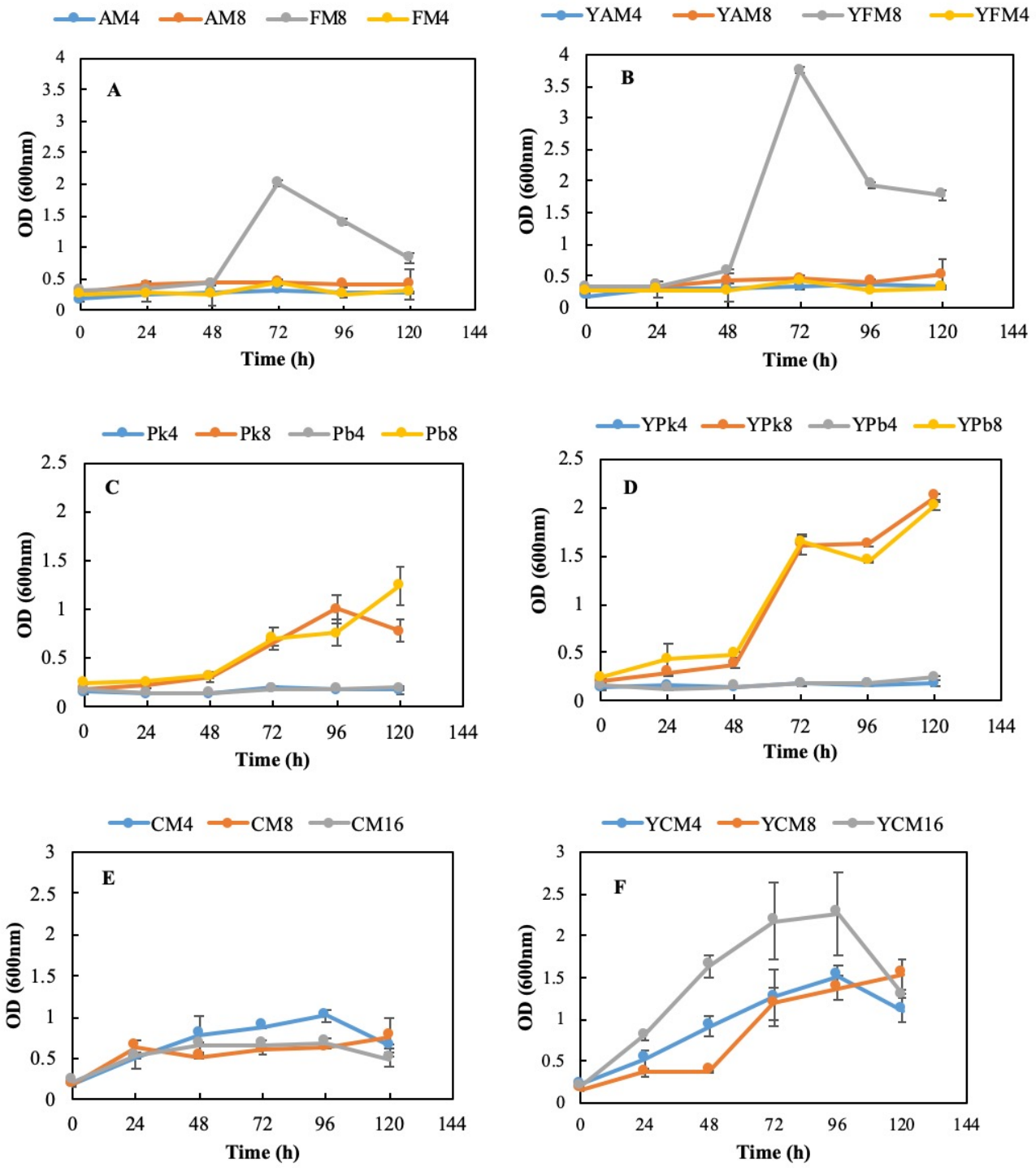


Figure 7. MBMHs fermentation cell growth: (A) AM4, AM8, FM4, and FM8; (B) YAM4, YAM8, YFM4, and YFM8; (C) Pk4, Pk8, Pb4, and Pb8; (D) YPk4, YPk8, YPb4, and YPb8; (E) CM4, CM8, and CM16; (F) YCM4, YCM8, and YCM16.

Final cell mass concentration and AX content were shown in **Figure 8**. In enzymatic hydrolyzation groups, YFM8 achieved the maximal OD of 10.18, and AX content of 0.21 mg AX/g biomass. However, YPk8 achieved a maximal AX content of 0.71 mg AX/g biomass and OD of 5.99. In alkaline hydrolyzation groups, YCM16 achieved the maximal OD of 11.47, and AX content of 0.75 mg AX/g biomass. However, YCM4 achieved a maximal AX content of 1.02 mg AX/g biomass and OD of 4.66.

The percentage of glucose consumptions were shown **in Figure 9**. The initial glucose concentration was 40 g/L. Among enzymatic hydrolyzation groups, YPk8 consumed maximal of glucose in the medium, which is 87.1%. Among alkaline hydrolyzation groups, YCM16 consumed maximal of glucose in the medium, which is 86.7%. Groups with YNB supplement consumed more glucose than the groups without YNB.

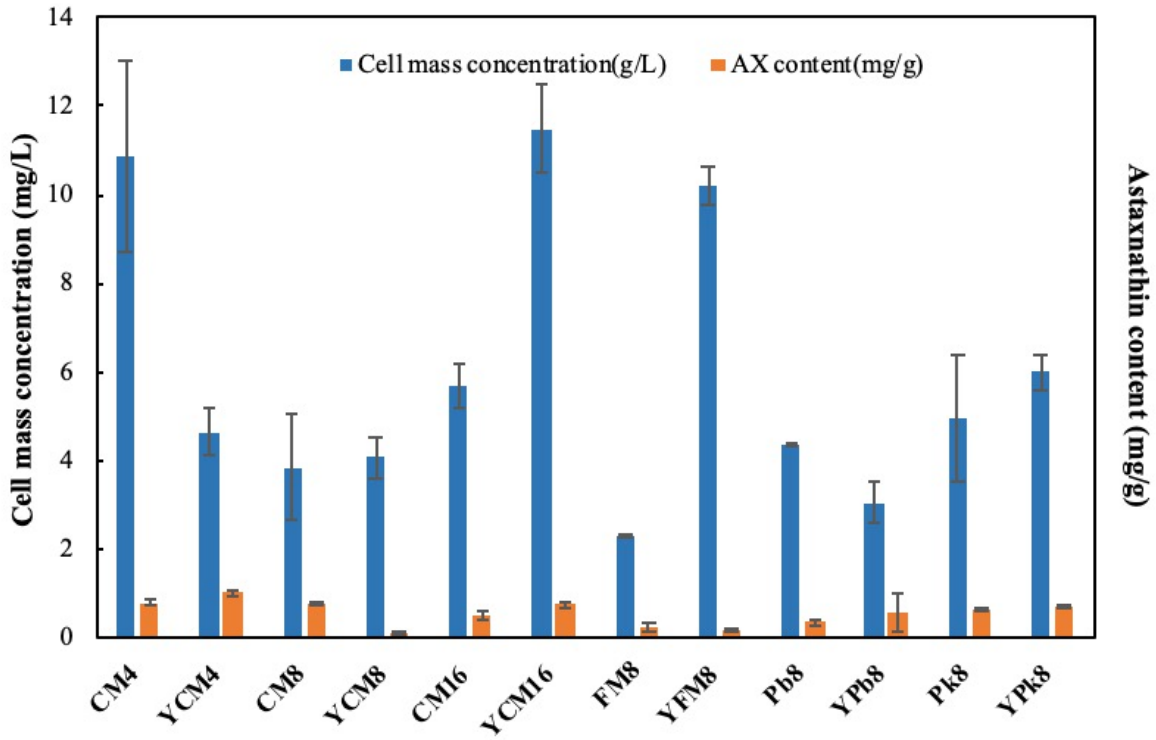


Figure 8. MBMHs fermentation: Cell mass concentration and AX content

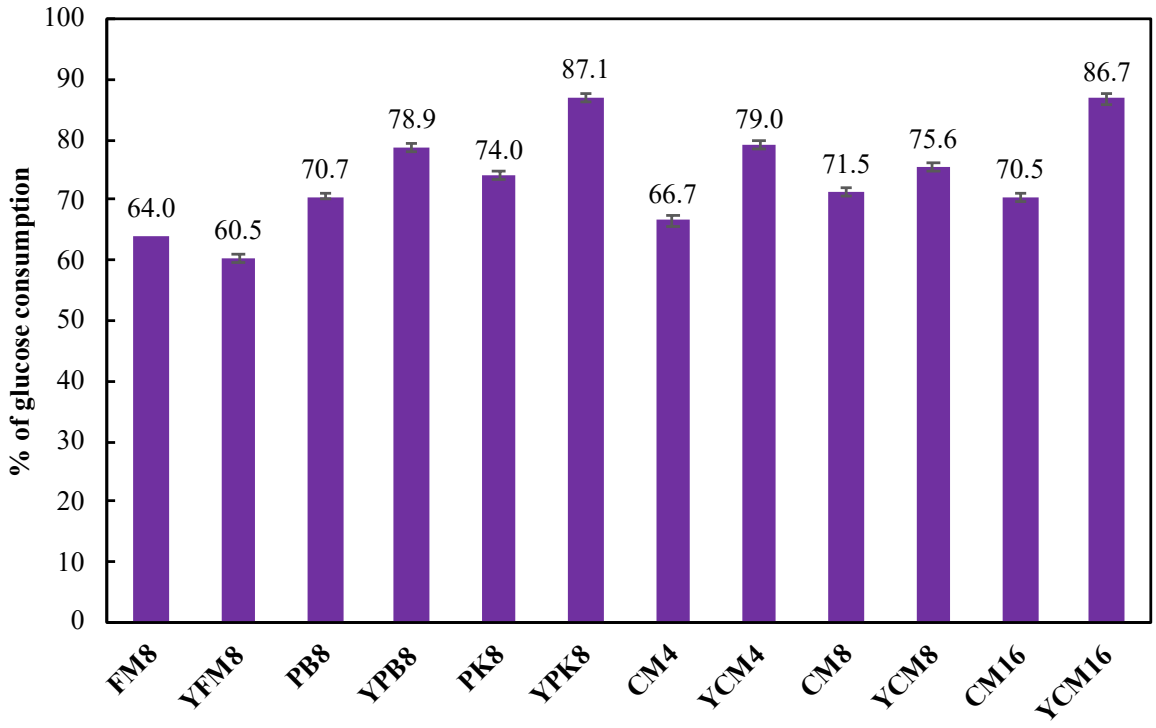


Figure 9. **MBMHs fermentation: percentage of glucose consumption**

3.4 Conclusions

MBMHS from alkaline hydrolyzation for 16 h achieved the highest final AX content with YNB supplement (YCM16), which was 1.02 mg AX/g biomass. The final AX content reached only half to one-third of the red yeast fermented in YMB medium, but the result showed a potential to utilize MBM as an inexpensive alternative nitrogen source. Secondly, extension of enzymatic hydrolysis time resulted higher nitrogen content in MBMHs, but did not show significant effect for alkaline hydrolysis. Finally, supplement (YNB) could enhance the cell growth, but may not enhance the AX production.

Chapter 4 - Astaxanthin production from corn and wheat starches by *Phaffia rhodozyma*

4.1 Introduction

Starch is a polysaccharide that plays an important role in human food consumption. It is widely consisted in many crops, such as rice, corn, potatoes, wheat, and cassava. In addition to the use for food, it has numerous other applications in chemicals, materials, biofuel production and more. In EU, over 30% of starch was used in paper, carboard, and corrugating industries, 54% was used as food applications, and 16% was used for other non-food applications (Sullivan, 2009). The starch has been oversupplied since 2009, which has called for new market outlets for starch to keep the starch-related agriculture profitable (Kim, Choi, Kim, & Baik, 2011).

Starch is a heterogenous polymer of amylose and amylopectin whose contents are 20~30% and 70~80% in starch, respectively (Shit & Shah, 2014). Amylose is a linear polysaccharide composed of glucose unit connected by α-1, 4 linkages, while amylopectin is highly branched polysaccharide of glucose connected by both α-1, 4 and α-1, 6 linkages. Compared to cellulose, starch is more amenable to enzyme degradation. Both amylase and glucoamylase are common starch degradation enzymes. Enzymatic hydrolysis of starch includes two major steps that are (1) liquefaction where long-chain polysaccharide is degraded into short-chain saccharides; and (2) saccharification where short-chain polysaccharide is further hydrolyzed into monosaccharides and disaccharides. The resultant starch hydrolysate mainly contains glucose and maltose which are fermentable by *P. rhodozyma* to produce astaxanthin. Therefore, it could be possible to use *P. rhodozyma* to directly degrade and ferment starch into astaxanthin without using external enzymes, which could make the starch fermentation process simpler and cheaper.

In this study, corn and wheat starches were liquified and then saccharified by α-amylase and amyloglucosidase in sequence, respectively. The hydrolysate of corn and wheat starches were diluted by different percentage of distilled water as a carbon source for *P. rhodozyma* fermentation. Also, *P. rhodozyma* amylolytic enzyme productivity was studied, because two different strains were demonstrated to produce amylolytic enzymes, which is CECT1690 (Díaz, Sieiro, & Villa, 2003) and CBS 5905^T (Libkind, Moliné, De García, Fontenla, & Van Broock, 2008). However, it is not clear if the strain in this study *P. rhodozyma* ATCC 74219 can produce Amylolytic enzymes.

4.2 Materials and Methods

4.2.1 Test of the extracellular amylolytic activity of *P. rhodozyma*

The amylolytic activity was determined on selective medium (SC) plates containing 2% of soluble starch. The SC plate also contained 0.67% yeast nitrogen base (YNB, Difco), 0.5% arbutin (ICN) and 2% agar, and the SC pH was adjusted to pH 5.0 before autoclave. Additional 2 mL of 1% ferric citrate ammonium was sterilized by filtration and added to 100 mL of autoclaved SC medium before poured into the plates. A volume of 100 μL seed culture was dropped in different area on the SC agar plate. The plates were incubated for 5 days at 22 °C, a clear zone around the colony would occur if the red yeast produced amylolytic enzymes (Strauss, Jolly, Lambrechts, & Van Rensburg, 2001).

4.2.2 Hydrolysis of corn/wheat starches

Enzyme dosages for α-amylase (Sigma, A3306) and amyloglucosidase (Sigma, A7095) were first determined prior to starch hydrolysis experiments. Both corn and wheat starch samples were

mixed with distilled water with a solid loading of 30% (w/v), as 0.5%, 1%, and 1.5% volume of α-amylase and amyloglucosidase that were added in sequence to hydrolyze corn and wheat starches. Hydrolysates were collected every 30 min during liquification process for 2 h, and then collected every 24 h for 2 days during saccharification process. We found that there was still significant amount of maltose in solution when 0.5% of enzymes applied. However, both enzyme dosages of 1% and 1.5% resulted in more complete starch saccharification than 0.5% enzyme dose, and they achieved similar glucose concentration, which means 1% of enzymes was sufficient to degrade starch. Thus, both 1% (v/v) α-amylase and amyloglucosidase were used for corn and wheat starch hydrolyzation.

Corn and wheat starch samples were mixed with distilled water separately with a solid loading of 30% (w/v). Starch slurry was liquefied by using 1% (v/v) α-amylase at 85 °C for 90 min in a shaking water bath. Cold distilled water was added to the water bath to decrease the temperature for the following saccharification. Consequently, saccharification was performed at 60 °C for 2 days with 1% (v/v) of glucoamylase being added. During starch hydrolysis, starch was decomposed into polysaccharides with lower degree of polymerization by α-amylase, and finally degraded into glucose by amyloglucosidase.

4.2.3 Corn/Wheat starch hydrolysate fermentation

Corn starch hydrolysate (CSH) and wheat starch hydrolysate (WSH) were diluted by distilled water of 0%, 25%, 50%, and 75%, respectively to adjust glucose concentrations to an appropriate level. *P. rhodozyma* seed culture was prepared by following the same procedure in **2.2.1**. 1% (v/v) of seed culture was inoculated in diluted CHS and WHS with different glucose

concentrations to initiate fermentation for 5 days. In the course of fermentation, 1 mL of broth was collected every 24 h for analysis. OD_{600}, glucose concentration and AX concentration were quantified by using the same methods in Sections **2.3.1**, **2.3.2**, and **2.3.3**.

4.3 Results and discussion

4.3.1 Detection of extracellular amylolytic activity of *P. rhodozyma*

A distinctive halo would appear around the microbial flora if the strain can produce amylolytic enzymes. As shown in **Figure 10**, no visible change was observed around the flora after incubation of 120 h, which suggested that this strain not be able to produce amylolytic enzyme.

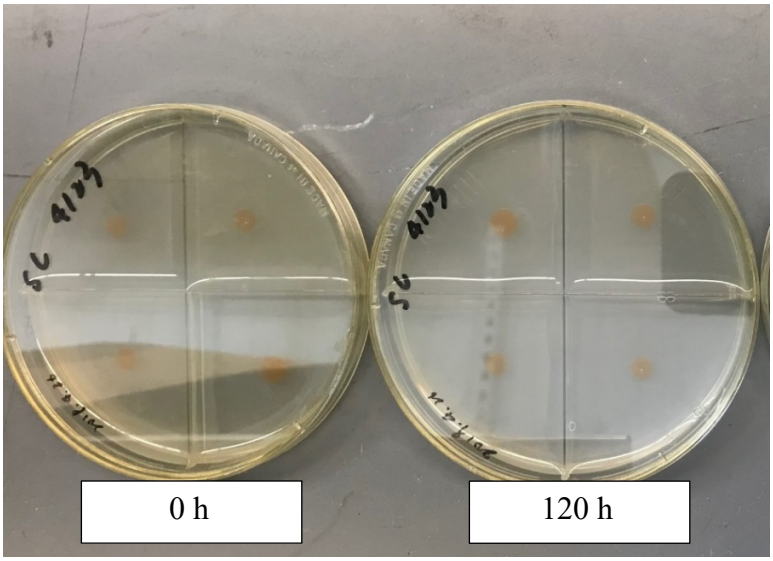


Figure 10. **Test of extracellular amylolytic activity of *P. rhodozyma* (A) SC plate at 0 h; and (B) SC plate at 120 h**

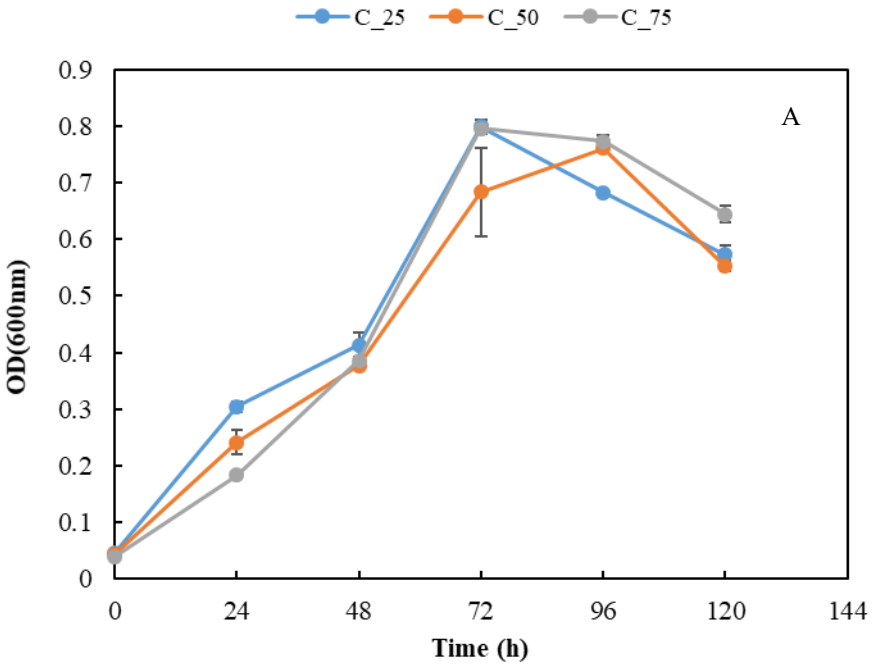
4.3.2 Corn and Wheat starch hydrolysis and fermentation

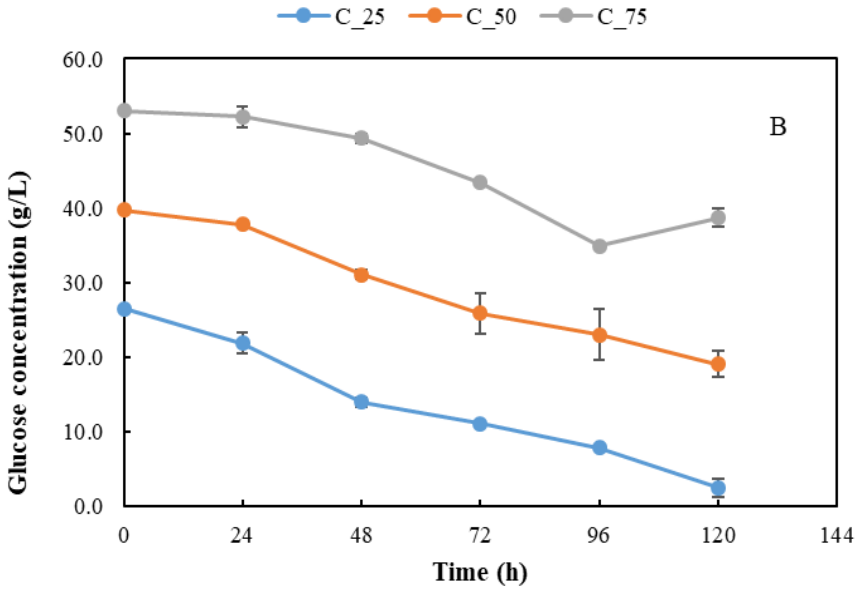
With 0.5% of enzyme loading, the resultant maltose was not completely converted into glucose (i.e., 0.314 g/L in WSH and 0.28 g/L in CHS) while little maltose was detected with 1% and 1.5% of enzymes being used, i.e., maltose derived from starch was completely degraded into

glucose. Therefore, 1% of enzyme loading was sufficient for starch hydrolysis. Thus, 1% enzyme loading was used for the following starch hydrolysis, and starch hydrolysate was used as a carbon source for *P. rhodozyma* fermentation.

Corn and wheat starches were hydrolyzed in a shaking water bath for 7 days, and the glucose concentration was measured every 24 h. Consequently, the time for hydrolysis process was determined to be 72 h when starch conversion to glucose reached steady state. At 72 h, the glucose concentration of corn and wheat starch hydrolysates reached 106 g/L and 145 g/L, respectively.

P. rhodozyma can grow in CSH and achieved different growth results. In **Figure 11A**, cells in C_25, C_50 and C_75 groups started to grow fast, without lag phase observed. C_25 and C_75 entered stationary phase from 72 h, and C_50 entered stationary phase at 96 h. OD of all groups started to decrease since cells achieved the maximal OD. In **Figure 11B**, all groups started to consume glucose in a relatively same speed during fermentation. In **Figure 11C**, C_25, C_50 and C_75 respectively reached an AX content of 1.71, 2.15, and 1.45 mg/g biomass, where C_50 achieved the maximum AX content.


C_25
C_50
C_75
A
OD(600nm)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
24
48
72
96
120
144
Time (h)


C_25
C_50
C_75
B
Glucose concentration (g/L)
60.0
50.0
40.0
30.0
20.0
10.0
0.0
0
24
48
72
96
120
144
Time (h)

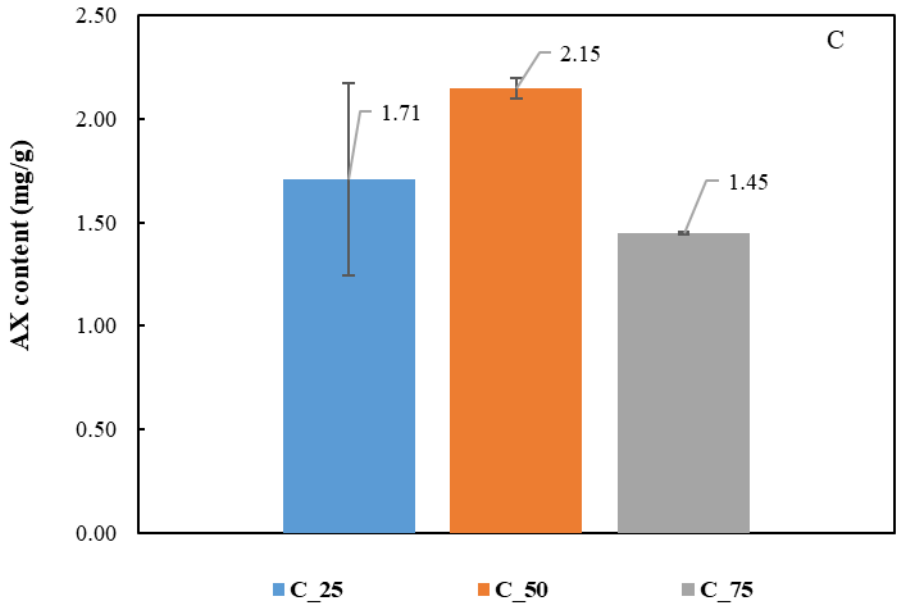
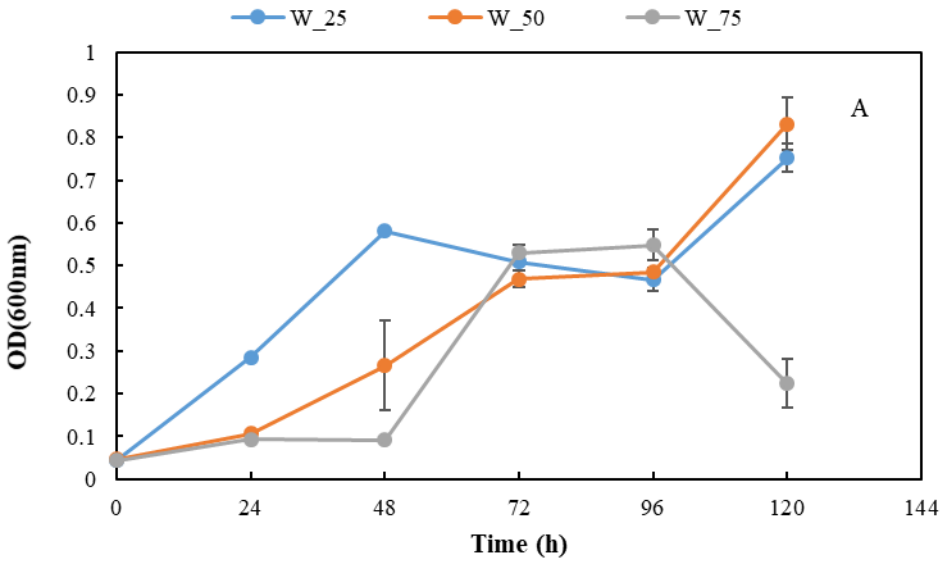
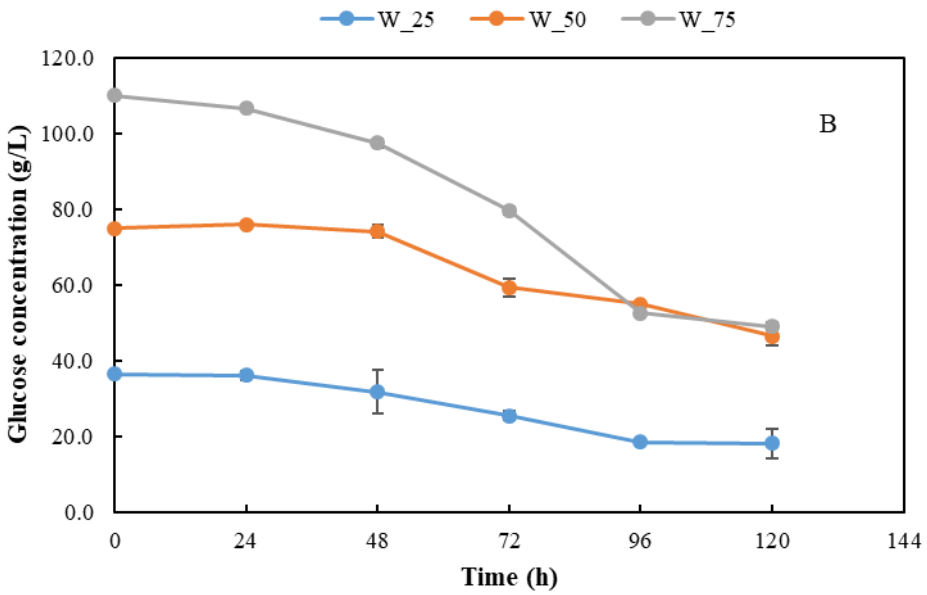


Figure 11. CHS fermentation: (A) Cell growth; (B) Glucose consumption; and (C) Final AX content

P. rhodozyma can grow in WSH and achieved different growth results. In **Figure 12A**, W_25, W_50 and W_75 entered exponential phase from the 0 h, 24 h, and 48 h, separately. W_25, W_50 and W_75 entered stationary phase from 48 h, 72 h, and 72 h. W_25 and W_50 groups had another increasing tendency of biomass growth after they entered stationary phase. OD of W_75 decreased drastically after stationary phase, and its curvature showed a typical four-phase cell growth curve. In **Figure 12B**, all groups started to consume glucose as quicker as the cells went into exponential phase and the consumption rate decreased after 96 h. Among three groups, W_75 had the highest glucose consumption speed during fermentation. In **Figure 12C**, W_25, W_50 and W_75 respectively achieved an AX content of 3.62, 2.43, and 1.5 mg/g biomass, of which W_25 was the best in terms of AX content.


W_25
W_50
W_75
A
OD(600nm)
Time (h)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
0
24
48
72
96
120
144


W_25
W_50
W_75
B
Glucose concentration (g/L)
Time (h)
0.0
20.0
40.0
60.0
80.0
100.0
120.0
0
24
48
72
96
120
144

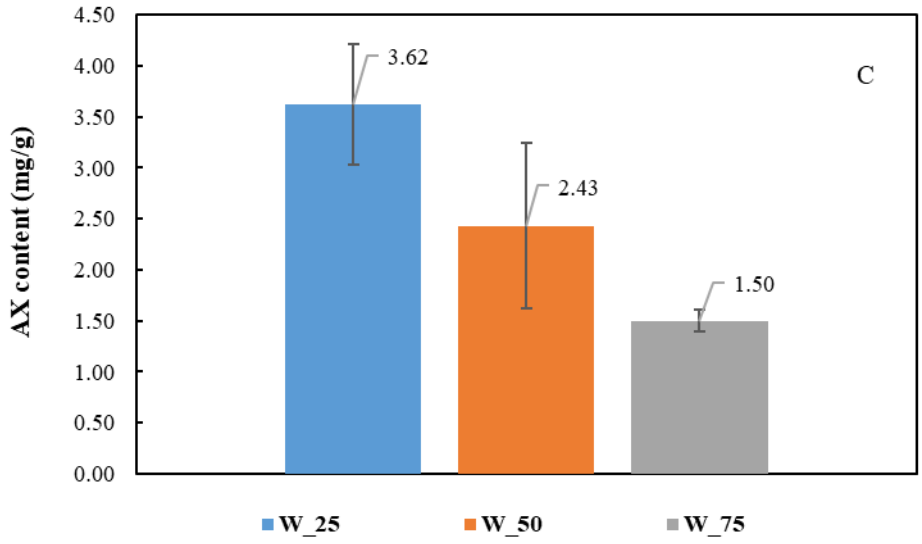


Figure 12. WHS fermentation: (A) Cell growth; (B) Glucose consumption; and (C) Final AX content

4.4 Conclusions

The corn and wheat starches were hydrolyzed by 1% amylase and glucoamylase into glucose with little maltose remained. The CHS and WHS were fermentable and could support *P. rhodozyma* growth and AX production. In CHS fermentation groups, a dilution ratio of 50% (C_50) reached the highest AX content of 2.15 mg/g cell biomass. In WHS fermentation groups, a dilution ratio of 25% (W_25) achieved the highest AX content 3.62 mg/g biomass. In addition, *P. rhodozyma* strain ATCC 74219 was found to be unable produce extracellular amylolytic enzymes for starch degradation.

Chapter 5 - Summary

Microbial fermentation is a major pathway to obtain natural AX. To meet the increasing market demand of natural AX, improving the AX productivity of microorganisms is necessary. Microbial fermentation is affected by carbon and nitrogen source loadings, an optimized fermentation medium formula is important. *P. rhodozyma*, a red yeast was the AX producer, and one of its strains ATCC 74219 was studied in this project. In **Chapter 1**, the carbon (glucose) and nitrogen (yeast extract and peptone) loading were studied to find out the optimal conditions for AX production by ATCC 74219. It was found that glucose, yeast extract, and peptone were 40, 3 and 5 g/L, the AX content was the highest with a final AX content of 3.5 mg/g biomass.

MBM is a rendering industry waste, which is rich in animal protein and lipid. MBM was considered as a potential nitrogen source for microbial fermentation. In **Chapter 2**, MBM was hydrolyzed by Alkalase 2.4L, Flavourzyme, Protenase K, Protenase from bacteria, and calcium hydroxide for different time. In order to enhance AX productivity, supplement (YNB) was added into fermentation broth to enhance MBMHs fermentation. MBM hydrolyzed for 16 h and with YNB supplement (YCM16) achieved the highest AX content of 1.02 mg/g biomass. Enzymatic hydrolysis time and YNB supplement were necessary to produce high AX content of cells. Although, the final AX production is not as high as ATCC 74219 fermented in medium contain yeast extract and peptone, but the MBM was found to show a potential as a nitrogen source in microbial fermentation for AX production.

Corn starch, and wheat starch as low-cost glucose sources are all able to be utilized by *P. rhodozyma* to produce high-value AX. With hydrolysis of starches by 1% amylase and

glucoamylase the resultant glucose can support the growth *P. rhodozyma* to accumulate AX, i.e., CHS and WHS achieved the maximum AX of 2.15 and 3.62 mg/g cell biomass

The future work can be focused on: (1) optimization of MBM hydrolysis and profile amino acid compositions and find out the correlation between AX production and amino acid compositions; (2) starch hydrolysis optimization; (3) AX fermentation optimization; (4) study the combination of MBM nitrogen with corn and wheat starch for AX production; and (5) develop mathematic model to simulate and predicate AX production by *P. rhodozyma* under different conditions that will provide a useful tool for process optimization and reactor design in the future.

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