CAROTENOID VALUE ADDITION TO DISTILLERS DRIED GRAIN WITH SOLUBLES BY RED YEAST FERMENTATION

by

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AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Grain Science and Industry
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Abstract

Distillers Dried grain with Solubles (DDGS) is a co-product of grain-based ethanol and is primarily used as livestock feed. With increasing production of DDGS, it is imperative to produce value-added products and/or find new applications of DDGS to help sustain the biofuel industry. Carotenoids are expensive yet essential feed additives. Since animals cannot synthesize carotenoids and animal feeds including DDGS are generally poor in carotenoids, about 30-120 ppm of total carotenoids is added to animal feed to improve animal health. The objectives of this study were to 1) produce carotenoid (astaxanthin and β-carotene)-enriched DDGS by *Phaffia rhodozyma* and *Sporobolomyces roseus* monoculture and mixed culture submerged fermentation of whole stillage, 2) optimize fermentation media by response surface methodology (RSM) and mixture design followed by validation, 3) evaluate the nutritional profile of carotenoid-enriched DDGS, 4) improve carotenoid production by the use of precursors, and 5) develop carotenoid-enriched feeds namely, wheat bran, rice bran and soybean products. Carotenoid-enriched DDGS was produced from both monoculture and mixed culture fermentation with yields ranging from 17-233 µg/g. Upon media optimization, astaxanthin and β-carotene yields, especially in *P. rhodozyma* were enhanced by 177% and 164% to yield 98 and 275 µg/g respectively. Nutrition profiling of the carotenoid-enriched DDGS showed that the secondary fermentation resulted in low fiber, protein and %N and enhanced fat. Fiber was reduced by 77% and 66% by *P. rhodozyma* and *S. roseus* respectively, whereas the crude fat increased by 80% in mixed culture fermentation. Additionally, abundant vaccenic acid, a monounsaturated fatty acid was seen in *S. roseus* and mixed culture fermented DDGS. Vaccenic acid is a precursor of conjugated linolenic acid which is known to confer numerous health benefits. Fermentation of milo DDGS, wheat

bran, rice bran and soybean products also resulted in carotenoid enrichment, with the best astaxanthin yield of 80 μg/g in rice bran, and best β-carotene yield of 837 μg/ g in soy flour. Precursors like mevalonic acid, apple pomace and tomato pomace increased carotenoid yield in DDGS and other substrates, with the yield increment depending on the substrate. Mevalonic acid resulted in the best astaxanthin and β-carotene yield increment by 140% and 236% resulting in 220 μg/g and 904 μg/g respectively in corn DDGS. Apple pomace and tomato pomace resulted in 29% carotenoid yield increment. Numerous studies thus far have used cheap agricultural substrates to produce carotenoids especially astaxanthin using *P. rhodozyma* with the intent of extracting the carotenoids for use in animal feed. However, by fermenting the animal feed directly, carotenoid-enriched feed can be produced without the need for extraction. By this simple yet novel carotenoid value addition, premium feeds or feed blends can be developed. Apart from carotenoid enrichment, low-fiber DDGS can help expand the market base of DDGS for use in non-ruminant feeds. Carotenoid value addition of DDGS can not only help sustain the biofuel industry but can also capture the aquaculture feed base which heavily relies on astaxanthin supplementation.

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Dedication

To my dear wife Keeru

CHAPTER 1 - An overview of distillers dried grain with solubles and carotenoids

Distillers Dried grain with Solubles (DDGS)

Corn distillers dried grains with solubles (DDGS) is a co-product of fuel ethanol industry and is obtained from dry-grind processing. Typically, one bushel of corn (25.4 kg corn) generates 2.7 gallons (11.8 L) of ethanol, 18 pounds (7.7 kg) of DDGS (non-fermentable residue) and 18 pounds of carbon dioxide (US Grains Council, 2007). A schematic representation of DDGS production by dry-grind processing is outlined in Fig. 1.1. According to the Renewable Fuels Association (Jan 2010), currently in the US, there are 187 ethanol plants in operation with a total ethanol production capacity of 13,028.4 million gallons per year. Additionally, 15 more plants are under construction or in expansion with a capacity of 1,432 million gallons per year. With increasing number of ethanol plants, annual DDGS production has steadily increased over the years (Fig. 1.2).

Nutritional profile

DDGS is rich in nutrients especially protein and energy. Since DDGS is primarily used as animal feed, numerous reports have documented the compositional analysis of DDGS. Data from some of the recent reports are outlined in Table 1.1. There is considerable variability in the nutrient content of DDGS due to many reasons (US Grains Council, 2007), including the inherent differences in the corn varieties, and the differences in the nutrients and blending proportions of condensed distillers solubles and grains (Shurson and Noll, 2005).

Crude fat, crude protein and crude fiber are the chief nutrients in DDGS. About 55% (range 47-69%) of crude protein is made up of ruminally undegradable protein (RUP) and is a

good energy source (Schingoethe, 2006). Large amounts of readily digestible fiber (NDF) are present in DDGS which also contribute to the high energy in DDGS (Schingoethe, 2006). Additionally, DDGS has high phosphorous (0.75-0.89%; www.ddgs.umn.edu, Spiehs et al., 2002; Chapter 4), high sulfur (0.7%, Chapter 4) and low calcium (0.06%; Spiehs et al., 2002). In the absence of standard DDGS composition, a subjective color evaluation is used to grade DDGS, with 'golden' DDGS preferred over darker varieties (Shurson and Noll, 2005). However, color may not be the most accurate indicator of protein quality (Belyea et al., 2004).

Applications of DDGS

DDGS is primarily used as livestock-feed. In fact, the US beef cattle industry is the major consumer of both wet and dried corn distillers co-products (US Grains Council, 2007). To a lesser extent, DDGS is also used as feed for lactating cows, poultry, and swine.

DDGS is an excellent source of protein and energy for beef cattle, and is used at 40-50% of ration dry matter (Schingoethe, 2006; Shurson and Noll, 2005). However, it provides excess protein and phosphorous for finishing feedlot cattle. Due to its high phosphorous content, DDGS can be used as a supplement in forage based diets (US Grains Council, 2007; Shurson and Noll, 2005). DDGS is a good protein source for dairy cattle and used at 20-40% of total dry matter ration along with forage supplements to provide adequate fiber (Schingoethe, 2006; Shurson and Noll, 2005). However, lysine is the first limiting amino acids in DDGS for lactating cows. In swine diets, about 10% DDGS is normally used although higher amounts of up to 50% can be used depending on the growth stage (Shurson and Noll, 2005). Apart from lysine, tryptophan limitation is also seen in diets with more than 10% DDGS. High phosphorous availability in DDGS is ideally suited for swine diets. DDGS at 10-15% are used in poultry diets and provide energy, amino acids and phosphorous (Shurson and Noll, 2005). DDGS contains about 40 ppm

of xanthophylls which can significantly enhance egg yolk color of laying hens and skin color of broilers (Shurson and Noll, 2005). Fish meal is the feed of choice in aquaculture, but compared to DDGS, is expensive, and has more phosphorous and protein resulting in excess nitrogen and phosphorous in fish farm effluents. DDGS supplemented with other plant protein sources like soybean meal or cottonseed meal are being explored as aquaculture feed, and the maximum dietary inclusion rates of DDGS is 10% in salmon to 82% in tilapia with or without lysine and methionine supplementation (US Grains Council, 2007).

In order to sustain the biofuel industry and stabilize the DDGS prices, efforts are underway to improve the quality of DDGS and find additional uses for DDGS. Tucker et al. (2004) by dilute-acid treatment converted the residual starch and fiber of distillers grains (DG) for ethanol production and used the resultant hydrolyzed distillers grains (HDG) with higher protein and lower fiber as poultry feed. Srinivasan et al (2005) developed a high fiber product, and another with low fiber, increased fat and protein by sieving and elutriation of DDGS. The low fiber product has potential application as non-ruminant feed. Additionally, the high protein and fat, low fiber product can fetch higher price (Srinivasan et al., 2006). DDGS has also been evaluated as biofillers in plastics, with DDGS affecting the physical and mechanical properties of molded specimens, and the biodegradability increasing from 0% to 38% with increasing DDGS (Tatara et al., 2007, 2009). Proteins were extracted by aqueous ethanol, alkaline-ethanol and aqueous enzyme treatments of DDGS to obtain a high-value protein-rich product and a carbohydrate-rich residue (Cookman and Glatz, 2009). DDGS, especially with aflatoxin contamination can be used as fertilizer. Nelson et al. (2009) used DDGS as a fertilizer source for corn production, increasing grain yield by 1.41 and 1.56 kg ha^{-1} for every kg ha^{-1} of DDGS applied in medium and high yield environments. Also, DDGS application did not affect the corn

development, SOM, P, K, Ca or Mg concentration or weed control. DDGS as animal feed is the only application of DDGS that has received maximum attention with numerous reviews and studies detailing its effect on animal diets and their products (US Grains Council, 2007). As more ethanol plants are commissioned with increased ethanol production, augmenting current uses and finding new applications of DDGS as value-added animal feed, human foods and manufactured products is the need of the hour (Rosentrater, 2008). Saunders and Rosentrater (2009) surveyed 23 ethanol plants to obtain suggestions from plant managers regarding potential product applications. Some of the suggested ideas include fuels, non-ruminate animal feeds, pelletizing, high protein products, pet and human food, extruded aquaculture feeds, plastics, construction and building materials, corn oil and biodiesel. All these suggestions are promising and may yield tangible results if thoroughly investigated.

Carotenoids

Carotenoids are widely distributed in nature and produced by plants, algae, fungi and bacteria. As many as 600 carotenoids have been isolated and characterized from natural sources (Pfander, 1987). Carotenoids are isoprenoids or terpenoids and are generally C_{40} tetraterpenoids (Fig. 1.3). Hydrocarbon carotenoids are called carotenes (β-carotene, lycopene) and their oxygenated derivatives are xanthophylls (e.g. astaxanthin, lutein, zeaxanthin; Rodriguez-Amaya and Kimura, 2004). Carotenoids exist as a mixture of *cis* and *trans* isomers, with majority in all-*trans* configurations (Rice-Evans et al., 1997), and can be inter converted by thermal, light or chemical energy (Stahls and Sies, 1993). The health benefits of carotenoids in humans and animals are well documented (Duffossé et al., 2005; Surai et al., 2001) of which the pro-vitamin A activity is extensively studied. Only 10% of the 600 carotenoids are known to have pro-vitamin A activity in mammals (Rock, 1997), of which only 10 are significant (Davison et al.,

1993). β-carotene, α-carotene and β–crytoxanthin are the major pro-vitamin A carotenoids (Olson, 1989), among which β-carotene is by far the most important. Astaxanthin has 10 times more antioxidant activity than β-carotene and is 500 times more effective than α-tocopherol (Duffossé et al., 2005 and references therein). Apart from pro-vitamin A activity, carotenoids function as antioxidants, mainly by their ability to quench singlet oxygen and interact with free radicals (Palozza and Krinsky, 1992); anticarcinogens; immunomodulators; natural colorants; cell membrane stabilizers and other functions in fertility (Surai et al., 2001). Castenmiller and West (1998) suggested that carotenoid bioavailability was influenced by nine factors: species of carotenoids, amount of carotenoids consumed, molecular linkage, matrix in which carotenoid is incorporated, compounds affecting absorption and bioconversion, nutrient status of the host, host genetics, and host-nutrient interactions.

Due to growing 'chemophobia' among consumers, natural carotenoids are preferred over synthetic carotenoids. Purified natural β-carotene from *Dunaliella* sp. is accompanied by other carotenoids accounting for 15% of β-carotene (Duffossé et al., 2005). In fact, a mixture of natural carotenoids containing different steroisomers is more beneficial than a single isomer present in synthetic carotenoids (Ben-Amotz and Levy, 1996). Synthetic carotenoids, on the other hand are exclusively made up of all-*trans* isomers (Surai et al., 2001). Low dosages of natural astaxanthin or β-carotene are as potent as synthetic carotenoids (An et al., 2004; Ben-Amotz et al., 1988a, b). Synthetic carotenoids instead of providing the health benefits can sometimes be harmful. Synthetic all-*trans* β-carotene can possibly lead to carcinogenicity in male smokers (The Alpha-tocopherol, Beta-carotene Cancer Prevention Study Group, 1994). Fish pigmentation also varies depending on natural or synthetic dietary carotenoids (Kop et al., 2010). Other beneficial effects of natural carotenoids from red yeasts are reviewed by Frengova

and Beshkova (2009). In spite of all these benefits, synthetic carotenoids seem to be preferred especially in animal diets as they are cheaper. Natural β-carotene and astaxanthin are priced at $2,000/ kg and $7,000/kg respectively, whereas synthetic β-carotene and astaxanthin cost about $800/kg and $2000/kg respectively (Caswell and Zilberman, 2000; www.algatech.com).

BCC Research (2005) estimated the worldwide market value of all commercially used carotenoids to cross $1 billion by 2009, with astaxanthin and β-carotene market shares of $257 and $253 million, respectively. Other important carotenoids include lutein, canthaxanthin and other minor carotenoids with market shares of $187, $156 and $170 million respectively. β-carotene is used primarily in foods followed by feeds, to improve fish, broiler skin and egg color, astaxanthin and canthaxanthin in aquaculture feed, and lutein to color egg yolks and broiler skin (BCC Research 2005). The market share is controlled predominantly by synthetic carotenoids.

Commercial production of natural astaxanthin and β-carotene is mostly achieved by microalgae *Dunaliella* sp. and *Hematococcus pluvialis*, respectively (Duffossé et al., 2005). Astaxanthin and other nutrients from *P. rhodozyma* fermentation are also commercially available and used in salmonid feed (Frengova and Beshkova, 2009).

Carotenoids and Animal health

Usually animal feeds are poor in carotenoids (Nys, 2000: Holden et al., 1999) and are added as feed supplements. Animals are incapable of producing carotenoids but are able to assimilate the ingested carotenoids (Eonseon et al., 2003). Carotenoids are beneficial to animals as they i) act as antioxidants and precursors of vitamin-A (Yang and Tume, 1993), ii) improve cell communication and enhance immune response in ruminants (van den Berg et al., 2000) and dogs (Chew et al., 2000), iii) reduce incidence of mastitis in dairy cows (Chew, 1995), iv) assimilate into milk as vitamin-A, thereby improving the keeping quality of milk (Noziere et al.,

2006), and v) improve reproductive efficiency (Chew, 1995; Hurley and Doane, 1989). Astaxanthin is vital in aquaculture feed: it improves the egg quality and fry survival, protects against oxidation of lipids in salmon which contain high levels of polyunsaturated fatty acids, has pro-vitamin A activity, improves fish liver histology and improves shrimp and prawn survival rates (Sanderson and Jolly, 1994 and references therein). Additionally, Amar et al. (2004) found that innate defense mechanisms of fish were modulated by dietary carotenoids from *P. rhodozyma* and *Dunaliella salina*.

The recommended dosages of carotenoids are 1-50 mg/day to enhance immune response (Hayek, 2000), 40mg astaxanthin/ kg feed in egg laying hens to enhance color of egg yolk and flesh of poultry (An et al., 2006), 40-70 mg astaxanthin /kg of feed (Decker et al., 2000), or 30-120mg/kg of total carotenoids (Venugopal, 2009) in aquaculture.

Carotenoid production by yeasts

Carotenoid production by yeasts namely, *Phaffia rhodozyma*, *Rhodotorula* sp., *Sporobolomyces roseus*, and their teleomorphs namely *Xanthophyllomyces dendrorhus* Golubev, *Rhodosporidium*, and *Sporidiobolus* respectively, and *Candida utilis* is documented. Among these, astaxanthin production by *P. rhodozyma* has received most attention and is the subject of numerous reviews and patents.

Phaffia rhodozyma M.W. Miller, Yoneyama & Soneda 1976 was originally isolated from slime exudates of Betulaceae from Japan and Pacific Northwest region of North America, but has since been isolated from other locations (Lukács et al., 2006). It is the only known red yeast that produces astaxanthin (Weber and Davoli, 2003). In fact, astaxanthin contributes to 80-90% of its total carotenoids (Tinoi et al., 2006). Carotenoids are produced during late log phase or stationary phase (Johnson and Lewis, 1979) by the mevalonate isoprenoid pathway. Andrewes et

al (1976) provided the first scheme for astaxanthin production (Fig. 1.4). Frengova and Beshkova (2009) have reviewed the astaxanthin yields of *P. rhodozyma* on both synthetic media and agricultural substrates: the yields have been highly variable ranging from 174 µg/g on Eucalyptus hydrolysates (Cruz and Parajo, 1998) to 7200µg/g on hydrolyzed corn syrup (Jacobson et al., 2000), with intermittent production on various substrates. The variability in yield is due to the inherent variability in the *P. rhodozyma* strains used and/or the carbon source in the media (Ngheim et al., 2009). Palágyi et al. (2001) evaluated the ability of 11 *P. rhodozyma* strains to utilize 99 different compounds as the sole carbon source. Overall, an exhaustive list of substrates has been evaluated for carotenoid production by *P. rhodozyma*. Physical factors like temperature, aeration, pH, light and media components like C source, C/N ratio, minerals, and nitrogen source affecting carotenoid production have also been evaluated extensively (see review by Frengova and Beshkova, 2009).

Sporobolomyces roseus Kluyver & van Niel 1924 has a worldwide distribution and is commonly found on phylloplanes of different types of plants and has been isolated from other substrates like air, water and skin of humans and animals (Valério et al., 2008; Davoli and Weber, 2002 and references therein). The major carotenoids produced by *S. roseus* are β-carotene, torulene and torularhodin (Davoli and Weber, 2002). About 82 µg/g total carotenoids was produced by *S. roseus* with 33 µg/g of torulene, 23 µg/g torularhodin and 12 µg/g β-carotene along with other minor carotenoids on glucose-yeast extract synthetic medium (Buzzini et al., 2007). However, about 412 µg/ g of total carotenoids was produced by another strain of *S. roseus* on yeast extract-dextrose medium (Davoli et al., 2004). Total carotenoid production by three species of *Sporidiobolus* were variable ranging from 34 to 184 µg/ g of yeast dry mass (Buzzini et al., 2007). Typically, β-carotene yield by *S. roseus* on yeast extract based synthetic medium

has ranged from as low as 11.8 µg per gram of yeast cells (Buzzini et al., 2007) to 230 µg/g (Yurkov et al., 2008), with intermittent production of 101 µg/g (Davoli et al. 2004) and 118 µg/L on YM broth (Maldonade et al., 2008).

Co-cultivation of microbes

Mixed culture fermentation or co-cultivation has been often employed for enhanced carotenoid production or effective substrate utilization or both. For effective substrate utilization, Frengova et al. (1994) cultivated lactose negative *Rhodotorula glutinis* with lactose fermenting bacteria, *Lactobacillus helviticus* on whey ultrafiltrate. The *Lactobacillus* converts lactose to lactic acid which can be used by *R. glutinis*. About 268 µg/g dry cells of total carotenoids was produced by *R. glutinis*, of which 182 µg/g was torularhodin, 44 µg/g of β-carotene and 23 µg/g of torulene. Co-cultivation of these two organisms can also yield caroteno-protein and exopolysaccharide (Frengova et al., 1997). Similarly, high β-carotene producer *Rhodotorula rubra* was co-cultivated with *Lactobacillus casei* on whey ultrafiltrate (Frengova et al., 2003). Oligosaccharides and dextrins of low hydrolyzed corn syrup can be hydrolyzed to maltose and glucose by starch-assimilating yeast *Debaryomyces castellii* and the sugars can be utilized by *R. glutinis* for carotenoid production (Buzzini, 2001). Under co-cultivation, *R. glutinis* produced three times the total carotenoid yield compared to its monoculture.

For enhanced carotenoid production, Dong and Zhao (2004) co-cultivated two astaxanthin overproducing strains namely *P. rhodozyma* and microalga *Haematococcus pluvialis* and found that astaxanthin yield was greater compared to that in monocultures of the two organisms. The higher yield was attributed to algal utilization of yeast CO_2 and yeast utilization of algal O_2. Similarly, co-cultivation of an *Aspergillus sp*. or the incorporation of its dried extract (80 µg/ml) into the fermentation of *Phycomyces blakesleeanus* resulted in a 5-fold increase of β-

carotene (Margalith, 1993). In lieu of co-cultivation, addition of fungal elicitors has enhanced carotenoid production. *Epicoccum nigrum* extract was used to enhance astaxanthin production of *X. dendrorhus* (Echavarri-Erasun and Johnson, 2004). Of the six fungal elicitors tested, extracts from *R. glutinis* and *R. rubra* showed greatest improvement in astaxanthin production of *X. dendrorhous* (Wang et al., 2006). Addition of regular yeast extract to the fermentation of high astaxanthin producing industrial strain of *P. rhodozyma* improved astaxanthin production (Nghiem et al., 2009; Meyer and du Preez, 1994). Though the specific carotenoid triggering mechanism is unknown, it is believed that some of the biochemical intermediates of red yeasts and *Aspergillus* may serve as precursors in carotenoid producing microbes. Co-cultivation of microbes is thought to improve yield due to 1) efficient substrate utilization by both microbes, 2) compatibility of microbes, and 3) product of one microbe being used by the other as precursor or elicitor.

Co-cultivation has also been used to disrupt yeast cell wall to make the carotenoids available for animal absorption. Okagbue and Lewis (1983) co-cultivated *Bacillus circulans* and *P. rhodozyma* on yeast nitrogen base (YNB) medium supplemented with 10 different carbohydrates or sugar sources and evaluated the effect of lytic enzyme produced by *Bacillus* on yeast cell wall disruption. Sucrose supported the best astaxanthin production of 1.43 μg/ml and the best extractability of 96.5%. Similarly, 97% extractability of total carotenoids was achieved when the same co-cultivation was conducted using two-stage batch fermentation (Fang and Wang, 2002). For the sake of comparison, schematic production of carotenoids from synthetic route is also provided (Fig. 1.5).

DDGS as a substrate for carotenoid production

Carotenoids, especially astaxanthin production from *P. rhodozyma* have been evaluated on numerous substrates in an effort to find a low-cost medium for optimal astaxanthin production. This has been coupled with strain improvement, screening for high yielding strains, media optimization, and metabolic engineering to obtain maximum carotenoid yield (see reviews by Frengova and Beshkova, 2009; Lukács et al., 2006). The ultimate goal of all these studies was to produce astaxanthin, extract the same, and use the product primarily as feed supplement along with other food applications. To reduce the cost of the product, it would be ideal to directly produce the carotenoids on the animal feed, thus avoiding the expensive extraction steps, and the use of corrosive chemicals. Since carotenoids from *P. rhodozyma* have been successfully produced on corn thin stillage and other products of corn wet-milling (Hayman et al., 1995), red yeast fermentation of corn whole stillage and/or DDGS predominantly used as an inexpensive animal feed, can provide carotenoid-enriched animal feed. If the yields are higher than the recommended dietary dosage of carotenoids, the enriched product can be used to make feed blends. The proposed (Fig. 1.4) carotenoid enrichment allows a novel, yet simple value-addition to DDGS and can help sustain the biofuel industry. If the proof-of-concept is established in DDGS, then similar products can be developed from other animal feeds like rice bran, wheat bran and soybean products.

Table 1.1 Nutrition profile of DDGS

Study	Details	%Crude fat	%Crude protein	%Crude fiber	%ADF	%NDF	%Ash
Chapter 4	Abengoa, KS (2009)	14.59	27.77	5.31	7	22.25	
Saunders & Rosentrater, 2009	Average of 5 plants	10.3	27.41	13.51	11.53	Na	4.71
Kim et al., 2008	Big River Resources LLC (West Burlington IA); Forage/feed nutritional compositional analyses	14.5	27.3	13.5			4.7
Kleinschmit et al., 2006			30.3		16	44	4.58
Belyea et al., 2004	Samples from DG ethanol plant, MN; average of 5 year sampling 1997-2000	11.9	31.3	10.2	17.2		4.6
Spiehs et al., 2002	Average of 118 samples from 10 plants (8MN, 2SD) from 1997-1999	10.9	30.2	8.8	16.2	42.1	5.8
Cromwell et al., 1993	Average from 9 plants, 7 beverage alcohol (IA), and 2 fuel alcohol (KY, OH)	10	26.9		14.4	35.1	4.8
www.ddgs.umn.edu	Averages from 32 US corn DDGS sources	10.7	30.9	7.2			6.0

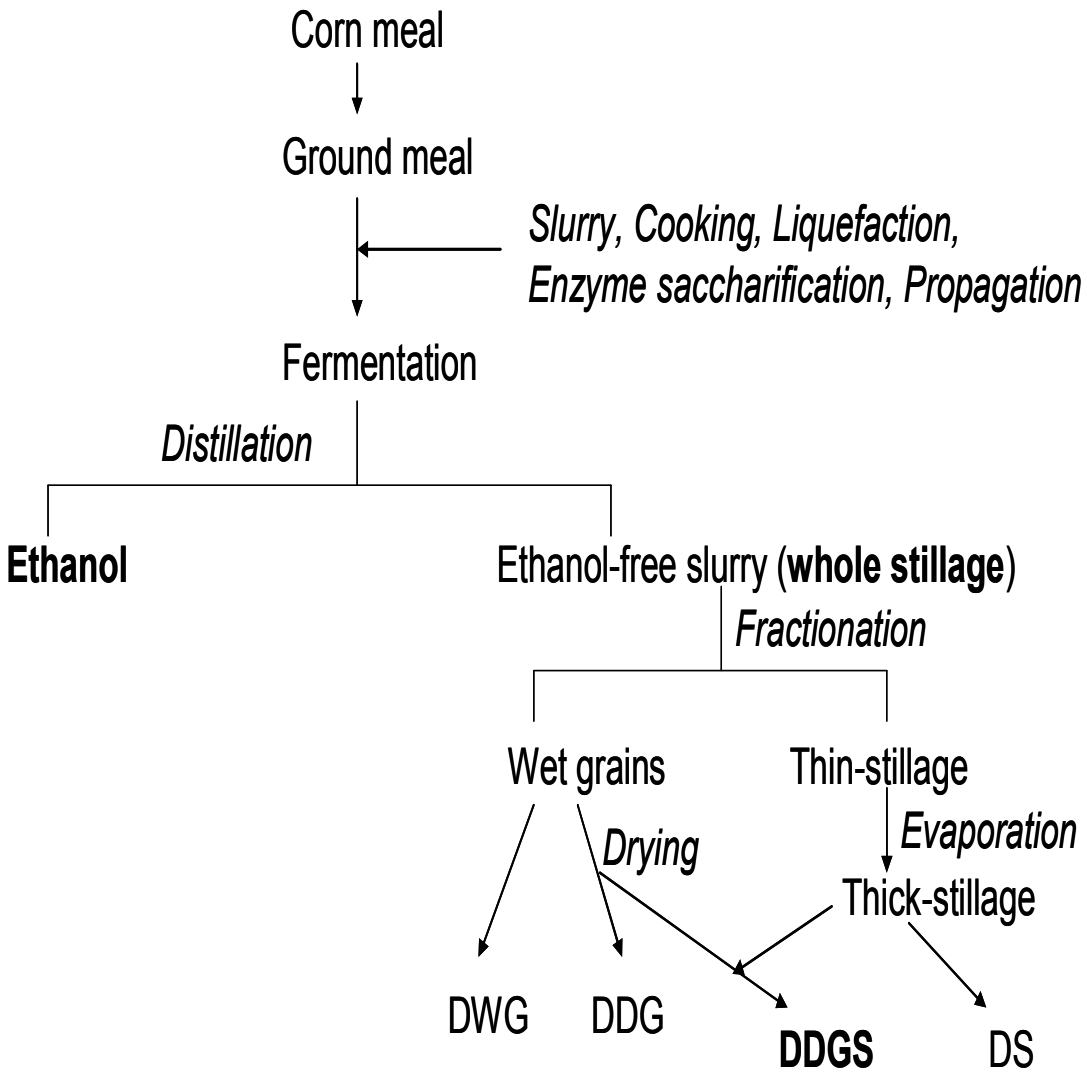

Figure 1.1 Schematic of ethanol dry-grind processing.

Modified from Rosentrater (2008).

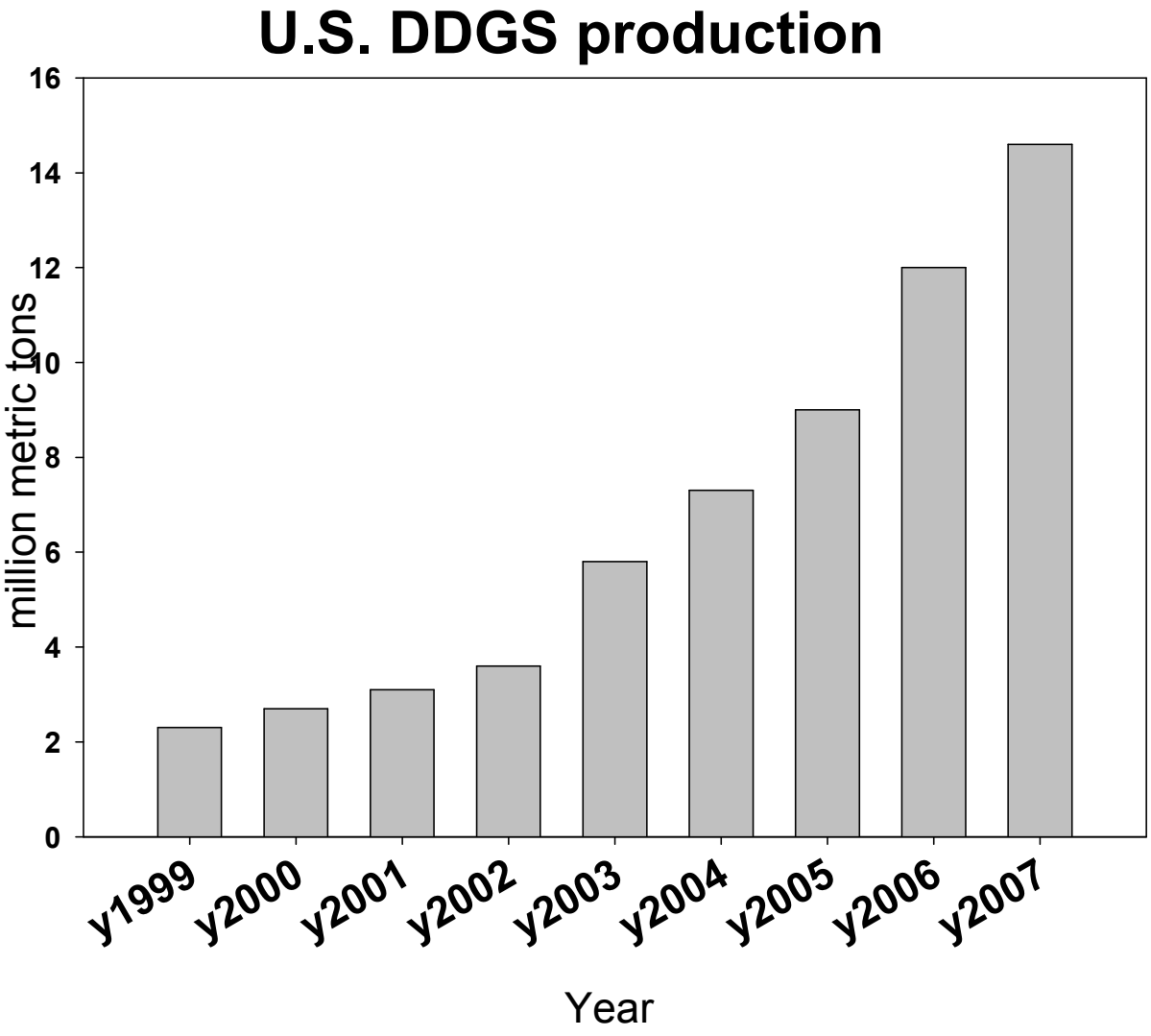

Figure 1.2 Annual DDGS production in the U.S. Source: Renewable Fuels Association (2008).

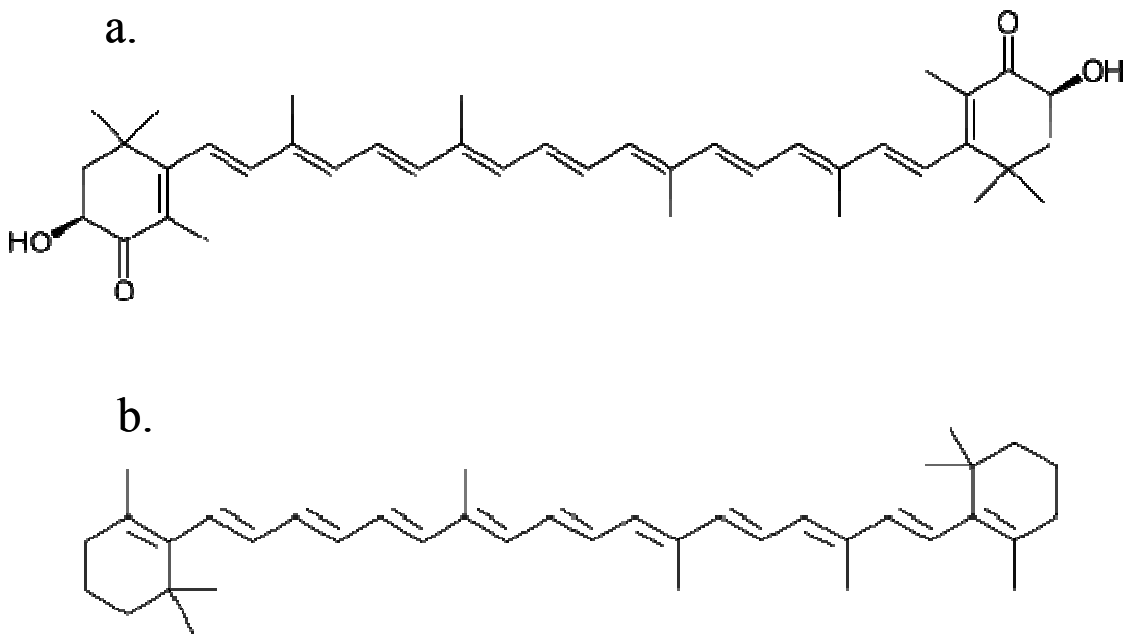

Figure 1.3 Structure of carotenoids.

a. Astaxanthin b. Beta-carotene. Source: Wikipedia (http://en.wikipedia.org/wiki/File:Astaxanthin.svg, http://en.wikipedia.org/wiki/File:Beta-carotene-2D-skeletal.svg)

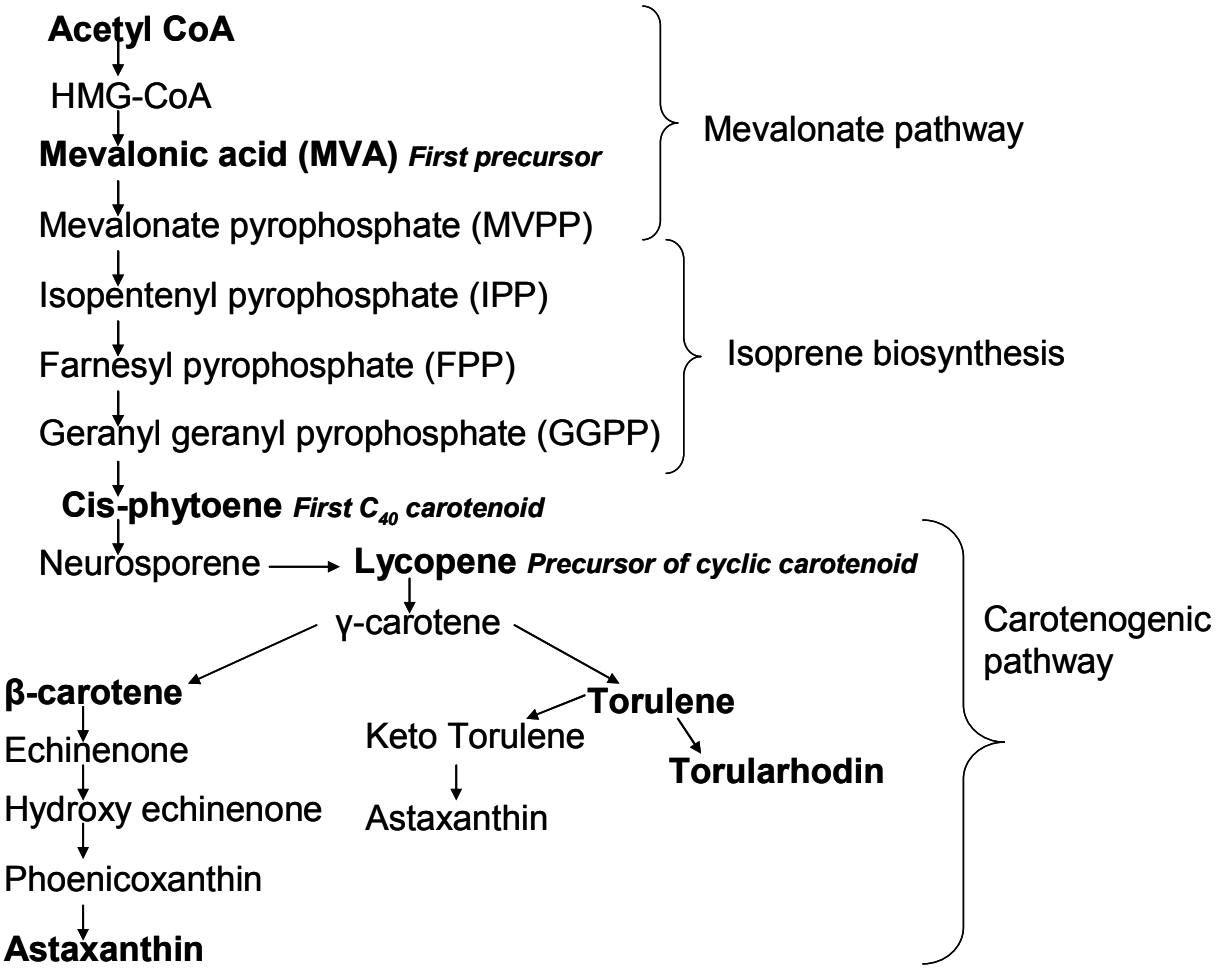

Figure 1.4 Schematic of carotenoid production in *P. rhodozyma*, *S. roseus* and *Rhodotorula* sp.

Adapted and modified from Frengova and Beshkova (2009) and Andrewes et al. (1976).

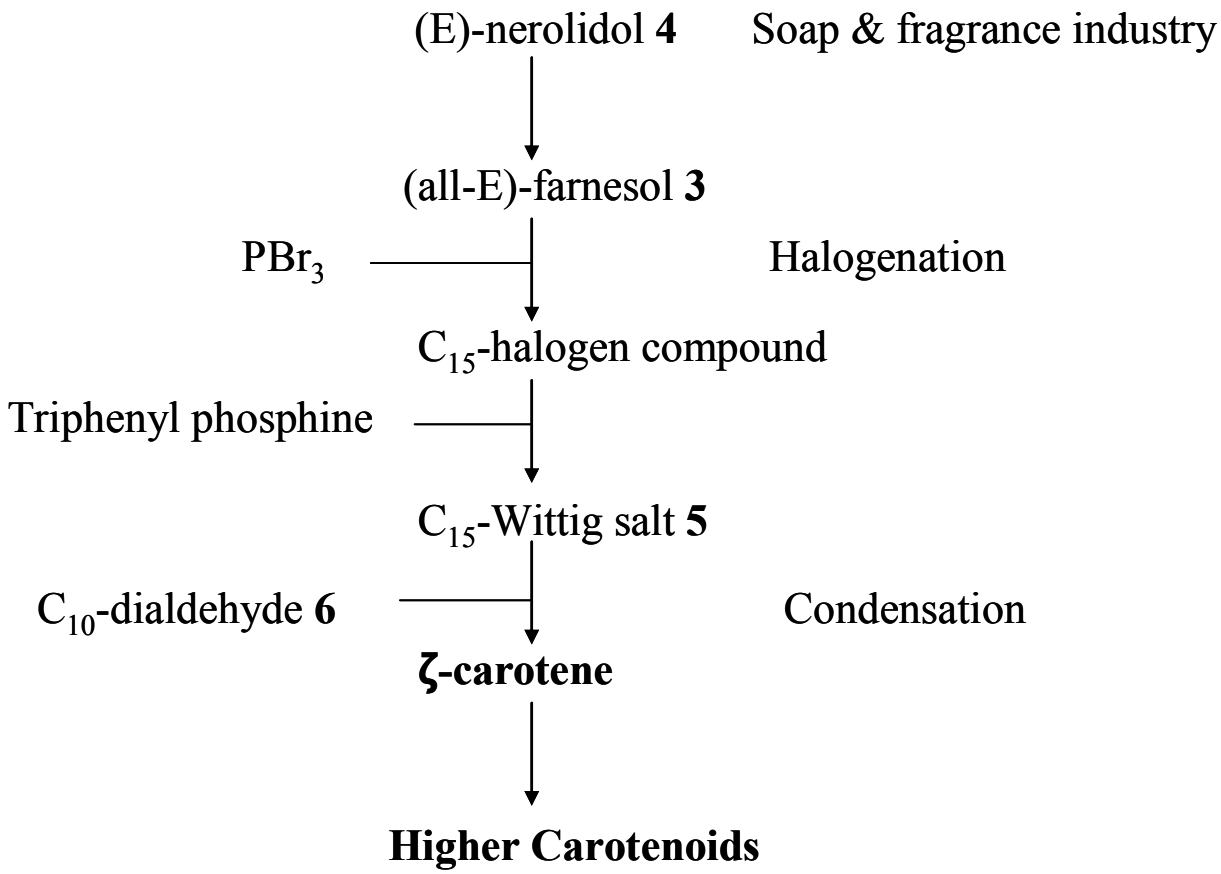

Figure 1.5 Schematic of carotenoid production by synthetic route.

Adapted from Fujita et al 1975.

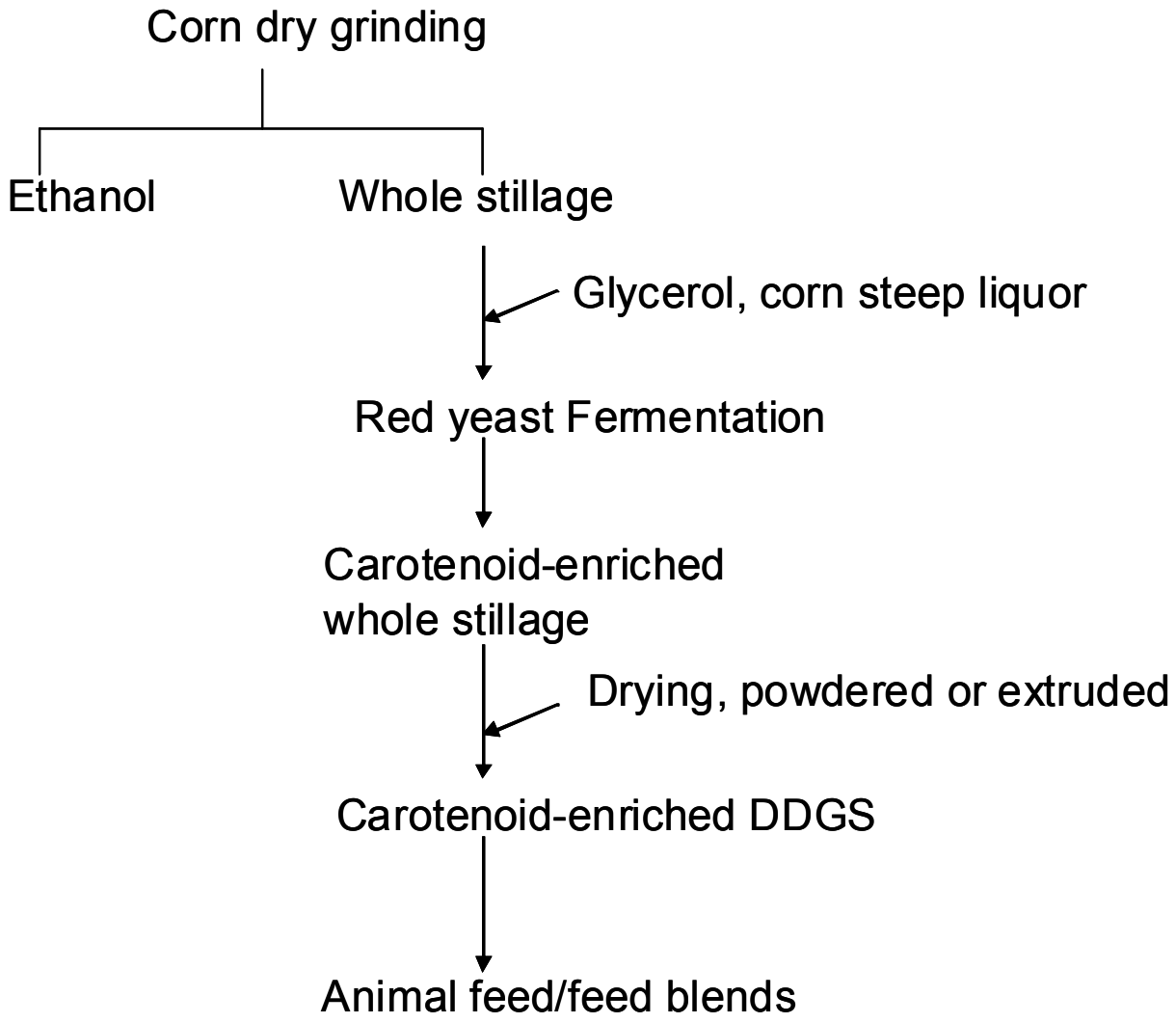

Figure 1.6 Proposed carotenoid value addition to DDGS.

CHAPTER 2 - [1]Production of carotenoid-enriched Distillers Dried Grains with Solubles (DDGS) by *Phaffia rhodozyma* and *Sporobolomyces roseus* fermentation of whole stillage

Abstract

Whole stillage a co-product of grain-based ethanol is used as an animal feed in the form of dried distillers grain with solubles (DDGS). Carotenoids are expensive yet essential feed additives. Since animals cannot synthesize carotenoids and animal feed is generally poor in carotenoids, about 30-120 ppm of total carotenoids is added to animal feed to improve animal health and enhance meat color and quality, and vitamin-A levels in milk and meat. The main objective of this study was to produce carotenoid (astaxanthin and β-carotene)-enriched DDGS by submerged fermentation of whole stillage. Mono- and mixed cultures of red yeasts, *Phaffia rhodozyma* (ATCC 24202) and *Sporobolomyces roseus* (ATCC 28988) were used to produce astaxanthin and β-carotene. The astaxanthin and β-carotene yields in mixed culture and *P. rhodozyma* monoculture were 17.4 and 187.9, and 35.7 and 104.7 μg/g, respectively, while *S. roseus* produced 232.9 μg/g of β-carotene. This study shows that whole stillage is an excellent substrate for carotenoid production. Furthermore, mixed culture fermentation seems more valuable than monoculture fermentation in terms of providing higher amount of total carotenoids. Since the carotenoid yields are in the range used in animal feed, the carotenoid-enriched DDGS has potential application as 'value-added animal feed'.

Introduction

[1] Chapter 2 is published as a part of Ananda and Vadlani (2010) Journal of Industrial Microbiology and Biotechnology 37:1183-1192

Distillers Dried grain with Solubles (DDGS) is a co-product of grain-based ethanol. With a three-fold increase in the number of ethanol plants in the US (Renewable Fuels Association, Jan 2009), production of ethanol co-products has also increased with DDGS production around 10 million metric tons (Shurson and Noll, 2005). DDGS is used as livestock feed since it is rich in fiber, protein, water-soluble vitamins and minerals (Schingoethe, 2006). During ethanol fermentation of corn, *Saccharomyces cerevisiae* utilizes glucose derived from corn starch, leaving the fiber untouched. In fact, the fiber concentration in DDGS is enhanced by a factor of three compared to corn (Shurson and Noll, 2005). Due to its nutrition profile, whole stillage makes an excellent substrate for secondary fermentation. Abundant production of whole stillage and/or DDGS offers unlimited opportunities for value-addition, with subsequent utilization of the value-added product in animal feed, human food and manufactured products (Rosentrater, 2008).

Usually animal feeds are poor in carotenoids (Holden et al., 1999; Nys 2000) and DDGS is no exception. Animals are incapable of producing carotenoids but are able to assimilate the ingested carotenoids (Eonseon et al., 2003). Carotenoids are beneficial to animals as they confer many health benefits (Chapter 1). Astaxanthin and β-carotene are important carotenoids in animal feed, especially in aquaculture and poultry. The recommended dosages are between 1 to 120 mg/day (Venugopal, 2009; An et al., 2006; Decker, 2000; Hayek, 2000). Whole stillage though abundantly produced has not been used as a substrate for carotenoid production.

Many red yeasts and filamentous fungi produce carotenoids. Astaxanthin is commonly produced by red yeast *Xanthophyllomyces dendrorhous* or *Phaffia rhodozyma* on various substrates (see review by Frengova and Beshkova, 2009) and it contributes to 80-90% of its total carotenoids (Tinoi et al., 2006). β-carotene is also produced by *P. rhodozyma*. However, yeasts

like *Rhodotorula glutinis* and *Sporobolomyces roseus* produce abundant β-carotene (Maldonade et al., 2008).

Apart from monoculture fermentation, mixed culture fermentation or co-cultivation of microorganisms has also been employed for enhanced carotenoid production (Chapter 1).

Hypothesis 1.1: Monocultures of *P. rhodozyma* and *S. roseus* can produce carotenoids on whole stillage. Co-cultivation of yeasts, *P. rhodozyma* and *S. roseus* on corn whole stillage will allow the production of carotenoid-enriched DDGS, rich in both astaxanthin and β-carotene.

Hypothesis 1.2: Co-cultivation would enhance the carotenoid yields of respective red yeasts due to stimulatory effects of the co-cultured yeast.

Specifically, the objectives of this study were to produce carotenoid-enriched whole stillage by monoculture and mixed culture fermentation of *P. rhodozyma* and *S. roseus*. Additionally, carotenoid fermentation in synthetic medium will also be carried out.

Materials and methods

Microbial cultures

Lyophilized cultures of *P. rhodozyma* (ATCC 24202) and *S. roseus* (ATCC 28988) were obtained form American Type Culture Collection (ATCC, Manassas, VA), revived on yeast extract malt extract agar (YMA) and incubated at 18°C for 10 d. After revival, cultures were inoculated into yeast extract malt extract broth (YMB) and incubated at 18°C on an orbital shaker at 180 rpm for five days. Cultures were then inoculated on YMA slants, incubated for 10 d and later stored at –80°C for long term preservation. Additionally, yeast cells from YMB were centrifuged and re-suspended in 20% glycerol and stored at –80°C in one ml aliquots. For routine experiments freshly prepared slants were used.

Phaffia rhodozyma ATCC 24202 is a known carotenoid producer along with xylose metabolizing ability (Ngheim et al., 2009; Vasquez et al., 1997). Since DDGS is rich in fiber and *P. rhodozyma* is known to degrade corn fiber (Leathers, 2003; Hayman et al., 1995), a *P. rhodozyma* strain that not only produces astaxanthin but also metabolizes corn fiber was chosen.

Inoculum generation

From each fungal strain, a loopful of cells from respective slants was inoculated into sterile 100 ml YMB in 500 ml flasks. Flasks were incubated at 18°C, 180 rpm for 72 h. Development of orange and red color in *P. rhodozyma* and *S. roseus* flasks, respectively, indicated good fungal growth. A 10% (v/v) inoculum was used for monoculture fermentation, while 5% of each strain was used in mixed culture fermentation.

Media preparation

Corn whole stillage was procured from Abengoa Bioenergy (Colwich, KS, USA). Apart from whole stillage, the medium consisted of glycerol and corn steep liquor. The supplementation with glycerol and corn steep liquor was considered necessary as 1) whole stillage is poor in readily utilizable sugars and addition of glycerol and corn steep liquor provide readily available carbon, and reduce the lag phase, 2) glycerol can act as a carbon source for astaxanthin production by *P. rhodozyma* (Kusdiyantini et al., 1998) and β-carotene production by *B. trispora* (Mantzouridou et al., 2008), 3) carotenoid production is increased by the balanced and increased formation of acetyl Co-A, pyruvate and glyceraldehyde-3-phosphate, all of which can be produced by glycolysis of glycerol (Das et al., 2007), and 4) glycerol is a cheap and abundantly produced co-product of biodiesel and soap industry, and evaluated as effective supplements for β-carotene production by *B. trispora* (Mantzouridou et al., 2008).

Whole stillage medium: A liter of the fermentation medium contained 25% (w/v) whole stillage, 2% corn steep liquor, 5% glycerol and minerals: 1g KH_2PO_4, 0.5g $MgSO_4$, 0.5g $MnSO_4$ and $ZnSO_4$. Medium pH was about 6.0 before sterilization and was not adjusted any further since pH 6 is ideal for the growth of P. rhodozyma (Meyer and du Preez, 1994).

Synthetic medium: Modified medium composition of Kusdiyantini et al (1998) was used. Briefly, a liter of the medium contained 1% yeast extract, 1% soy peptone, 2.7% glycerol, traces of KH_2PO_4, $MgSO_4$, $ZnSO_4$ and pH was adjusted to 6.0 before sterilization. About 50ml of respective media in 250ml flasks were sterilized at 121°C for 30min.

Fermentation conditions

Submerged fermentation of *P. rhodozyma* and *S. roseus* mono- and mixed cultures were conducted. Flasks were inoculated and incubated at 18°C, 180 rpm for nine days. Control flasks without inocula for both media were maintained. Two replicates per treatment were employed. Samples were harvested on 5^{th}, 7^{th} and 9^{th} day of fermentation, centrifuged and the supernatant discarded. Pellets were freeze dried for 24 h and stored at –80°C until further analyses. In case of synthetic medium, pellet consisted of yeast cells only, while the pellet in whole stillage was a mixture of yeast cells and solids from whole stillage.

Extraction, quantification and identification of carotenoids

Known quantity of freeze dried sample was weighed into a mortar, 0.2 g of acid washed sand (40-100 mesh size) added and carotenoids extracted by grinding the mixture in dichloromethane solvent. Samples were centrifuged at 5000 rpm for 5 min and supernatant filtered into 1.5 ml HPLC vials using 0.2 μm filters.

High performance liquid chromatography (HPLC) was used for quantification of carotenoids. Astaxanthin and β-carotene standards were obtained from Sigma Aldrich (St Louis,

MO, USA). A Shimadzu HPLC equipped with LC-20AB pump, SIL -20AC auto sampler, SPD-M20A PDA detector and CTO-20A column oven was used. Phenomenex Prodigy C_{18} column (150 mm length and 4.6mm internal diameter) along with a C_{18} guard column was used for the separation of carotenoids. Acetonitrile and methanol (80:20) was used as the mobile phase. Flow rate was maintained at 2.0 ml/min and the column was maintained at 40°C. About 20 µl of the sample was injected using autosampler. HPLC data was acquired using Lab Solutions software. Carotenoid yield was expressed as µg/g of freeze dried whole stillage sample instead of yield per gram of yeast dry weight as it was impossible to separate yeast cells from the whole stillage solids. Total carotenoids were calculated as the sum of astaxanthin and β-carotene yields.

Mass Spectroscopy (MS) of Carotenoids

To confirm HPLC detection of astaxanthin and β-carotene, samples of mono and mixed culture fermentation of whole stillage were subjected to MS analyses.

About 2 µL of the sample was mixed with 8 µL of 30 mg/mL super dihydroxybutyrate (DHB) (Bruker Daltonics, Germany) dissolved in 33% acetonitrile/ 0.1% trifluoroacetic acid, and 2 µL of this mixture was applied to Bruker aluminum target plate for MALDI/TOF and TOF/TOF analyses. Mass spectra and tandem mass spectra were obtained on a Bluker Ultraflex II TOF/TOF mass spectrometer. Positively charged ions were analyzed in the reflector mode. MS spectra were analyzed with Flex analysis 3.0 software (Bruker Daltonics). Measurements were externally calibrated with eight different peptides ranging from 757.39 to 3147.47 (Peptide Calibration Standard I, Bruker Daltonics).

Statistical analyses

Data were analyzed using SAS (version 9.1.3). PROC GLM was used to compare multiple treatments and when necessary pair-wise comparisons were made using Tukey-Kramer at P=0.05.

Results

Synthetic medium was used as a baseline to evaluate carotenoid production of the two red yeasts. However, it is emphasized that the carotenoid production on synthetic and whole stillage media are incomparable as the yields were evaluated on different scales (see below). The pH profiles for carotenoid fermentation on different media are provided in Fig.2.1.

Predictably, both *P. rhodozyma* monoculture and mixed culture fermentations produced astaxanthin and β-carotene, while *S. roseus* monoculture produced only β-carotene.

Production profile of astaxanthin and β-carotene

The ANOVA statistics for carotenoid production profile are provided in table 2.1. Astaxanthin yield on days 5, 7 and 9 of fermentation by *P. rhodozyma* and mixed culture respectively showed an increasing trend in both media. Astaxanthin yield in *P. rhodozyma* fermentation did not vary over time in whole stillage medium (Table 2.2), but mixed culture fermentation yield on day 9 was significantly greater than that on days 5 and 7 (Table 2.2). The astaxanthin yield from *P. rhodozyma* and mixed culture respectively did not vary significantly on synthetic medium (Table 2.2).

On whole stillage medium, β-carotene yields in all three treatments showed an increasing trend: *S. roseus* yields on days 5, 7 and 9 were significantly different from each other, *P. rhodozyma* did not vary significantly and yield from mixed culture fermentation was the greatest on day 9 and significantly different from that on days 5 and 7 (Table 2.2). On synthetic medium

(Table 2.2), β-carotene yield on days 5, 7 and 9 of fermentation by *P. rhodozyma* showed an increasing trend and the yields were significantly different from each other. The β-carotene yield by *S. roseus* was the highest on day 7 and decreased on day 9 but did not vary significantly (Table 2.2). Mixed culture fermentation showed an increasing trend, with yields on days 7 and 9 being significantly greater than that on day 5.

In whole stillage, total carotenoid production in mixed culture and *S. roseus* monoculture were similar but were significantly greater than that in *P. rhodozyma* (Table 2.2). However, on synthetic medium, total carotenoid production in mixed culture and *P. rhodozyma* monoculture were similar but were significantly greater than that in *S. roseus* (Table 2.2).

Monoculture versus mixed culture

In both media, overall astaxanthin yield in *P. rhodozyma* monoculture was significantly greater than that in mixed culture fermentation (Table 2.2). Overall production of β-carotene varied both in whole stillage and synthetic media (Table 2.2). In whole stillage medium, β-carotene yield in *S. roseus* monoculture and mixed culture fermentation were similar and both were significantly greater than that in *P. rhodozyma* monoculture (Table 2.2). However, in synthetic medium, β-carotene yield in *P. rhodozyma* monoculture and mixed culture fermentation were similar and both were significantly greater than that in *S. roseus* monoculture (Table 2.2).

Mass spectrometry of carotenoids

MALDI/TOF mass spectroscopy positively identified astaxanthin and β-carotene in all the tested samples. MS spectrum from mixed culture fermentation is shown (Fig. 2.2). While the DHB matrix assisted mixture showed good detection of astaxanthin in all the tested samples and standard, β-carotene signals were poor in the samples as well as in the standard.

Discussion

This study demonstrated the successful production of carotenoid-enriched whole stillage rich in both astaxanthin and β-carotene, supporting hypothesis 1.1. However, hypothesis 1.2 was not supported. Over-production of astaxanthin or β-carotene in mixed culture fermentation was not observed suggesting a lack of stimulatory effect of either yeast on carotenoid production of the co-cultivated yeast. However, mixed culture yielded the highest amount of total carotenoids. Since the carotenoid levels in carotenoid-enriched whole stillage were in the range that is generally used in animal feed, carotenoid-enriched DDGS has potential application as 'value-added animal feed'.

Astaxanthin

Wild-type strains of *P. rhodozyma* typically yield around 200-300 µg/g of yeast of astaxanthin (Johnson, 2003). A pentose utilizing strain was used instead of an astaxanthin overproducing strain of *P. rhodozyma*. Compared to the published estimates, astaxanthin yield in DDGS may appear low. However, as mentioned earlier the yield was calculated per gram of freeze dried whole stillage and not per gram of yeast cells as seen in most studies, leading to an underestimation of the yield. Frengova and Beshkova (2009) have reviewed the astaxanthin yields of *P. rhodozyma* on both synthetic media and agricultural substrates: the yields have been highly variable ranging from 174 µg/g on Eucalyptus hydrolysates (Cruz and Parajo, 1998) to 7200µg/g on hydrolyzed corn syrup (Jacobson et al., 2000), with intermittent production on various substrates. The variability in yield may be due to the inherent variability in the *P. rhodozyma* strains used and/or the carbon source in the media (Ngheim et al., 2009). It should be noted that in most studies yield was recorded at optimal fermentation conditions, unlike this study where the process is yet to be optimized. As far as utilizing biofuel co-products go,

Hayman et al. (1995) evaluated six co-products of corn wet-milling for astaxanthin production by *P. rhodozyma* and found that thin stillage and corn condensed distillers solubles (CCDS) supported maximum yield of 4.1 and 3.1 µg/ml respectively. The evaluated co-products are rich in corn fiber, arabinoxylan, a complex cross-linked structure not easily degraded by enzymes. Their study clearly demonstrated the ability of *P. rhodozyma* to degrade corn fiber without any pre-treatment of the substrates. Ngheim et al. (2009) also evaluated corn fiber for astaxanthin production by *P. rhodozyma*, but the corn fiber was pre-treated with enzymatic degradation to yield the respective sugars. Incidentally, arabinose gave the highest astaxanthin yield.

In the case of synthetic medium, Kusdiyantini et al. (1998) reported astaxanthin yield of 33.7 mg/L on 3.78%, and 27.7 mg/L on 2.88% glycerol medium supplemented with YE and peptone. Surprisingly, in this study, the yield was ten times lesser at 120 µg/g on similar synthetic medium (2.7% glycerol medium + 1%YE). It is believed that this may due to the inherent variability in the *P. rhodozyma* strains used.

Contrary to improved astaxanthin yield in mixed culture fermentation (Dong et al., 2006; Dong and Zhao, 2004), the astaxanthin yield in mixed culture fermentation of DDGS was lower than that in *P. rhodozyma* monoculture. This is interesting since *S. roseus* did not produce astaxanthin and mixed culture fermentation should have been a reflection of *P. rhodozyma* monoculture. The yield reduction may be due to the 1) competition for carbon and other resources by two organisms occupying the same niche, 2) lower aeration due to growth of two organisms affecting astaxanthin production, and 3) slower growth of *P. rhodozyma* compared to *S. roseus*. Since astaxanthin is very sensitive to aeration, it is plausible that growth of two organisms severely affected the oxygen levels affecting astaxanthin production. Since fungal extracts are known to enhance astaxanthin yield of *P. rhodozyma* (Wang et al., 2006; Echavarri-

Erasun and Johnson, 2004; Margalith, 1993), it will be interesting to evaluate the effect of *S. roseus* culture extract on the astaxanthin production of *P. rhodozyma*.

β-carotene

Sporobolomyces roseus strain used in this study predominantly produced β-carotene and the maximum yield was about 278 μg/g of freeze dried whole stillage. Typically, β-carotene yield by *S. roseus* on YE based synthetic medium has ranged from as low as 11.8 μg/g (Buzzini et al., 2007) to 230 μg/g (Yurkov et al., 2008), with intermittent production of 101 μg/g (Davoli et al., 2004) and 118 μg/L on YM broth (Maldonade et al., 2008).

Usually astaxanthin accounts for 80-90% (Tinoi et al., 2006) or even 100% (Parajo et al., 1997) of the total carotenoids of *P. rhodozyma*. However, under microaerophilic conditions β-carotene is accumulated at the expense of astaxanthin (Ramirez et al., 2006; Johnson and Lewis, 1979). In DDGS, β-carotene production by *P. rhodozyma* accounted for 75% of its total carotenoids, indicating that the medium was probably microaerophilic. The macro ingredients probably increased the medium viscosity leading to lesser diffusion of oxygen. In mixed culture fermentation, the β-carotene yield was comparable to that of *P. rhodozyma* and *S. roseus*, and was not cumulative of that of the two strains.

Total carotenoids

Astaxanthin and β-carotene constitute the total carotenoid pool in this study. However, *S. roseus* produces other carotenoids such as torulene and torularhodin (Daevoli et al., 2004; Davoli and Weber, 2002). Total carotenoid content of *S. roseus* on synthetic medium has ranged from 82.3 μg/g (22.9 μg/g of torularhodin and 33.2 μg/g of torulene; Buzzini et al., 2007) to 237 μg/g (10 μg/g of torularhodin and 71 μg/g of torulene; Maldonade et al., 2008). Similarly, *P. rhodozyma* is also known to produce torulene and torularhodin (Frengova and Beshkova, 2009

and references therein). Whereas these additional carotenoids were not evaluated in the carotenoid-enriched whole stillage, it is likely that they are produced by both *P. rhodozyma* and *S. roseus* strains. The total carotenoid content in our value-added DDGS will be further enhanced if these carotenoids are accounted for. In whole stillage medium, mixed culture fermentation provided the highest amount of total carotenoids. Usually, about 30-120 µg/g of total carotenoids is added to aquaculture feed (Venugopal, 2009). In DDGS, both mixed culture and *P. rhodozyma* monoculture fermentations were able to provide the prescribed amount of total carotenoids.

Potential applications

According to the Global market for Carotenoids (BCC Research, 2005), the worldwide market value of all commercially used carotenoids in 2009 is set to cross $1 billion of which astaxanthin and β-carotene share $257 and $254 million respectively. The feed industry has a huge demand for astaxanthin due to its pigment and anti-oxidant properties, and β-carotene for mostly its pigment properties. Since DDGS is predominantly sold as livestock and poultry feed, carotenoid-enriched DDGS can not only provide value-added animal feed, but also can improve the market base of DDGS. Aquaculture, especially salmonid and crustacean aquaculture are dependent on astaxanthin to provide the visually appealing, characteristic pink color and is the principal market driver for astaxanthin (Venugopal, 2009). Astaxanthin is the most expensive ingredient in salmonid feed (Johnson, 2003). Since DDGS as aquaculture feed is being explored (US Grains Council, 2007), carotenoid-enriched DDGS can prove to be 'cost-effective, naturally pigmented' aquaculture feed.

Carotenoid value addition of DDGS has many advantages apart from being cost-effective: 1) the whole stillage need not be transported to a separate facility and secondary submerged fermentation can be carried out at the same ethanol plant without any procedural

modifications, 2) other media ingredients are cheap additives like glycerol allowing the sustainability of biodiesel industry, and corn-steep liquor a product of corn wet-milling, 3) whole stillage is not rich in fermentable glucose thereby preventing the Crabtree effect in *P. rhodozyma* (Reynders et al., 1997) and allowing the accumulation of *P. rhodozyma* biomass and carotenoids, 4) precludes the addition of expensive N source namely yeast extract as the whole stillage is rich in residual yeast, 5) either all or a portion of whole stillage can be fermented to produce carotenoid-enriched feed depending on the requirement without the need for chemical extraction, 6) overcomes the addition of expensive carotenoids in animal feed, 7) is visually appealing for improved marketability (Fig. 2.4), and 8) value added DDGS can fetch premium price. Additionally, biological astaxanthin has more advantages than synthetic astaxanthin. Firstly, biological astaxanthin at 50% concentration of synthetic astaxanthin gives similar effects. For example, An et al. (2004) showed that synthetic astaxanthin at 45 mg/kg feed and biological astaxanthin at 22.5 mg/kg feed provide similar levels of pigmentation in egg laying hens. Secondly, biological astaxanthin is also associated with higher lipid synthesis in yeasts, thereby allowing greater absorption of carotenoids (An et al., 2004).

Conclusions

Carotenoid-enriched whole stillage, a unique product is not only visually appealing, but also provides astaxanthin and β-carotene, the predominant carotenoids in animal feed. Depending on the type of carotenoids required in the feed, mono- or mixed culture fermentation can be employed. The carotenoid-enriched DDGS can not only be used in livestock, but can also capture the aquaculture feed base due to its inherent requirement of carotenoid pigments.

Table 2.1 ANOVA results for carotenoid yield on different days of fermentation

Treatment	**Astaxanthin**				**β-carotene**			
	Synthetic		Whole stillage		Synthetic		Whole stillage	
	F	*P*	F	*P*	F	*P*	F	*P*
Mixed culture	0.69	0.5688	33.0	*0.0091*	26.99	*0.0121*	66.85	*0.0033*
P. rhodozyma	4.04	0.1982	5.89	0.0914	624.73	*0.0016*	4.86	0.1145
S. roseus	-	-	-	-	7.3	0.0704	155.67	*0.0009*

Significant *P* is italicized

Table 2.2 Carotenoid yields on whole stillage and synthetic media

Media [a]	Carotenoids [b]	Treatment [c]	Day 5	Day 7	Day 9
Whole stillage					
	Astaxanthin				
		Mx	11.26±0.8 [B]	12.59±0.49 [B]	17.41±0.17 [A]
		PR	25.95±2.9[A]	31.21±0.99[A]	35.73±1.64[A]
		SR	-	-	-
	β-carotene				
		Mx	135.58±5.12 [B]	135.92±3.74 [B]	187.89±0.6 [A]
		PR	76.28±8.95[A]	89.92±4.48[A]	104.72±4.96[A]
		SR	149.97±1.34 [c]	192.72±4.98 [b]	232.99±2.55 [a]
	Total [d]				
		Mx	146.84	148.51	205.3
		PR	102.33	121.13	140.45
		SR	-	-	-
Synthetic					
	Astaxanthin				
		Mx	69.11±5.2[A]	73.53±2.1[A]	75.53±3.96[A]
		PR	109.77±4.88[A]	111.77±4.25[A]	131.24[A]
		SR	-	-	-
	β-carotene				
		Mx	239.01±2.4[B]	408.79±3.1[A]	475.54±3.5[A]
		PR	338.03±9.33[C]	556.75±0.36[B]	724.0[A]
		SR	103.99±2.62[A]	204.66±3.28[A]	174.21±2.98[A]
	Total [d]				
		Mx	308.12	482.32	551.07
		PR	447.8	668.52	855.24
		SR	-	-	-

[a] Carotenoid yield: whole stillage-µg/g of freeze dried whole stillage, synthetic medium- µg/g of yeast

[b] Means and standard errors are provided; Significance was set at $P \leq 0.05$. Significantly different treatments across days do not share a letter (upper-case);

[c] Mx-mixed culture, PR-*P. rhodozyma*, SR-*S. roseus*;

[d] Total carotenoid is the sum of the respective astaxanthin and β-carotene yields.

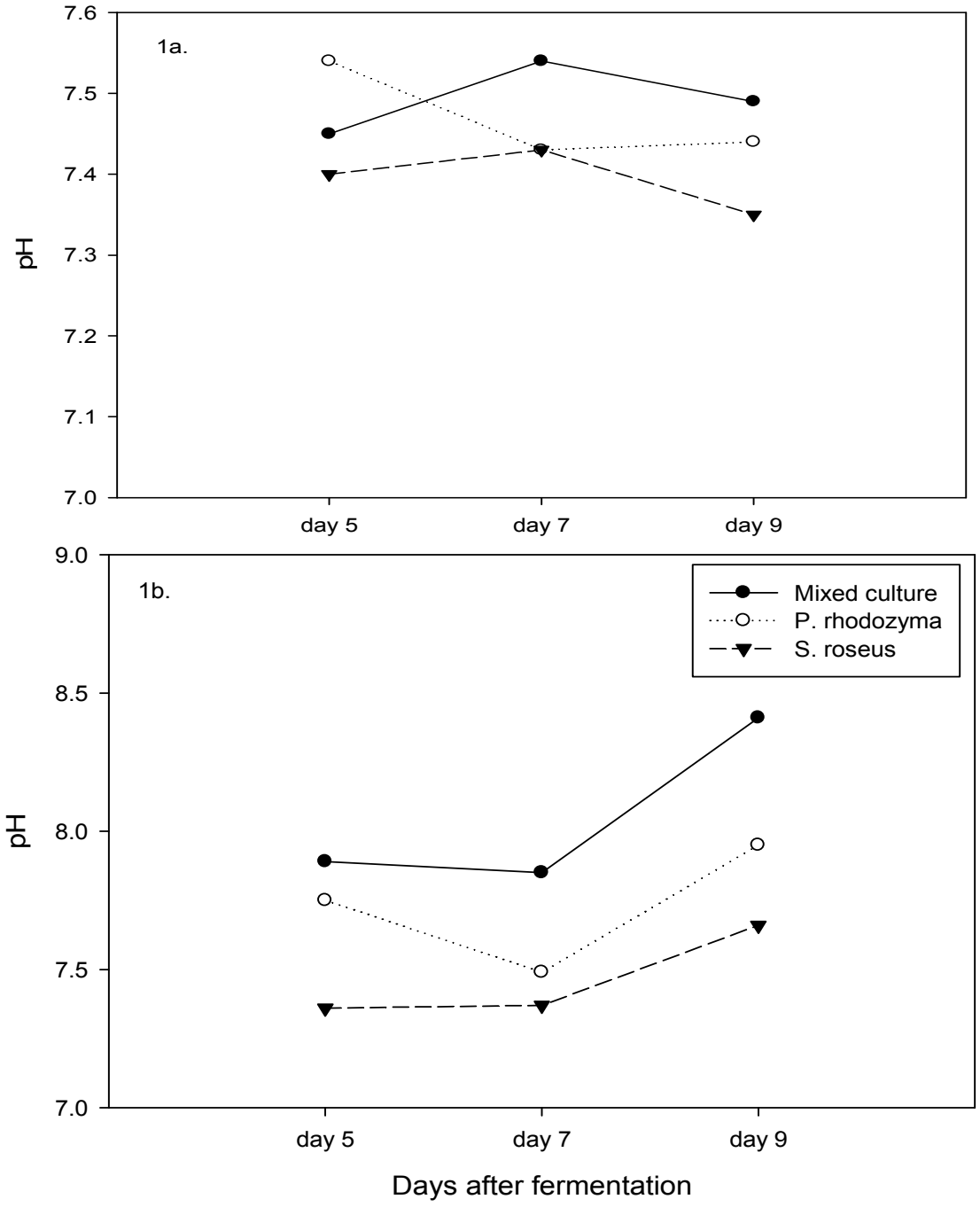

Figure 2.1 pH profile for carotenoid fermentation.

a) Whole stillage medium b) Synthetic medium.

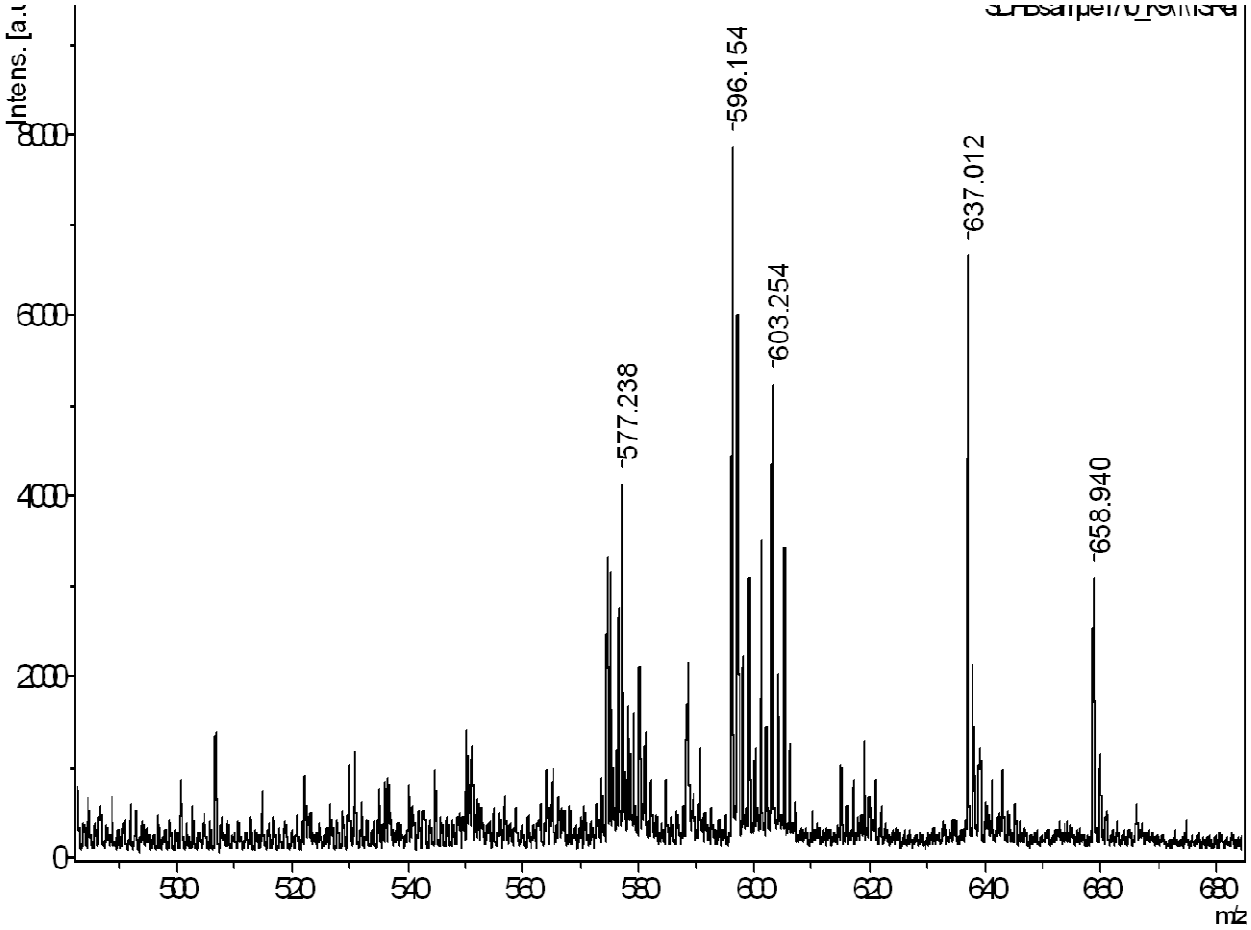

Figure 2.2 MALDI/TOF MS spectrum for carotenoids on mixed culture fermentation.

Astaxanthin is indicated by the peak at 596.15. β-carotene peak was very feeble and therefore not visible. (Molecular weights of astaxanthin and β-carotene are 596.84 and 536.87 respectively).

Figure 2.3 Carotenoid-enriched DDGS.

a) Freeze dried control b) Freeze dried carotenoid-enriched DDGS from mixed culture fermentation. Similar products are available from *P. rhodozyma* and *S. roseus*.

CHAPTER 3 - [2]Media optimization for the production of carotenoid-enriched Distillers Dried Grains with Solubles (DDGS) by *Phaffia rhodozyma* and *Sporobolomyces roseus* fermentation of whole stillage

Abstract

Carotenoid-enriched dried distillers grain with solubles (DDGS) was produced by the fermentation of whole stillage. In the absence of media optimization, the carotenoid yield (17-233 μg/g) from both, monoculture and mixed culture fermentation was in the range that is normally provided in animal feed. To further enhance the yield, this study used response surface methodology (RSM) and mixture design for media optimization. Macro ingredients whole stillage, corn steep liquor and glycerol, and minerals were fitted to a second-degree polynomial in RSM and mixture design respectively. Media optimization suggested that the previously used concentrations of all macro ingredients, except glycerol should be reduced to enhance the yields of astaxanthin and β-carotene. Although statistically not significant, minerals had a positive influence on both carotenoids. Validation studies indicated that media optimization resulted in enhanced carotenoid yields. Astaxanthin and β-carotene yields in mixed culture and *P. rhodozyma* monoculture were 5 and 278, 97 and 275 μg/g, respectively, while *S. roseus* produced 278 μg/g of β-carotene. Apart from HPLC detection, NMR spectroscopy of the samples confirmed beyond doubt the presence of astaxanthin and β-carotene. Carotenoids in the samples were stable for a period of six months and storage temperature did not affect their

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stability. Since the carotenoid yields were almost twice the quantity used in animal feed, the carotenoid-enriched DDGS has potential application as 'value-added animal feed or feed blends'.

Introduction

As animal feeds are poor in carotenoids (Nys 2000; Holden et al., 1999) and distillers dried grain with solubles (DDGS) is no exception, carotenoid-enriched DDGS was produced by the secondary fermentation of whole stillage (Chapter 2, Ananda and Vadlani, 2010). While carotenoids, especially astaxanthin have been produced on a variety of cheap substrates (Frengova and Beshkova, 2009), and the production process optimized, the use of an animal feed DDGS for carotenoid production is admittedly unique. Just as any new process is optimized for maximum output, the secondary fermentation of whole stillage also needs to be optimized for maximum carotenoid yield. In this study, the focus was on optimizing only media ingredients for maximum carotenoid yield because 1) cheap products of corn biofuel (whole stillage, corn steep liquor) and biodiesel (glycerol) were used as substrates and 2) parameters like temperature, aeration, pH, light etc. have been optimized in various studies and their effects on carotenoid production are well documented. Most carotenoid optimization studies have relied on powerful designs namely, factorial design (Park et al., 2005; Bhosale and Gadre, 2001; Ramírez et al., 2001), mixture design (Ni et al., 2007), Plackett-Burman design (Valduga et al., 2009; Chen et al., 2006), and response surface methodology (Choudhari and Singhal, 2008; Vázquez and Martin, 1997).

Optimization of astaxanthin production by *Phaffia rhodozyma* has been achieved by altering physical factors like temperature, aeration, pH, light, and media components like C source, C/N ratio, minerals, and nitrogen source. Most optimization studies have relied on powerful statistical designs and response surface methodology (Valduga et al., 2009; Park et al.,

2005; Vasquez and Martin, 1997). For example, suggested optimum temperatures are 15, 18, 19.7, 22 °C (Ramirez et al., 2001; Vasquez and Martin, 1997; Meyer and du Preez, 1994; Fang and Cheng, 1993; Johnson and Lewis, 1979), and pH are 4.0-7.0 (Fang and Cheng, 1993) or 5.0, 6.0 and 6.9 (Ramirez et al., 2001; Vasquez and Martin, 1997; Meyer and du Preez, 1994). A positive influence of organic N sources like yeast extract, beef extract or peptone (Ramirez et al., 2001; An et al., 1996; Fang and Cheng, 1993) or inorganic N sources like urea, KNO_3, ammonium salts (Ni et al., 2007; Parajo et al., 1997; An et al., 1996; Fang and Cheng, 1993) is well documented. Optimization of whole stillage fermentation to produce carotenoid-enriched DDGS is a necessity for producing cost-effective value added animal feed.

Hypothesis 2.1: Media optimization will enhance the yield of carotenoids, both in monoculture and mixed culture fermentation.

Hypothesis 2.2: Since, both *P. rhodozyma* and *Sporobolomyces roseus* are red yeasts, results from the optimization of *P. rhodozyma* monoculture are applicable for *S. roseus* optimization, as well as that of their mixed culture.

The objectives of this study were 1) optimization of media ingredients using response surface methodology and mixture design, 2) validation of the optimization in shake flasks, 3) confirmation of carotenoids by NMR and 4) evaluation of product stability.

Materials and methods

Microbial cultures

Culture maintenance and inoculum generation of *P. rhodozyma* and *S. roseus* are outlined in chapter 2. A 10% (v/v) inoculum was used for monoculture fermentation, while 5% of each strain was used in mixed culture fermentation.

Media preparation

Optimized medium: A liter of the fermentation medium contained 15% whole stillage, 1.5% corn steep liquor, 7.7% glycerol and mineral salts (0.6g KH_2PO4, 0.3g $MgSO_4$, 0.3g $MnSO_4$ and 0.7g $ZnSO_4$). Corn whole stillage was procured from Abengoa Bioenergy (Colwich, KS, USA). Media pH was about 6.0 before sterilization and was not adjusted any further. Flasks with 50 ml of whole stillage medium were sterilized at 121°C for 30 min.

Fermentation conditions

The conditions were similar to that followed for unoptimized media (Chapter 2). Submerged fermentation of *P. rhodozyma* and *S. roseus* mono- and mixed cultures were conducted. Flasks were inoculated and incubated at 18°C, 180 rpm for nine days. Control flasks without inocula were maintained. Samples for optimization were harvested only on day 7, whereas, for validation, samples were harvested on 5^{th}, 7^{th} and 9^{th} day, centrifuged and supernatant discarded. Pellets were freeze dried for 24 h and stored at –80°C until further analysis. Two replicates per treatment were employed.

Experimental design for optimization

Media optimization was carried out in two phases- response surface methodology for the optimization of major ingredients namely, whole stillage, glycerol and corn steep liquor and mixture design for the optimization of minerals, KH_2PO_4, $MgSO_4$, $MnSO_4$ and $ZnSO_4$. Design expert 7.1. 6 (Stat-Ease Inc., Minneapolis, MN, USA) was used to generate experimental designs, estimate the responses of dependent variables and also generate the contour and/or response surface plots.

Response surface methodology

The three independent variables and their levels for a rotatable central composite design (CCD) are given in table 3.1. The CCD consisted of six central points and 14 non-central points. The experiment consisted of 20 runs with no blocking and the design matrix is provided in table 3.2. The relation between coded and actual values is according to the following equation

$$x_i = \frac{X_i - X_0}{\Delta X} \qquad (1)$$

where x_i is the coded value of the independent variable (x_1=whole stillage, x_2=corn steep liquor, x_3=glycerol), X_i is the real value of the independent variable, X_0 is the real value of the independent variable at the center point and ΔX is the step change value

The relationship between independent variables and dependent variables was obtained as the sum of the contributions of the three factors through first order, second order and interaction terms according to the quadratic polynomial function in equation 2.

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i X_j + \sum i \sum_{i<k} j \beta_{ij} X_i X_j \qquad (2)$$

where Y is the predicted response, β_i is the linear coefficient, β_{ii} the squared coefficient and β_{ij} the interaction coefficient and k the number of factors.

Data were square root transformed prior to analyses as the min to max ratio was greater than 10. Astaxanthin and β-carotene produced were the two response variables.

Mixture design

A D-optimal mixture design with constraint (KH_2PO4+$MgSO_4$+$MnSO_4$+$ZnSO_4$+Macro ingredients ≤ 322.5g/L) was applied. The levels of all the ingredients used for mixture design are provided in table 3.3. The design matrix consisted of 25 runs–15 model points, five to estimate lack of fit and additional five replicates (Table 3.4). The influence of the various factors on the response variables are described by the quadratic polynomial equation 2. Astaxanthin and β-

carotene were the response variables. The fermentation conditions for optimization were the same as listed above. However, samples were harvested only on day 7.

Only *P. rhodozyma* monoculture was used for optimization by RSM and mixture design since i) it produces both astaxanthin and β-carotene, ii) astaxanthin is a high value product and iii) optimization of all treatments (*S. roseus* and mixed culture) is laborious due to the volume of the experiments.

Validation of optimized conditions

The optimized medium was formulated based on the RSM and mixture design results and used for validation. Both, monoculture and mixed culture fermentations were carried out using the optimized medium. Since both *P. rhodozyma* and *S. roseus* are red yeasts, it was assumed that the optimal medium for the former would be applicable for the latter and also for their mixed culture. Three replications were carried out per treatment. Fermentation conditions, sample collection and data analyses were carried out as previously described.

Nuclear magnetic resonance (NMR) for carotenoids

Phaffia rhodozyma ***fermentation in fermenter***

Phaffia rhodozyma fermentation was carried out using a 2-L BBraun Biostat-B fermenter. About 1.5-L of the fermentation medium was sterilized in the fermenter at 121 °C for 30 min. Batch fermentation was carried out for seven days at 20 °C, pH 6.0, 500 rpm and 1 vvm sterile air. Dissolved oxygen and pH were monitored for every 2 h. The entire fermentation broth was harvested on day 7, aliquoted into five bottles and freeze dried for five days. After freeze drying, samples were pooled and blended using a coffee blender. Samples were stored at –20 °C until further analyses.

Purification and concentration of carotenoids

About 10 g of freeze dried sample from *P. rhodozyma* fermentation was ground using 40-100 mesh sand and carotenoids were extracted using 20 ml dichloromethane. The procedure was repeated until the entire sample was extracted. The extracts were pooled and centrifuged. Supernatant was collected in a round bottom flask and subjected to rotary vacuum drying. Dried samples were re-dissolved in 3 ml dichloromethane and purified by following the HPLC method described in Chapter 2. However, for purification C_{18} semi-prep Phenomenex Luna column (250 mm × 10 mm) was used. About 100 µl of the sample was injected each time and fraction between 1.2 to 2.0 min was collected for astaxanthin, and fraction at 16.3 to 17.3 min was collected for β-carotene. This procedure was repeated until the entire sample was utilized. Respective fractions of astaxanthin and β-carotene were pooled and concentrated to dryness using rotovap.

Identification of purified carotenoids was carried out by MALDI/TOF MS (Bluker Ultraflex II TOF/TOF mass spectrometer) and proton NMR (Varian Inova, 400MHz) at the Department of Chemistry, KSU.

Carotenoid extraction and analyses

High performance liquid chromatography (HPLC) was used for quantification of both carotenoids, and is outlined in Chapter 2.

Evaluation of product stability

Dried samples of carotenoid-enriched DDGS from shake flasks were stored at four temperatures namely, room temperature, 4, –20 and –80 °C. Samples were subjected to HPLC estimation on a monthly basis for six months to determine the stability of carotenoids.

Statistical analyses

Data after validation were analyzed using SAS (version 9.1.3). PROC GLM was used to compare multiple treatments and when necessary pair-wise comparisons were made using Tukey-Kramer at *P*=0.05. Optimization data were analyzed by Design expert 7.1.6.

Results

Optimization

Response surface methodology

A central composite design of 20 experiments was carried out to evaluate the effect of three independent macro ingredients on astaxanthin and β-carotene production. Second order polynomial equation was used to correlate the independent variables with astaxanthin and β-carotene production, respectively. The actual and predicted values of the response variables are provided in table 3.2.

Table 3.5 provides the ANOVA for astaxanthin production. The model was significant with F value of 26.02. The coefficient estimates and their corresponding *P* values suggest that all the variables and the interaction of glycerol and corn steep liquor are significant. The different variables were correlated with astaxanthin production by multiple regression according to the equation 2. The final equation in coded terms is given below

$$\text{Sqrt (Astaxanthin)} = 7.69 - 0.71*A - 0.81*B - 1.50*C - 0.56*B*C - 1.30*C^2 \quad (3)$$

The R^2 for equation 2 was 0.91 indicating that 91% of the variation in astaxanthin production is explained by the quadratic polynomial.

The response surface and contour plots for astaxanthin production were generated (Fig. 3.1). At optimal point of corn steep liquor, three-dimensional plot of two factors whole stillage and glycerol versus astaxanthin production were drawn along with the corresponding contour plot (Fig. 3.1a, b). Based on equation 3 and confirmed by contour plot, all three variables negatively influenced the astaxanthin production indicating that lower concentrations of these ingredients in the medium would result in higher production of astaxanthin. According to the contour plot, mean astaxanthin production was 78 μg/g of freeze dried whole stillage (Fig. 3.1b).

ANOVA for β-carotene is provided by table 3.5. Model significance is indicated by F=12.53. The coefficient estimates and corresponding *P* values are provided in table 6. The final equation in coded terms after multiple regression analysis:

Sqrt (β-carotene) = 12.67 – 1.85*A – 0.48*B + 1.59*C – 0.95*A*C –1.40*B*C – $1.69*C^2$ (4)

The goodness of fit for equation 3 is given by the coefficient of determination, R^2, of 0.85, indicating that 85% of the variability in β-carotene production is explained by the model.

Similarly to astaxanthin production, response surface and contour plots were generated for β-carotene (Fig. 3.2a, b). From equation 4 and the contour plot, β-carotene production was negatively influenced by whole stillage and positively by glycerol. Corn steep liquor negatively influenced β-carotene production although it was not statistically significant. However, the interaction of glycerol and corn steep liquor had a significant effect. The mean β-carotene production as seen in the contour plot was 257 μg/g of freeze dried whole stillage (Fig. 3.2b). Overall, the optimal medium constituents were 150g/L of whole stillage, 15g/L of corn steep liquor and 7.7g/L of glycerol.

Mixture design

Table 3.6 provides the ANOVA for astaxanthin production. The model was significant at F=4.08. The coefficient estimates for the main effects and interactions are provided in Table 8. The predictive model in coded terms is as follows:

Astaxanthin = +122.05*A +53.46*B +37.62*C +122.08*D +69.02*E –307.86*A*D – 72.01*A*E (5)

The R^2 for equation 5 is 0.58 and is lesser than the suggested value of 0.75. This is most likely due to outliers. Based on coefficient estimates from table 8, it is evident that all the minerals had a positive influence on astaxanthin production. However, the mineral main effects were not statistically significant (Table 3.6). The contour plot for astaxanthin production is provided (Fig. 3.3) and the mean astaxanthin production was 72µg/g when Zn and all other macro ingredients were kept constant.

ANOVA for β-carotene production is provided in table 3.6. The model was significant at F=5.95. The coefficient estimates for the main effects and interactions are provided in table 3.7. From multiple regression analysis, the final equation for the actual terms for β-carotene production is provided by equation 6 as follows

β-carotene = +189.22*A +98.30*B +52.07*C +2342.31*D +153.67*E +395.73*A*B – 3093.91*A*D –2821.81*B*D –2199.00*C*D –2562.99*D*E (6)

The goodness of fit R^2 for the quadratic polynomial is 0.78 suggesting that the proposed model is suitable for β-carotene production by *P. rhodozyma*. Figure 3.4 provides the contour plot for β-carotene production where the maximum production was 166µg/g. Linear mixture of mineral and the K*Mg interaction positively influenced β-carotene production, whereas all other interactions of Zn had a negative influence.

After establishing the models for each response, numerical optimization was chosen to maximize production of both astaxanthin and β-carotene. The highest desirability was 0.949 at which the optimal mineral composition was: 0.6 g/L of K, 0.3g/L Mg and Mn, and 0.7g/L of Zn.

Validation

The astaxanthin and β-carotene yields from mono- and mixed culture fermentations of optimized medium are provided in table 3.8. The astaxanthin and β-carotene yields by *P. rhodozyma* on day 7 were 67 and 265 μ/g, respectively, and both were comparable to the predicted values of 78 and 257μg/g, respectively from the contour plots of macro ingredients.

Media optimization improved *P. rhodozyma* astaxanthin yield by 119% and β-carotene yield by 197% on day 7 (Table 3.8). Astaxanthin yield in *P. rhodozyma* increased by 177% on day 9 confirming the enhanced astaxanthin production in late log phase or exponential phase. Although the optimized conditions of *P. rhodozyma* were applied to the *S. roseus* monoculture and mixed culture fermentations, only marginal increase in carotenoid production was observed except in the astaxanthin yield of mixed culture where a yield reduction of 71% was observed. This indicates that *S. roseus* monoculture and mixed culture fermentations require separate optimization studies.

NMR

NMR spectra indicated that the astaxanthin and β-carotene in the *P. rhodozyma* carotenoid-enriched DDGS (Figs. 3.5 and 3.6) were a perfect match to the respective standards (Figs. 3.7 and 3.8).

Discussion

This study demonstrated the successful media optimization for carotenoid production from secondary fermentation of whole stillage, thus supporting hypothesis 2.1. The optimization

results from *P. rhodozyma* were applicable for *S. roseus* and mixed culture fermentation supporting hypothesis 2.2. However, the yields in *S. roseus* and mixed culture did not appreciate as much as that seen in *P. rhodozyma*, indicating that separate optimizations for *S. roseus* and mixed culture would vastly improve the yields in the respective fermentations.

Prior to media optimization, total carotenoid production in our study followed a trend similar to that of β-carotene, with mixed culture fermentation providing the highest amount of total carotenoids (Chapter 2). However, *P. rhodozyma* yielded the highest amount of total carotenoids after medium optimization. Usually, about 30-120 μg/g of total carotenoids is added to aquaculture feed (Venugopal, 2009). In this study, both mixed culture and *P. rhodozyma* monoculture fermentations were able to provide the prescribed amount of total carotenoids before optimization and nearly 2.5-3 times after optimization.

Overall, the optimization studies indicate that in shake flasks, lower concentrations of whole stillage, glycerol and corn steep liquor improve the carotenoid yield. The optimized medium had 40% lesser whole stillage, 25% lesser corn steep liquor and 54% higher glycerol. These results indirectly confirm that carotenoid production, especially astaxanthin production is influenced by aeration. As the medium viscosity increases, the amount of dissolved oxygen is reduced severely affecting astaxanthin production. The glycerol concentration was increased since it positively influenced β-carotene production. It is likely that a reduction of glycerol would further increase astaxanthin production at the cost of β-carotene production.

Although mineral salts are added in trace amounts, their optimization was deemed necessary. While the exact roles of inorganic salts have not been defined in carotenogenesis, their presence in the growth media have nevertheless improved carotenoid yields: after evaluating 11 different inorganic salts, carotenoid production by *Rhodotorula sphaeroides* was

enhanced by $MgSO_4$, Na_2HPO_4, $FeSO_4$ and Na_2CO_3 (Chen et al., 2006); KH_2PO_4 and $MgSO_4$ were required for β-carotene production in *Blakslea trispora* (Choudhari and Singhal, 2008); $(NH_4)_2SO_4$ and KNO_3 were nitrogen sources (Ni et al., 2007) and KNO_3 at low concentration was required for astaxanthin production in *P. rhodozyma* (Parajo et al., 1998). In most of these studies, inorganic nitrogen sources were incorporated in addition to organic sources like beef extract, peptone and/or yeast extract. In the present study, minerals like K, Mg, Mn and Zn had a positive influence on carotenoid production even though it was not statistically significant. Therefore, with an exception of Zn, minimum concentration of all the minerals was chosen. Zn is not known to have any effect on carotenoid synthesis (An et al., 2001). However, it is a known co-factor of superoxide dismutase and may enhance astaxanthin production under enhanced oxidative stress (Frengova and Beshkova, 2009). Mn salts can have a positive or negative influence on *P. rhodozyma* carotenoid production depending on its concentration and the type of C source in the medium (An et al., 1996). K_2HPO_4 does not affect carotenoid production in *R. glutinis* (Park et al., 2005), but its specific effect on *P. rhodozyma* is not known. However, many studies have routinely included K_2HPO_4 and other mineral salts in the *P. rhodozyma* growth media, even if the substrates were composed of complex plant products (Ramirez et al., 2006; Vustin et al., 2004; Ramirez et al., 2001; Reynders et al., 1997). DDGS is rich in minerals and has about 0.91% K, 0.68% P, 0.28% Mg, 0.84% S, 22 ppm Mn and 61 ppm Ze (Batal and Dale, 2003). In optimization of whole stillage medium, 0.06% of KH_2PO_4, 0.03% of $MgSO_4$ and $MnSO_4$ and 0.07% $ZnSO_4$ were added. Admittedly, the concentrations of the added minerals were too low to impact carotenogenesis one way or the other.

Factors like aeration, temperature, pH, inoculum size or N source were not optimized. The most suitable conditions were identified based on well documented studies. For example,

18°C, pH 6.0 and 10% inoculum were chosen. Since whole stillage is a fermented product with residual yeast, yeast extract or any other N source was not added. Also, the procured whole stillage sample had a pH of 6.0 and was not altered as it was well within the documented range. Further media optimization by lowering glycerol concentration, including factors like aeration/dissolved oxygen and temperature in the statistical design coupled with strain improvement or the use of over-producing strains can enhance the astaxanthin yield in *P. rhodozyma* monoculture.

Conclusions

Media optimization improved carotenoid yields both from monoculture and mixed culture fermentation of whole stillage. To further enhance the yield, high yielding wild-type strains and/ or mutant strains of *P. rhodozyma* and *S. roseus* can be utilized. Depending on the type of carotenoids required in the feed, mono- or mixed culture fermentation can be employed. As the process is scaled-up, further optimization steps are required to obtain the best yield.

Table 3.1 Macro ingredient variables and their levels tested in central composite design

Factor	Nutrient [a]	Low Actual (-1)	Mean (0)	High Actual (+1)	+α	– α
A	WSL (g/L)	150	325	500	619.314	30.68
B	CSL (g/L)	15	32.5	50	61.93	3.068
C	GLY (g/L)	30	65	100	6.13	123.86

[a] WSL-whole stillage, CSL-corn steep liquor, GLY-glycerol

Table 3.2 Experimental design matrix for macro ingredients and carotenoid yields

Run	A	B	C	Astaxanthin		β-carotene	
				Actual	Predicted	Actual	Predicted
1	150	50	100	18.15	17.81	196.28	181.81
2	325	61.93	65	45.76	40.10	192.87	140.65
3	30.69	32.5	65	83.95	78.81	296.89	249.30
4	500	15	100	38.23	30.82	192.95	135.91
5	500	15	30	63.94	55.20	66.42	57.21
6	325	32.5	65	56.40	59.14	139.79	160.57
7	500	50	30	39.56	48.20	73.28	88.46
8	325	32.5	65	68.58	59.14	152.93	160.57
9	619.31	32.5	65	48.54	42.29	115.80	91.28
10	325	32.5	65	67.36	59.14	139.18	160.57
11	325	3.07	65	67.64	81.86	136.60	181.80
12	325	32.5	65	58.64	59.14	131.96	160.57
13	150	15	30	79.78	78.17	100.90	88.00
14	325	32.5	123.86	18.30	17.72	102.80	111.62
15	150	15	100	47.50	48.48	288.67	297.78
16	150	50	30	74.10	69.79	102.06	125.94
17	500	50	100	5.17	7.89	37.34	62.19
18	325	32.5	6.14	41.26	42.74	31.87	27.25
19	325	32.5	65	48.94	59.14	154.35	160.57
20	325	32.5	65	47.89	59.14	175.48	160.57

A, B and C expressed as g/L; astaxanthin and β-carotene expressed in μg/g

Table 3.3 Mineral nutrients and their levels tested in mixture design

Component	Mineral nutrient (g/L)	Level	Low Level	High Level
A	K	1	0.6	1.4
B	Mg	0.5	0.3	0.7
C	Mn	0.5	0.3	0.7
D	Zn	0.5	0.3	0.7
E	All else	320	319	321

Table 3.4 Experimental design matrix for mineral nutrients in mixture design

Run	A:	B:	C:	D:	E:	Astaxanthin		β-carotene	
						Actual	Predicted	Actual	Predicted
1	0.6	0.7	0.7	0.3	320.2	55.10	59.63	116.04	122.28
2	1	0.3	0.7	0.3	320.2	59.18	64.70	133.73	140.46
3	0.6	0.7	0.7	0.7	319.8	81.00	70.38	158.32	154.13
4	0.6	0.3	0.7	0.3	320.6	64.90	62.74	138.13	133.35
5	0.8	0.5	0.4	0.6	320.2	65.18	70.32	145.89	142.97
6	1.4	0.3	0.3	0.7	319.8	69.99	64.83	165.05	153.07
7	1.4	0.3	0.7	0.3	319.8	70.74	72.43	150.91	147.57
8	1	0.3	0.3	0.3	320.6	72.77	68.10	156.06	160.78
9	1.4	0.5	0.3	0.3	320	78.62	74.27	186.49	178.18
10	1	0.3	0.7	0.7	319.8	67.59	66.02	156.01	161.43
11	1.4	0.7	0.5	0.3	319.6	71.78	75.34	158.14	178.32
12	0.6	0.3	0.3	0.7	320.6	77.96	79.77	183.81	181.32
13	1.4	0.7	0.5	0.3	319.6	80.74	75.34	201.02	178.32
14	0.6	0.3	0.3	0.7	320.6	77.73	79.77	177.70	181.32
15	1.4	0.3	0.7	0.7	319.4	62.08	64.32	139.51	147.31
16	1.4	0.7	0.3	0.5	319.6	74.36	71.54	145.21	150.26
17	1	0.7	0.3	0.7	319.8	66.60	69.19	149.56	161.60
18	0.6	0.3	0.3	0.3	321	70.63	69.02	156.10	153.67
19	1	0.7	0.7	0.3	319.8	65.37	64.47	148.41	145.22
20	0.6	0.7	0.3	0.3	320.6	67.93	65.91	144.58	142.60
21	1.4	0.7	0.3	0.5	319.6	62.78	71.54	150.57	150.26
22	1.4	0.7	0.7	0.7	319	71.16	66.97	161.14	157.54
23	0.8	0.5	0.4	0.6	320.2	68.51	70.32	146.36	142.97
24	1.4	0.5	0.3	0.3	320	73.15	74.27	170.11	178.18
25	1	0.7	0.7	0.7	319.4	61.14	65.79	160.09	155.84

A, B, C, D and E expressed as g/L; astaxanthin and β-carotene expressed in µg/g

Table 3.5 Astaxanthin and β-carotene responses from RSM: ANOVA for Response Surface Reduced Quadratic Model

Carotenoid	Source	Coefficient Estimate	Std Error	Sum of Squares	df	F Value	p-value Prob > F
Astaxanthin							
	Model or intercept	7.69	0.17	44.16	5	26.02	**< 0.0001**
	A	-0.71	0.16	6.81	1	20.05	**0.0006**
	B	-0.81	0.16	8.90	1	26.23	**0.0002**
	C	-1.50	0.19	20.81	1	61.31	**< 0.0001**
	BC	-0.56	0.21	2.54	1	7.49	**0.0169**
	C^2	-1.30	0.20	14.35	1	42.28	**< 0.0001**
β-carotene							
	Model or intercept	12.67	0.40	149.39	6	12.53	**< 0.0001**
	A	-1.85	0.38	46.93	1	23.61	**0.0003**
	B	-0.48	0.38	3.18	1	1.60	0.2280
	C	1.59	0.38	34.48	1	17.35	**0.0011**
	AC	-0.95	0.50	7.14	1	3.60	0.0804
	BC	-1.40	0.50	15.75	1	7.93	**0.0146**
	C^2	-1.69	0.37	41.89	1	21.07	**0.0005**

Significant *P* values (<0.05) are boldfaced

Table 3.6 Astaxanthin and β-carotene responses from mixture design: ANOVA for Mixture Reduced Quadratic Model

Carotenoids	Source	Sum of Squares	df	Mean Square	F Value	p-value
Astaxanthin						
	Model	631.38	6	105.23	4.08	**0.0093**
	Linear Mixture	198.88	4	49.72	1.93	0.1496
	AD	431.43	1	431.43	16.72	**0.0007**
	AE	102.67	1	102.67	3.98	**0.0614**
β-carotene						
	Model	6105.72	9	678.41	5.95	**0.0013**
	Linear Mixture	1852.87	4	463.29	4.06	**0.0199**
	AB	785.56	1	785.56	6.89	**0.0191**
	AD	1834.61	1	1834.61	16.09	**0.0011**
	BD	1473.89	1	1473.90	12.93	**0.0026**
	CD	743.62	1	743.62	6.52	**0.0220**
	DE	1250.31	1	1250.31	10.97	**0.0047**

Significant *P* values (<0.05) are boldfaced

Table 3.7 Regression coefficients for astaxanthin and β-carotene

Carotenoid	Component	Coefficient Estimate	Std Error
Astaxanthin			
	A	122.05	17.09
	B	53.46	11.63
	C	37.62	10.80
	D	122.80	16.01
	E	69.02	3.19
	AD	-307.86	75.29
	AE	-72.01	36.10
β-carotene			
	A	189.22	21.08
	B	98.30	40.26
	C	52.07	31.17
	D	2342.31	624.54
	E	153.67	7.37
	AB	395.73	150.72
	AD	-3093.91	771.11
	BD	-2821.81	784.65
	CD	-2199	860.86
	DE	-2562.99	773.78

Table 3.8 Validation of optimization: Carotenoid yields from optimized medium

Carotenoids [a]	Treatment [b]	Day 5	Day 7	Day 9
Astaxanthin				
	Mx	5.91±0.93 [a] (–54%)	5.076±0.33 [a] (–58%)	5.08±0.31[a] (–71%)
	PR	47.86±2.07[c] (88%)	67.77±4.22[b] (116%)	97.71±1.59[a] (177%)
	SR	-	-	-
β-carotene				
	Mx	212.47±8.04 [b] (57%)	244.96±15.01 [ab] (80%)	278.86±9.65[a] (48%)
	PR	241.83±2.97[a] (217%)	265.77±23.63[a] (197%)	275.20±16.38[a] (164%)
	SR	243.39±6.28 [a] (63%)	237.52±9.95 [a] (23%)	278.58±28.00[a] (20%)
Total				
	Mx	218.38±8.32[b] (48%)	250.03±15.34[ab] (68%)	283.94±9.36[a] (38%)
	PR	289.69±4.89[b] (183%)	333.53±27.65[ab] (175%)	372.91±15.63[a] (165%)
	SR	-	-	-

[a] carotenoid yield μg/g of freeze dried whole stillage; Means and standard errors are provided; Treatments across days for a treatment are significantly different if they do not share a letter; Total carotenoid is the sum of the respective astaxanthin and β-carotene yields; % in parentheses is the percent increase in the yield compared to that from unoptimized medium (Table 2.2).

[b] Mx-mixed culture, PR-*P. rhodozyma*, SR-*S. roseus*;

ANOVA: Astaxanthin: Mx- F=0.64, *P*=0.5578; PR-F=76.6, *P*=<0.0001;

β-carotene: Mx- F=8.63, *P*=0.0172; PR- F=1.06, *P*=0.4025, SR- F=1.60, *P*=0.2768;

Total carotenoids: Mx- F=8.22, *P*=0.0191; PR- F=5.04, *P*=0.052;

Table 3.9 Evaluation of product stability

Months	Temp [a]	Mx		PR		SR
	°C	Astaxanthin	β-carotene	Astaxanthin	β-carotene	β-carotene
Sep		4.87	282	98.3	278	285
Oct						
	RT	5.02	276	96	275.11	276.09
	4	4.95	279.1	96.1	276.19	269
	–20	4.94	288.62	98.7	278.41	288.11
	–80	5.01	283	98.4	276	288.97
Nov						
	RT	4.91	268.41	97.1	261	268.99
	4	4.99	272.04	97.41	268.21	256.41
	–20	5.25	287.68	97.62	279.58	286.52
	–80	4.88	284.66	99.12	282.11	284.62
Dec						
	RT	4.01	256.58	94.22	246	254.33
	4	4.22	267.55	96.41	255.13	253.88
	–20	4.87	286.09	96.98	273.14	286.77
	–80	5.01	284.67	98.11	279.33	292.11
Jan						
	RT	4.77	251.27	95.26	243.58	241.08
	4	4.51	255.45	95.22	256.36	248.08
	–20	4.92	282.34	97.41	281	283.41
	–80	4.77	284	98.19	276.45	289.06
Feb						
	RT	4.21	247.97	94.99	246.66	-
	4	4.39	250.97	95.27	251	-
	–20	5.01	279.64	95.21	276	-
	–80	4.96	285.61	98.67	277.28	-
Mar						-
	RT	4.23	247.14	93.59	239.55	-
	4	4.5	245.22	95.82	241	-
	–20	4.94	277.55	97.11	277	-
	–80	5.06	287.66	97.28	281.01	-

[a] RT-room temperature; - sample insufficient for analysis; carotenoids µg/g

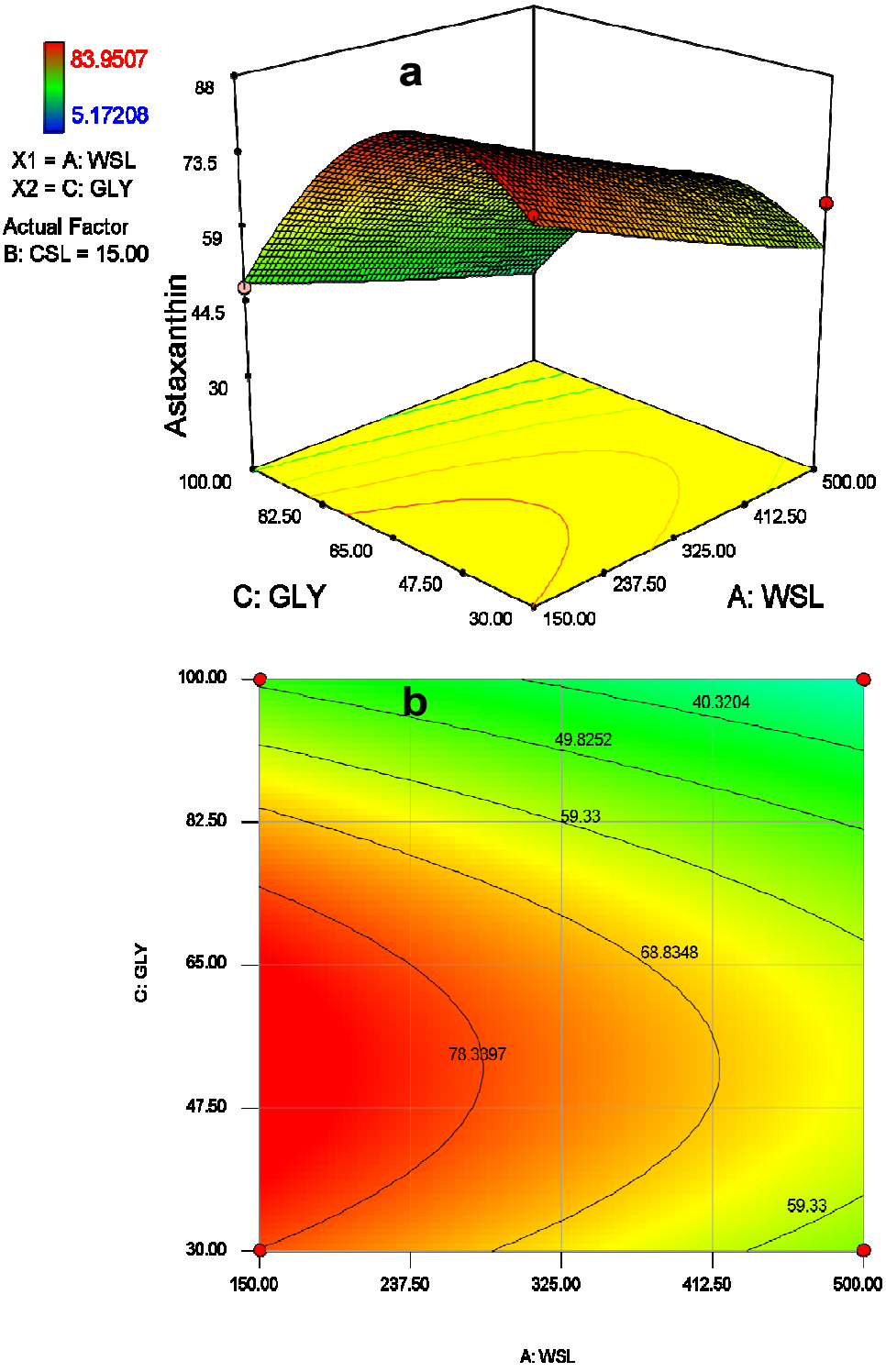

Figure 3.1 RSM for astaxanthin production using macro ingredients.

a) Response surface plot b) Contour plot

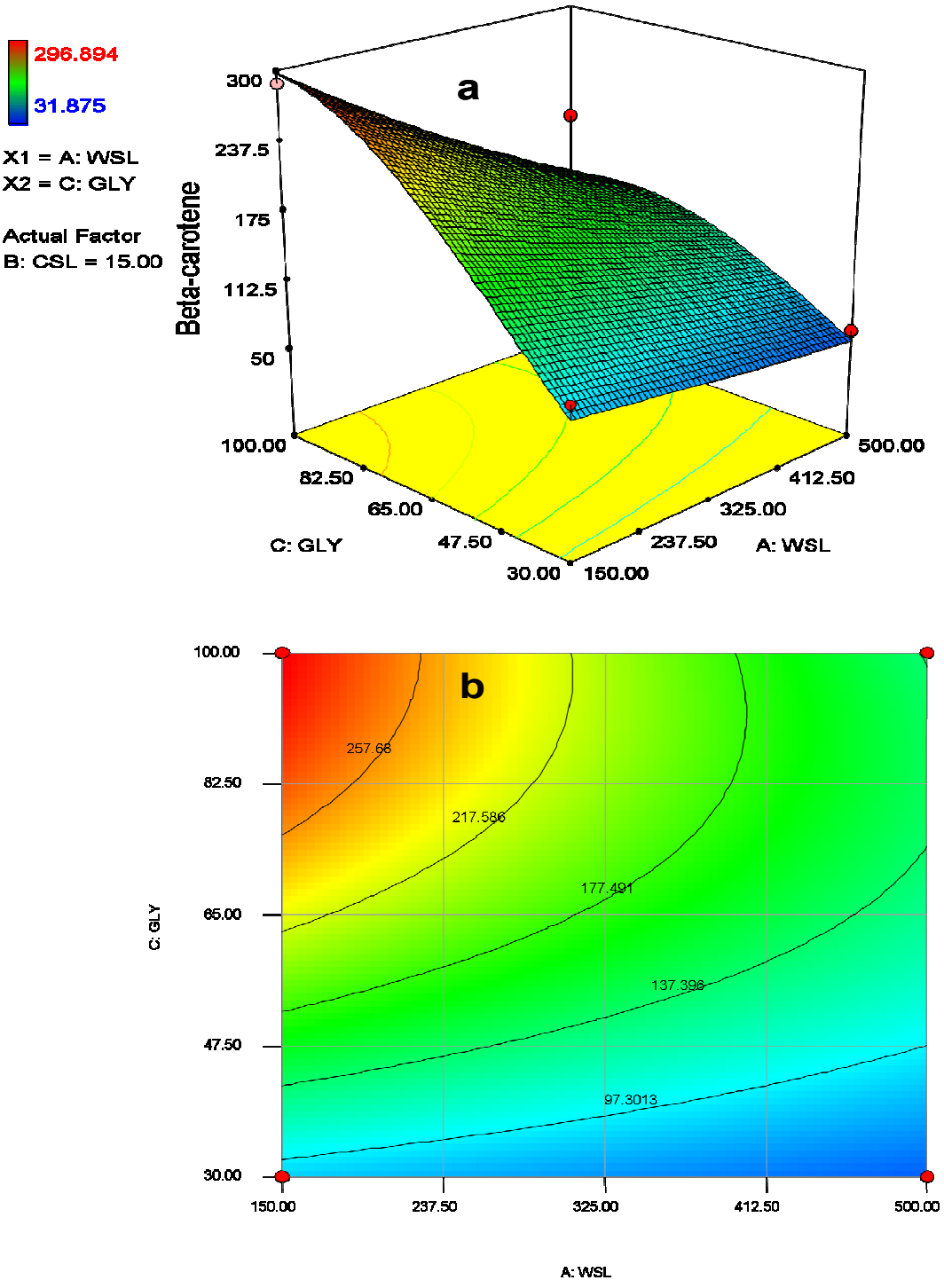

Figure 3.2 RSM for beta-carotene production using macro ingredients.

a) Response surface plot b) Contour plot.

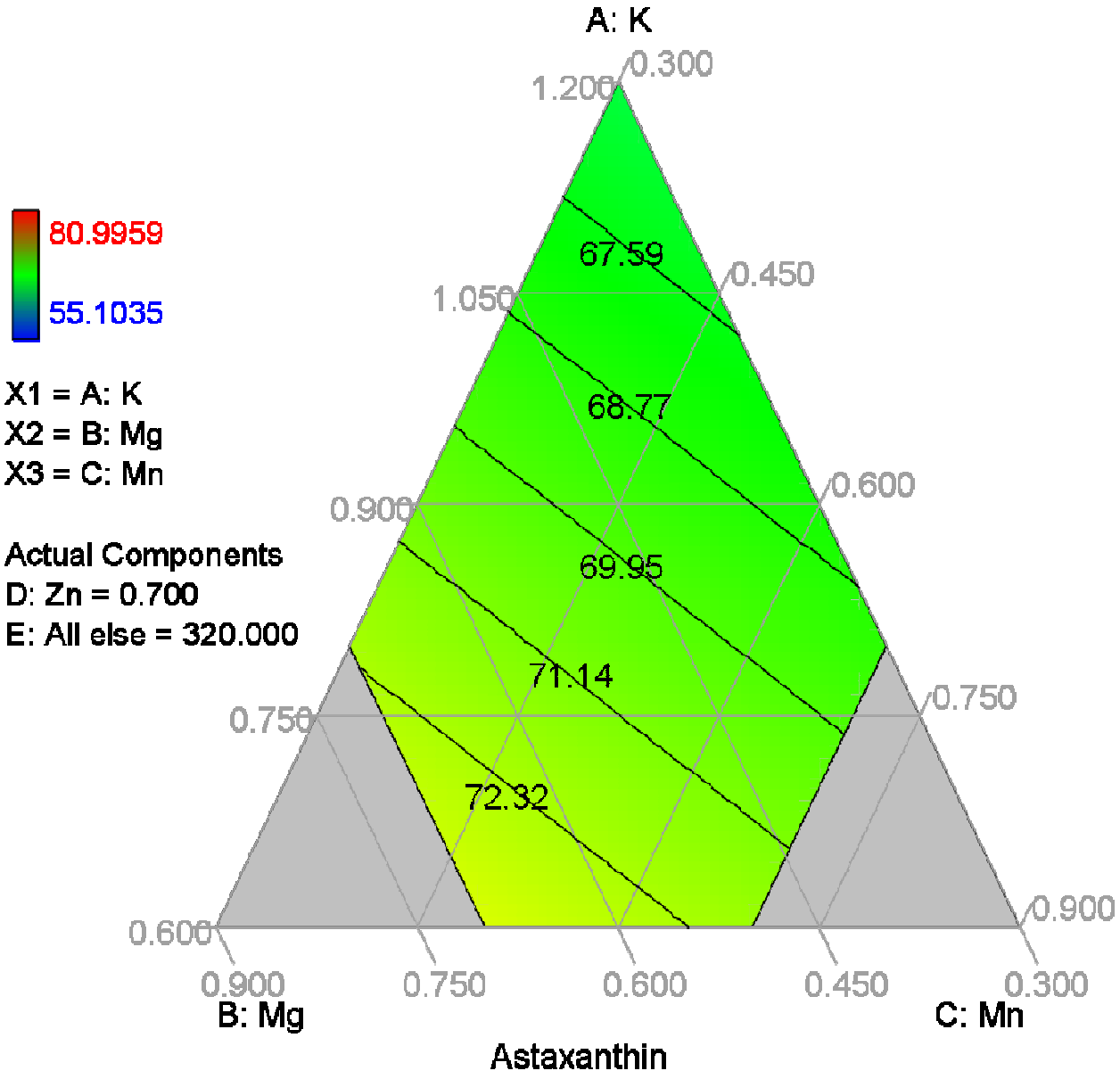

Figure 3.3 Contour plot for astaxanthin production based on minerals

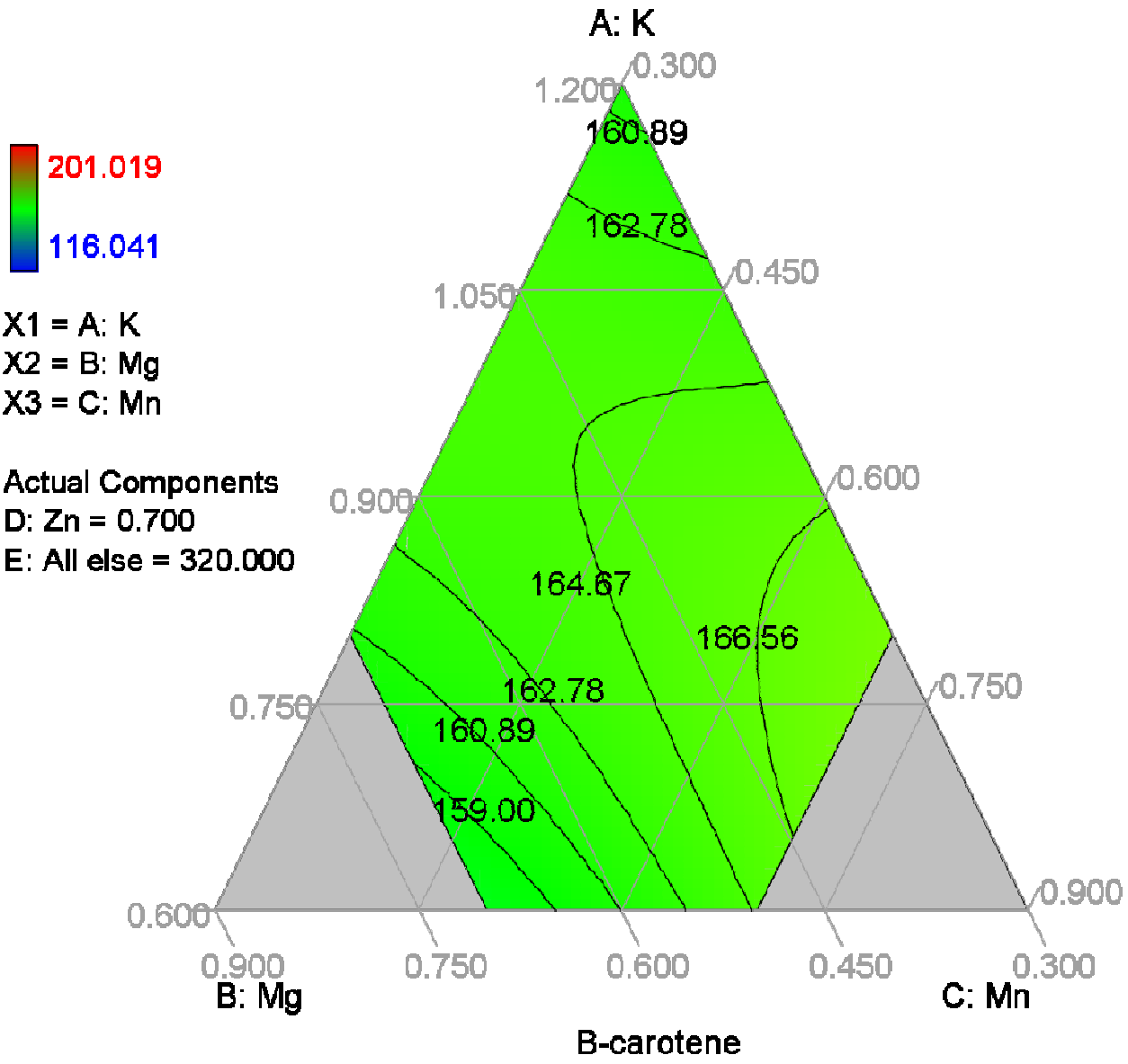

Figure 3.4 Contour plot for beta-carotene production based on minerals.

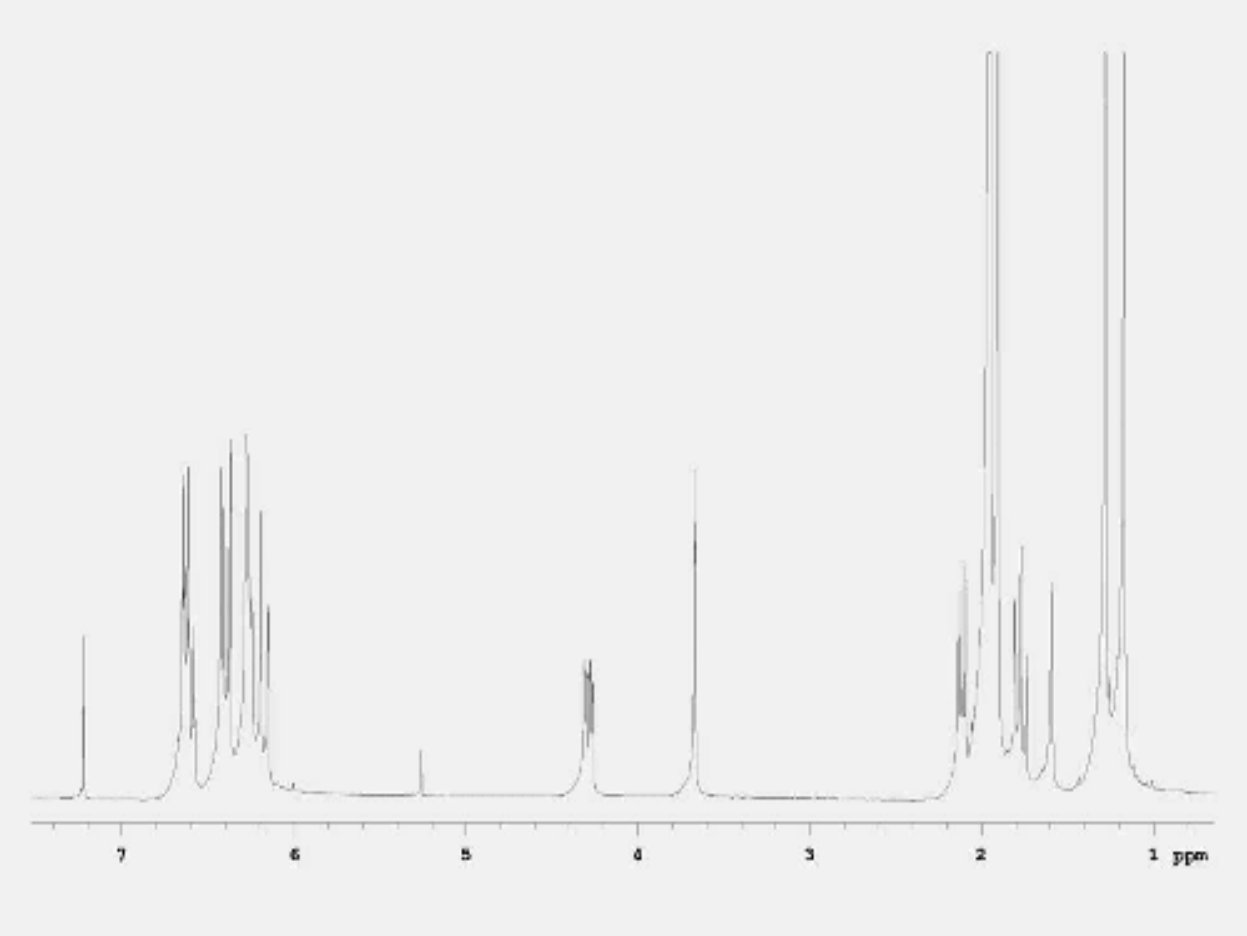

Figure 3.5 Proton NMR spectrum of astaxanthin from *P. rhodozyma* carotenoid-enriched DDGS

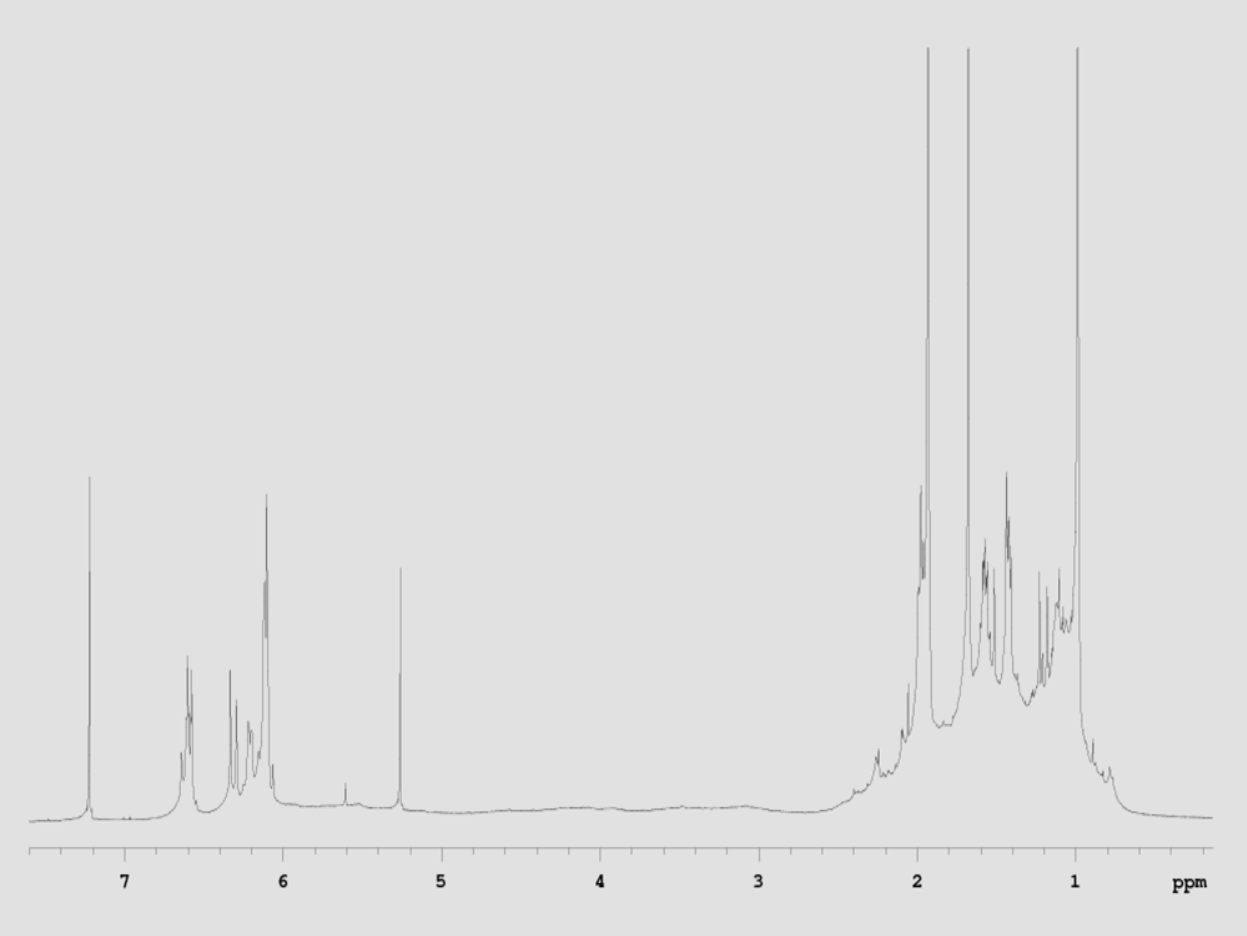

Figure 3.6 Proton NMR spectrum of beta-carotene from *P. rhodozyma* carotenoid-enriched DDGS

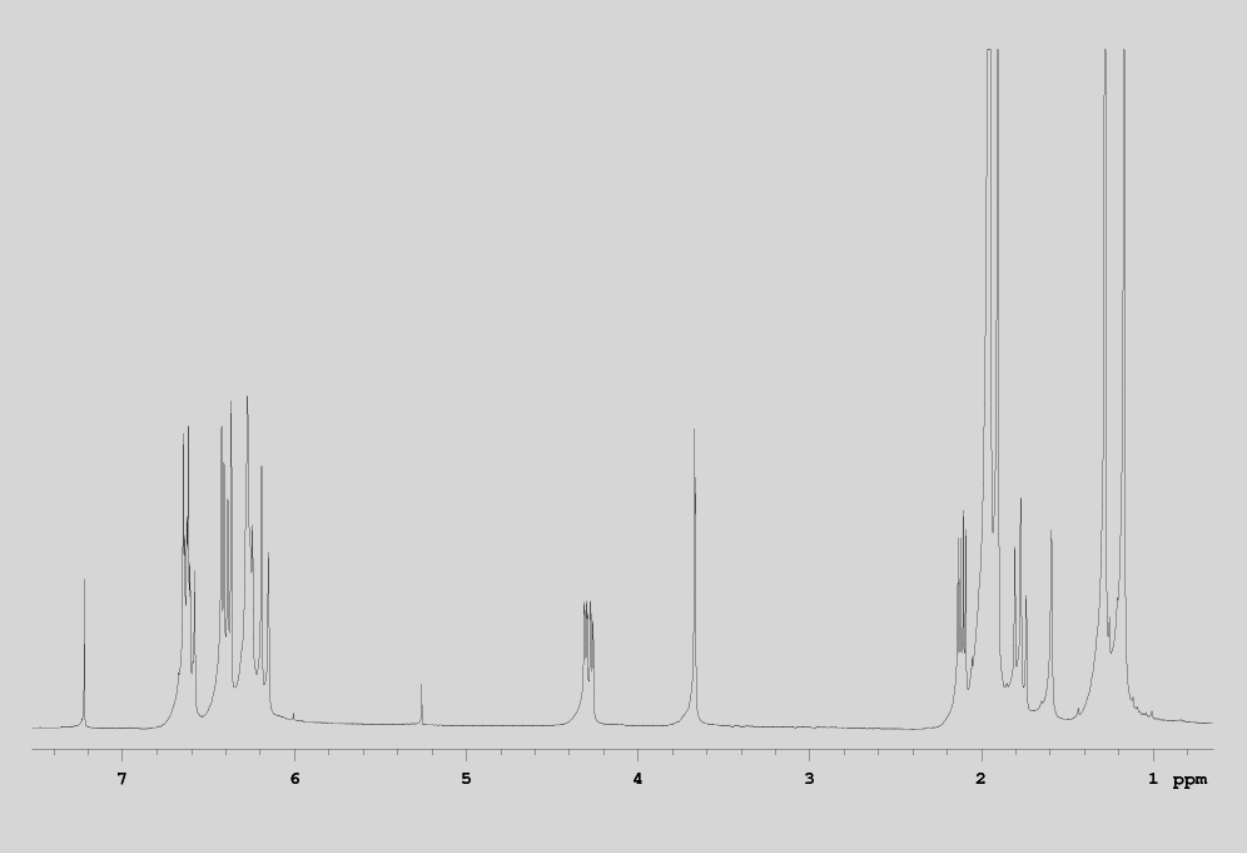

Figure 3.7 Proton NMR spectrum of standard astaxanthin.

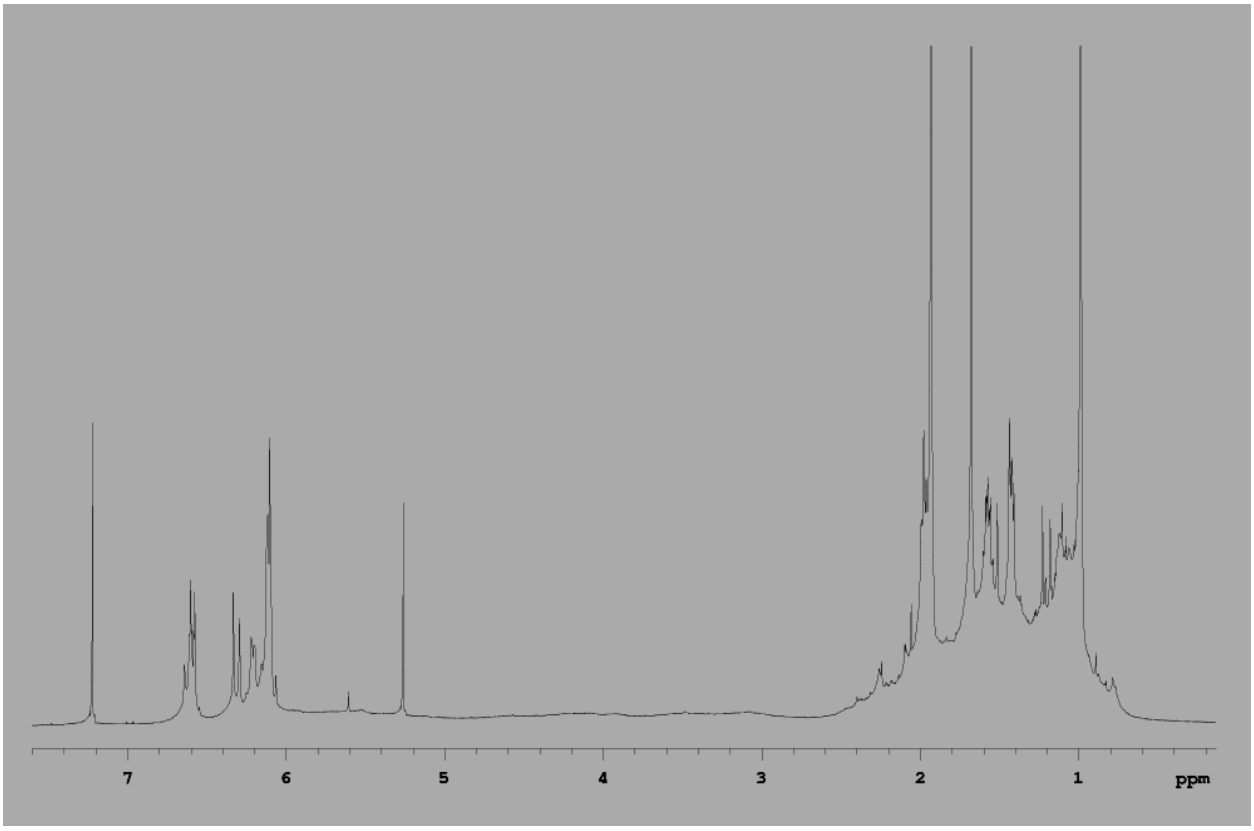

Figure 3.8 Proton NMR spectrum of standard beta-carotene.

CHAPTER 4 - [3]Nutritional profile of carotenoid-enriched DDGS produced by mono- and mixed culture fermentation of *Phaffia rhodozyma* and *Sporobolomyces roseus*

Abstract

Distillers dried grain with solubles (DDGS), a co-product of biofuel industry is primarily used as livestock feed. Carotenoid-enriched DDGS developed as a value-added animal feed to provide carotenoids, astaxanthin and β-carotene from mono- and mixed culture (Mx) fermentation of red yeasts, *Phaffia rhodozyma* (PR) and *Sporobolomyces roseus* (SR) were evaluated for their nutritional composition and compared to the control (C) DDGS. Apart from providing carotenoids, the secondary fermentation by red yeasts resulted in low fiber (C>PR>SR>Mx), enhanced crude fat (Mx>SR>PR>C), and decreased protein and amino acids (C>SR>Mx>PR). The %N was also low in value-added DDGS (C>SR>Mx>PR), while %P, S and K were similar compared to the control. Both *P. rhodozyma* and *S. roseus* were able to degrade corn fiber by 77% and 66%, respectively, in the absence of any pretreatment. The fatty acid profiles were different among the treatments. The predominant fatty acids in C and PR were linoleic acid, oleic acid, palmitic acid and stearic acid, whereas vaccenic acid, linoleic acid, palmitic acid and stearic acid were predominant in SR and Mx. Both, SR and Mx fermentation produced vaccenic acid, a monounsaturated fatty acid absent both in control and *P. rhodozyma* monoculture. DDGS with reduced fiber and nitrogen is highly desirable for non-ruminants and in aquaculture feed. Vaccenic acid can be useful for both lactating cows and beef cattle. Depending upon the animal feed requirements, the carotenoid-enriched DDGS can be used to make feed

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blends. This study shows that microbial modification of nutrient composition of DDGS could be explored to obtain tailor-made feeds/feed blends for specific animal diets.

Introduction

Distillers dried grain with solubles (DDGS) is primarily used as animal feed as it is rich in protein and energy. However, it does not provide optimal concentrations of all nutrients. For example, in lactating cows lysine is the limiting amino acid in DDGS; lysine, threonine and tryptophan in swine diets; and lysine and methionine in aquaculture feed (US Grains Council, 2007). DDGS diet is probably best suited for beef cattle; however, it can provide more protein and phosphorous than necessary (US Grains Council, 2007). DDGS with 0.4% sulfur is not advisable for beef cattle and results in poor animal performance (Tjardes and Wright, 2002). Overall, DDGS is ideal for beef cattle as it provides low starch and high fiber. However, high fiber is an impediment in using higher inclusion rates of DDGS in non-ruminant feed. The poor digestibility of dietary fiber in swine (43% apparent total tract digestibility of dietary fiber) is the primary reason for reduced digestibility of dry matter and subsequently reduced digestibility of energy (Stein and Shruson, 2009). Accordingly, based on the specific animal diets, DDGS is supplemented with soybean meal or other agricultural products to overcome any nutrient limitation (US Grains Council, 2007).

Recently, in an effort to bring about value addition to DDGS, carotenoid-enriched DDGS was developed by red yeast fermentation as a means to provide 'natural' and inexpensive carotenoids in animal feeds (Chapter 2, Ananda and Vadlani, 2010). Since carotenogenic yeasts are rich in fatty acids, especially polyunsaturated fatty acids, proteins and vitamins (PUFA; Libkind et al., 2008; Davoli et al., 2004; Sanderson and Jolly, 1994; Johnson et al., 1987), it is essential to evaluate the carotenoid-enriched DDGS for nutrients other than carotenoids. Of all

the nutrients, it is interesting to know if there is any reduction of fiber in DDGS. Previously, Hayman et al. (1995) were able to produce astaxanthin from *P. rhodozyma* on six co-products of corn wet-milling rich in corn fiber. Leathers (2003) pointed to the ability of yeasts like *Auerobasidium* and *P. rhodozyma* to break down corn fiber in the absence of any pretreatment. This ability is indeed valuable since corn fiber (composition of glucan 21.2%, xylan 17.2%, arabinan 12.9% galactan 4.1% and starch 17.5%; Ngheim et al., 2009) is a complex cross-linked structure not easily degraded by commercial enzymes.

Hypothesis 3.1: Apart from carotenoid-enrichment, secondary fermentation of whole stillage by red yeasts will reduce fiber and increase the fatty acid content.

Specifically, the objective of this study was to evaluate the nutritional composition of carotenoid-enriched DDGS from monoculture and mixed culture fermentation, and compare them with that of control DDGS.

Materials and Methods

Microbial cultures and inoculum generation

Culture maintenance and inoculum generation of *P. rhodozyma* and *Sporobolomyces roseus* are outlined in Chapter 2. A 10% (v/v) inoculum was used for monoculture fermentation, while 5% of each strain was used in mixed culture fermentation.

Media preparation

Optimized medium as outlined in Chapter 3 was used. A liter of the fermentation medium contained 15% whole stillage, 1.5% corn steep liquor, 7.7% glycerol and mineral salts (0.6g KH_2PO4, 0.3g $MgSO_4$, 0.3g $MnSO_4$ and 0.7g $ZnSO_4$). Corn whole stillage was procured from

Abengoa Bioenergy (Colwich, KS, USA). Media pH was about 6.0 before sterilization and was not adjusted any further.

Fermentation

Fermentation was carried out using a 2-L BBraun Biostat-B fermenter. About 1.5-L of the fermentation medium was sterilized in the fermenter at 121 °C for 30 min. Batch fermentation was carried out for seven days at 20 °C, 500 rpm and 1 (v/v) sterile air. Dissolved oxygen and pH were monitored for every 2 h. Three fermenter runs, one each for *P. rhodozyma* and *S. roseus* monocultures, and mixed culture fermentation were carried out. The entire fermentation broth was harvested on day 7, aliquoted into five bottles and freeze dried for five days. After freeze drying, samples were pooled and blended using a coffee blender. Samples were stored at –20 °C until further analyses. The control sample contained all the media ingredients except glycerol and freeze dried. Two representative samples from each treatment were subjected to nutritional profiling.

Carotenoid extraction and estimation

High performance liquid chromatography (HPLC) was used for quantification of carotenoids, astaxanthin and β-carotene and is outlined in Chapter 2.

Nutrition profiling

Nutrition composition ana1yses of the samples were conducted to include total amino acid profile, total fatty acid profile, crude fat and protein, crude fiber, %NDF and %ADF and %P, S and K. About 10 g of each representative sample from each treatment was sent to Agricultural Experiment Station Chemical Laboratories, University of Missouri (Columbia, MO) for estimating total amino acid profile (AOAC Official method 982.30 E (a, b, c; chapter 45.3.05, 2006)), total fatty acid profile (AOAC Official Method 996.06 AOCS Official Method

Ce 2-66, AOAC Official Method 965.49, AOAC Official Method 969.33), crude fat (acid hydrolysis, AOAC Official Method 954.02, 2006) and protein (Kjeldahl method, AOAC Official Method 984.13 (A-D), 2006). Estimation of %P, K, S and crude fiber, %NDF and %ADF was conducted at Analytical lab, Animal Science and Industry, KSU.

Results

The crude composition of DDGS and the secondary fermented products are presented in Table 4.1. Compared to the control, *P. rhodozyma*, *S. roseus* and mixed culture fermentation resulted in lesser protein, fiber and %N, and enhanced fat. Maximum reduction in % protein, % fiber and % N was seen in *P. rhodozyma*, and the best fat enhancement was seen in *S. roseus*. Both *P. rhodozyma* and *S. roseus* reduced fiber by an astounding 77% and 66% whereas mixed culture showed 63% reduction in crude fiber. %P, K and S did not reduce drastically compared to the control. However, *S. roseus* reduced %P and %K by 17% and 14% respectively, and *P. rhodozyma* reduced %S by 15%.

The amino acid profiles of all the treatments are presented in Table 4.2. Both monoculture and mixed culture fermentation resulted in lesser amino acids compared to the control. The highest amino acid reduction by 57% was brought about by *P. rhodozyma*, whereas 42% and 40% reduction was seen in mixed culture and *S. roseus*.

The fatty acid profiles of all the treatments are presented in Table 4.3. Both monoculture and mixed culture fermentation resulted in higher fatty acids compared to the control. Based on the abundance of different fatty acids (accounting for more than 2% of total fats), both, control and *P. rhodozyma* fermentation contained linoleic acid, oleic acid, palmitic acid and stearic acid. Linoleic acid in the control accounted for 52.7% whereas it accounted for only 34.6% in *P. rhodozyma*. Oleic acid, palmitic acid and stearic acid in *P. rhodozyma* fermented DDGS were

higher than that in the control (Table 4.3). Both, *S. roseus* and mixed culture showed similar fatty acid profiles with vaccenic acid, linoleic acid, palmitic acid and stearic acid being the most abundant in that order. Vaccenic acid was not seen in both the control and *P. rhodozyma* fermented DDGS (Table 4.3).

Discussion

The carotenoid-enriched DDGS from red yeast fermentation not only contained carotenoids, but also had reduced fiber and enhanced fat supporting hypothesis 4.1. Additionally, carotenoid-enriched DDGS had low protein and %N. Apart from carotenoids, other modifications to DDGS may or may not be beneficial depending on the specific needs of various animal diets. However, feed blends of carotenoid-enriched DDGS can provide the required daily dietary recommendation of carotenoids (1-120 ppm). Red yeast modifications in protein, fat and fiber content of DDGS are discussed as they can be exploited to develop tailor-made DDGS diets catering to the demands of different animal diets.

As noted earlier by Hayman et al. (1995) and Leathers (2003), in this study, *P. rhodozyma* was able to reduce DDGS fiber by 77% without any pretreatment. Additionally, *S. roseus* was also able to reduce fiber by 66%. Reduction in DDGS fiber can allow the expansion of the DDGS feed base, especially in non-ruminant feeds and aquaculture feeds. Srinivasan et al. (2005) developed 'eluseive', a process of sieving and elutriation to produce low fiber DDGS: sieving alone produced two fractions, one with low fiber and other with increased fat and protein; elutriation of these fractions further concentrated the fat and protein, and fiber, allowing the high fat and protein DDGS with low fiber could be used for non-ruminant feed. Additionally, Srinivasan et al. (2006) showed that the sieving and elutriation process reduced the quantity of DDGS but increased the value of DDGS, as high fat (13%) and high protein (33%) DDGS

fetches $5-$20 more per ton than DDGS with lower fat (11%) and protein (28%; Belyea et al., 2004). Secondary fermentation of whole stillage by red yeasts to reduce fiber is likely to be more economical than mechanical methods simply because additional processing or equipment costs are not accrued (eluseive costs $1.4 million with a payback in 2.5 to 4.6 years, Srinivasan et al., 2006), and is an added bonus in the production of a premium product, namely carotenoid-enriched DDGS. Low fiber DDGS may not be useful for livestock, but is definitely suitable for non-ruminants and aquaculture feed.

The protein and amino acid levels in DDGS were reduced substantially by red yeast fermentation. Also, the secondary fermentation did not alleviate the known deficiencies of amino acids like lysine, methionine, threonine or tryptophan required for lactating cows, swine, poultry or aquaculture feeds. However, in case of feed blends using protein rich sources like soybean, the low protein and fiber, and carotenoid-enriched DDGS may provide a balanced diet. Fish meal used as aquaculture feed is high in protein and nitrogen (US Grains Council, 2007). Carotenoid-enriched DDGS with low protein and fiber can be used as an ideal feed supplement along with fish meal to provide the necessary carotenoids, without providing excess proteins.

Red yeast fermentation increased the crude fat and fatty acid content and altered the fatty acid composition of DDGS. This alone should be able to fetch a higher price for DDGS. Soybean oil, oil seeds, vegetable oils, marine oils or animal fats are often used to supplement fat in animal feeds (US Grains Council, 2007; Chilliard et al., 2001). Instead, DDGS with enhanced fat can be used to supplement diets. Vaccenic acid, a monounsaturated fatty acid was produced in *S. roseus* and mixed culture fermentation. Vaccenic acid is primarily found in bovine milk and meat, accounting for 70% of *trans* fatty acids in ruminant-derived lipids (Cruz-Hernandez et al., 2007; Lock and Bauman, 2004). It is a known precursor of conjugated linoleic acid (CLA), and the

principal sources of CLA in human diets are dairy products and ruminant meat (Burdge et al., 2005). CLA is known to confer many health benefits to animals and humans (Burdge et al., 2005). Santora et al. (2000) studied the effects of feeding specific fatty acids and their fate in mice. They found that elaidic acid and *trans* vaccenic acid (TVA) from feed were incorporated similarly in mice, CLA found in mice fed with TVA was greater than that fed with CLA, and the conversion of TVA to CLA was about 11% of TVA or 50% of stored TVA. Additionally, CLA in the carcass was found only when CLA or TVA was fed to the mice. Chronic TVA dietary supplementation in obese dyslipidemic rats reduced plasma triglycerides along with improved dyslipidemia, without influencing food intake, body weight or glucose/insulin metabolism (Wang et al., 2009). Since vaccenic acid is abundant in *S. roseus* and mixed culture fermented DDGS, providing this to cattle may possibly increase the TVA and CLA levels in milk and meat especially since different types of forages and lipid supplementations have different effects on cow and goat milk fat composition and synthesis (Chilliard et al., 2001, 2003). If increased fat is not essential in animal feed, then *P. rhodozyma* fermentation of DDGS may be more suitable as it increased fat merely by 16%. On the other hand, if enhanced fat is required, mixed culture fermentation is suitable as it enhances fat by 80%.

Production of vaccenic acid in *S. roseus* is most likely due to the substrate namely DDGS. In the case of *S. roseus* grown in synthetic yeast extract dextrose (YED) broth, vaccenic acid production was not seen (Davoli et al., 2004). The significant fatty acids from yeast cells were linoleic acid (60-64% of total fats), followed by palmitic or stearic acid (16-20%) depending on aeration, with other fatty acids in trace amounts (<5%). However, fatty acid profiles of *P. rhodozyma* cells (Red Star® Phaffia Yeast from Red Star Speciality Products, Milwaukee, WI, USA in Sanderson and Jolly, 1994) were very similar to that seen in this study

with abundant fatty acids—linoleic acid (40%), oleic acid (32%) and palmitic acid (13%). Effect of culture media on fatty acid composition and relative abundance was seen in carotenogenic yeasts including *Sporobolomyces patagonicus* from Patagonia (Libkind et al., 2008). The major fatty acids were linoleic acid (40%), oleic acid (34%), palmitic acid (13%) and α–linoleic acid (9%) and their relative abundance was influenced by the media.

Libkind et al. (2008) hypothesized that carotenoids are lipid-based protection against oxidative stress and as more carotenoids are produced by the carotenogenic yeasts, more fatty acids, especially PUFA are produced. Similarly, Davoli et al. (2004) noted that fatty acid (from 14.4 to 42.2 mg/g) and carotenoid levels (from 109 to 412 μg/g) increased relative to biomass in *S. roseus* with enhanced aeration on synthetic YED medium. However, this may not be true for all red yeasts. *Rhodotorula gracilis* carotenoid and lipid levels were inversely related depending on C/N ratio of the synthetic media (Somashekar and Joseph, 2000). Similarly, *Rhodotorula glutinis* showed minimal increase in carotenoid levels (from 113 to 206 μg/g) upon aeration with unchanged levels of fatty acid at 19.6 mg/g in synthetic YED medium (Davoli et al., 2004). Apart from the hypothesis of Libkind et al. (2008), it is also likely that the higher fatty acid levels seen in some red yeasts and in the red yeast fermentation of DDGS are due to the antioxidant protection conferred by carotenoids that prevent lipid peroxidation.

It is probably convenient that the carotenoid-enriched DDGS is also enriched in fatty acids. Surai et al. (2001) have reviewed the uptake of carotenoids and the intrinsic role of fatty acids in carotenoid transport and absorption. Micelles formed from dietary lipids, transport and deliver carotenoids to the absorptive surfaces, implying the importance of the feed matrix. They also note that the amount and quality of dietary fat and fatty acids of varying chain length and saturation affect the transport and absorption of carotenoids.

The red yeast fermentation of DDGS altered the %N composition from 36-53%. This is probably useful in reducing nitrogen in animal wastes and fish farm effluents. The %P, K and S remained largely unchanged except for 17% and 14% reduction in %P and %K respectively by *S. roseus*, and 15% reduction of %S by *P. rhodozyma*. These reductions may not be significant and warrants further investigation.

Mycotoxins are found in corn and eventually find their way into corn DDGS. In an exhaustive survey, Zhang et al. (2009) evaluated mycotoxins namely aflatoxins, deoxynivalenol, fumonisins, T-2 toxin, and zearalenone in 235 DDGS samples from 20 ethanol plants in Midwestern U.S. and 23 export shipping containers from 2006 to 2008. The levels of these mycotoxins were either below the FDA guidelines for use in animal feed or were below the detection rate. Only 10% of the samples contained funonisin levels higher than the FDA guidelines for use in animal feed. It is indeed interesting to note that *P. rhodozyma* and *Xanthophyllomyces dendrorhous* are able to degrade more than 90% of ochratoxin A (OTA), one of the most important mycotoxins in about 7 days at 20 °C (Péteri et al., 2007). OTA was also adsorbed by the yeast cells after two days of fermentation and also by heat-treated (dead) cells. In the light of this finding, it is imperative to evaluate the carotenoid-enriched DDGS for mycotoxins. Since the levels of mycotoxins in the recent DDGS samples in the U.S. seem to be well within the FDA guidelines for use in animal feed, mycotoxins and their potential adsorption in *P. rhodozyma* and/or *S. roseus* does not seem to be a potential problem.

Conclusions

Secondary fermentation of corn whole stillage by red yeasts not only provides carotenoid-enriched DDGS, but also brings about two important changes: increase in fat and reduction in fiber. Additionally, there is reduction in protein and %N. The potential benefits of

the carotenoid-enriched DDGS should be thoroughly evaluated in animal studies. Carotenoid-enriched DDGS can be used to make feed blends to not only provide carotenoid, but also to balance the other nutrients like fat and protein. The use of microbial modification of DDGS to obtain tailor-made DDGS catering to the different animal diets is a definite possibility and should be explored to further the market of DDGS and eventually to sustain the biofuel industry.

Table 4.1 Nutrition profile of DDGS and carotenoid-enriched DDGS from read yeast fermentation

Fungus [a] Components	C	MX	PR	SR
%Crude Protein [b]	27.77	17.16 (38%)	12.95 (**53**%)	17.75 (36%)
%Crude Fat [c]	14.59	26.35 (**81**%)	17.07 (17%)	24.25 (66%)
%Crude fiber	5.31	1.99 (63%)	1.20 **(77%)**	1.81 (66%)
%NDF	22.25	9.68 (57%)	5.49 (**75**%)	8.42 (62%)
%ADF	7.00	4.61 (34%)	1.97 (**72**%)	3.66 (48%)
%N	4.44	2.74 (38%)	2.07 (**53**%)	2.84 (36%)
%P	0.81	0.85	0.81	0.67
%K	1.00	0.97	1.01	0.86
%S	0.70	0.67	0.59	0.66
Astaxanthin (ug/g)	0.00	2.73	50.91	-
β-carotene (ug/g)	0.00	240.00	79.86	119.99

[a] C-control DDGS, Mx-mixed culture, PR-*P. rhodozyma*, SR-*S. roseus*

[b] Kjeldahl

[c] acid hydrolysis

Numbers in parentheses indicate the % increase or decrease compared to the control and the maximum increase or decrease is boldfaced

Table 4.2 Amino acid profile

Fungus [a] Amino acids (w/w%)	C	MX	PR	SR
Taurine	0.04	0.04	0.03	0.04
Hydroxyproline	0.00	0.00	0.00	0.00
Aspartic Acid	1.78	1.32	0.81	1.36
Threonine	1.02	0.69	0.64	0.75
Serine	1.15	0.75	0.65	0.80
Glutamic Acid	3.81	1.94	0.98	1.87
Proline	2.02	0.98	0.76	1.02
Lanthionine	0.00	0.00	0.00	0.00
Glycine	1.18	0.98	0.62	1.01
Alanine	1.95	1.00	0.71	1.04
Cysteine	0.57	0.44	0.23	0.45
Valine	1.43	0.86	0.79	0.91
Methionine	0.58	0.27	0.20	0.29
Isoleucine	1.04	0.62	0.63	0.65
Leucine	2.99	1.17	1.12	1.24
Tyrosine	0.94	0.52	0.38	0.51
Phenylalanine	1.19	0.61	0.48	0.61
Hydroxylysine	0.00	0.00	0.00	0.00
Ornithine	0.04	0.04	0.01	0.04
Lysine	1.12	0.93	0.74	0.94
Histidine	0.82	0.47	0.45	0.49
Arginine	1.39	0.84	0.61	0.88
Tryptophan	0.22	0.16	0.13	0.18
Total	25.22	14.58 (42%)	10.91 (**57**%)	15.06 (40%)

[a] C-control DDGS, Mx-mixed culture, PR-*P. rhodozyma*, SR-*S. roseus*

Numbers in parentheses indicate the % decrease compared to the control and the maximum decrease is boldfaced

Table 4.3 Fatty acid profile

Fungus [a]	**C**	**MX**	**PR**	**SR**
Fatty acid (% of total fat)				
Myristic (14:0)	0.06	0.45	0.18	0.45
Myristoleic (14:1)	0.00	0.00	0.00	0.00
(C15:0)	0.00	0.13	0.09	0.14
Palmitic (16:0)	14.12	14.30	17.59	14.02
Palmitoleic (16:1)	0.22	0.84	0.16	0.69
(17:0)	0.08	0.12	0.23	0.12
(17:1)	0.05	0.12	0.05	0.12
Stearic (18:0)	2.53	2.98	10.10	4.07
Elaidic (18:1*t9*)	0.06	0.12	0.07	0.12
Oleic (18:1*n9*)	26.98	0.00	33.94	0.00
Vaccenic (18:1*n7*)	0.00	61.66	0.00	60.95
Linoleic (18:2)	52.70	15.73	34.64	15.41
Linolenic (ω18:3)	1.49	0.72	0.88	0.74
(ω18:4)	0.00	0.00	0.00	0.00
Arachidic (20:0)	0.44	0.30	0.85	0.34
(20:1n9)	0.25	0.62	0.09	0.66
(20:3 ω3)	0.00	0.00	0.00	0.00
Arachidonic (20:4*n6*)	0.00	0.00	0.00	0.00
Arachidonic (20:4 ω3)	0.00	0.00	0.00	0.00
(20:5 ω3; EPA)	0.00	0.00	0.00	0.00
Docosanoic (22:0)	0.23	0.45	0.45	0.53
Erucic (22:1*n9*)	0.00	0.06	0.00	0.07
(22:5 ω3; DPA)	0.00	0.00	0.00	0.00
(22:6 ω3; DHA)	0.16	0.09	0.03	0.11
Lignoceric (24:0)	0.34	0.79	0.32	0.93
Nervonic (24:1*n9*)	0.00	0.03	0.00	0.03
% Crude Fat	14.59	26.35	17.07	24.25

[a] C-control DDGS, Mx-mixed culture, PR-*P. rhodozyma*, SR-*S. Roseus*

CHAPTER 5 - Carotenoid value addition of cereal products by monoculture and mixed culture fermentation of *Phaffia rhodozyma* and *Sporobolomyces roseus*

Abstract

Carotenoid value addition of corn whole stillage by red yeast fermentation has successfully produced astaxanthin and β-carotene-enriched distillers dried grains with solubles (DDGS) animal feed. In this study commonly used animal feeds, rice bran, wheat bran, milo whole stillage, and soybean products were evaluated as substrates for carotenoid value addition. *Phaffia rhodozyma* and *Sporobolomyces roseus* monoculture and mixed culture submerged fermentation of these substrates supplemented with 5% glycerol were evaluated for astaxanthin, β-carotene, and residual glycerol. Among all the substrates, full fat rice bran and full fat soy flour resulted in the best astaxanthin (80 µg/g by *P. rhodozyma*) and β-carotene yields (836 µg/g by *S. roseus*). *Phaffia rhodozyma* produced the highest astaxanthin yield on each substrate, whereas depending on the substrate, either mixed culture or *S. roseus* monoculture produced the highest β-carotene yield. Soy hull was a poor substrate for carotenoid value addition. Both yeasts used glycerol as a carbon source for carotenoid production. Carotenoid value addition of these substrates provides as much or more than the required daily dosage of carotenoids in animal feed, allowing the production of feed blends. These carotenoid-enriched feeds could be particularly valuable in the poultry and aquaculture industry which require feed that contains carotenoid pigments.

Introduction

Astaxanthin and β-carotene are important carotenoids in animal feed. Aquaculture feed is especially dependent on astaxanthin and is the principal market driver for astaxanthin (Venugopal, 2009). The recommended daily dosage of carotenoids in animal feed ranges from 30 to 120 mg/kg feed (Venugopal, 2009; An et al. 2006; Hayek, 2000). Chapter 2 outlines the production of carotenoid-enriched DDGS by secondary fermentation of corn whole stillage using red yeasts *Phaffia rhodozyma* and *Sporobolomyces roseus*. Monoculture and mixed culture fermentations of corn whole stillage were carried out and the astaxanthin and β-carotene yields in mixed culture and *P. rhodozyma* monoculture were 17 and 188 µg/g and 36 and 104 µg/g, respectively, whereas *S. roseus* produced 233 µg/g of β-carotene (Chapter 2). The resultant value-added product could allow production of a feed blend because the enriched DDGS contained twice the prescribed concentration of total carotenoids. Many cereal products that are used chiefly as animal feed (e.g., milo whole stillage, rice bran, wheat bran, and soy products) are potential substrates for carotenoid value addition.

Hypothesis 4.1: Cereal based products used as animal feed can be subjected to carotenoid value addition similarly to that carried out for corn whole stillage, and can provide astaxanthin and β-carotene enriched feeds or feed blends.

Based on the proof of concept for carotenoid value addition (Chapter 2), the objectives of this study were to (1) enrich animal feeds, namely milo whole stillage, defatted rice bran, full fat rice bran, full fat soy flour, defatted soy flour, soy meal, soy hull, and wheat bran, with carotenoids by red yeast fermentation and (2) compare astaxanthin and β-carotene production by monocultures and mixed culture of *P. rhodozyma* and *S. roseus* on these substrates.

Materials and Methods

Microbial strains

Lyophilized cultures of red yeasts *P. rhodozyma* (ATCC 24202) and *S. roseus* (ATCC 28988) were obtained form American Type Culture Collection (ATCC, Manassas, VA). The selected strain of *P. rhodozyma* produces astaxanthin and β-carotene, whereas the *S. roseus* strain produces only β-carotene. Culture maintenance and long-term preservation procedures followed those described in chapter 2.

Inoculum generation

Inoculum was generated according to that described in Chapter 2. Briefly, inoculum for each fungal strain was prepared by inoculating a loopful of cells from respective slants into sterile 100 ml YMB in 500 ml flasks and incubated at 18°C and 180 rpm for 72 h. Development of orange and red color in *P. rhodozyma* and *S. roseus* flasks, respectively, indicated good fungal growth. A 10% (v/v) inoculum was used. For monoculture fermentation, 10 ml of each strain was used for media inoculation, and 5 ml of each strain was used for mixed culture fermentation.

Media preparation

Eight different substrates were used in this study: defatted rice bran and full fat rice bran (Nutracea, Phoenix, AZ, USA), milo whole stillage (Nesika Energy, Scandia, KS, USA), full fat soy flour (Barry farm, Wapakoneta, OH, USA), and defatted soy flour, soy meal, soy hull and wheat bran (Kansas State University Department of Grain Science and Industry, Manhattan, KS, USA). Defatted rice bran, full fat rice bran, wheat bran, soy meal and soy hull samples were ground with an Udy-grinder at setting 0. Ground samples were sieved (US standard sieves), and the <600 μm fraction was used. A liter of the basal fermentation medium contained 5% glycerol and the following minerals: 1g KH_2PO_4, 0.5g $MgSO_4$, 0.5g $MnSO_4$, and $ZnSO_4$. All substrates

except milo whole stillage were added to the basal media at 5% concentration because higher concentrations made the media highly viscous. Milo whole stillage was used at 25%, similarly to the corn whole stillage medium used in chapter 2. Media pH ranged from 5.5 to 6.0 before sterilization and was not adjusted further. Media were sterilized by autoclaving at 121°C for 30 min.

Fermentation conditions

About 100 ml of respective media were taken in 500 ml flasks per fungal treatment. Two replicates per treatment were maintained. The flasks were inoculated and incubated at 18°C and 180 rpm for 11 days. Samples were harvested on days 3, 5, 7, 9, and 11 and centrifuged. The supernatant was used for glycerol analyses, and the pellets were freeze-dried for 24 h and stored at –80°C until further analysis.

Extraction and detection of carotenoids by HPLC

The method outlined in Chapter 2 was used for carotenoid extraction and analyses. Briefly, freeze-dried samples were ground with 0.2 g of acid-washed sand, and carotenoids were extracted in dichloromethane solvent. Samples were centrifuged, and the supernatant was filtered into 1.5-ml HPLC vials through 0.2-µm filters.

A Shimadzu HPLC equipped with LC-20AB pump, SIL -20AC autosampler, SPD-M20A PDA detector, and CTO-20A column oven was used to quantify carotenoids. A Phenomenex Prodigy C_{18} column (150 mm length and 4.6 mm internal diameter) along with a C_{18} guard column was used to separate carotenoids. Astaxanthin and β-carotene standards were obtained from Sigma Aldrich (St Louis, MO, USA). Carotenoid yield was expressed as µg per gram of freeze-dried sample instead of per gram of yeast cells as it is impossible to sediment only yeast cells from the fermented sample.

Extraction and detection of glycerol

About 100 µl of the supernatant was diluted 1:1 with water and filtered using 0.45-µm syringe filters, and samples were analyzed with a Shimadzu HPLC equipped with refractory index detector and CTO-20A column oven at 80°C. Water was used as the mobile phase with a flow rate of 0.6 ml/min. A Rezex-Organic acid column was used to quantify glycerol.

Statistics

Pearson correlation between residual glycerol and carotenoid production for each treatment was carried out using PROC CORR at P=0.05 (SAS version 9.1.4).

Results

Carotenoid yields and glycerol levels in different media are outlined in Table 5.1. Overall, the best astaxanthin and β-carotene yields were produced by *P. rhodozyma* monoculture on rice bran (80 µg/g) and *S. roseus* on full fat soy flour (836 µg/g), respectively. *Phaffia rhodozyma* produced the highest astaxanthin yield on each substrate. The highest β-carotene yields were produced by mixed culture in milo whole stillage and rice bran, by *P. rhodozyma* monoculture in soy hull, and by *S. roseus* monoculture in full fat soy flour, defatted soy flour, soy meal, defatted rice bran and wheat bran. Soy hull was a poor substrate for value addition: mixed culture and *S. roseus* monoculture did not produce any astaxanthin and β-carotene, respectively.

Residual glycerol at each time point varied within and between treatments for each substrate (Table 5.1). For example, by day 5, all the glycerol was consumed by the mixed culture in wheat bran but much of it remained in soy hull. In most treatments, there was a negative correlation between residual glycerol and carotenoids, which suggests that more carotenoids were synthesized as the yeasts consumed more glycerol (Table 5.2). There was no correlation

between glycerol and carotenoid production in all treatments on defatted rice bran and soy hull and mixed culture on wheat bran. In defatted rice bran, unlike other substrates, the carotenoid production peaked on day 7 or 9 and then had a decreasing trend, while in soy hull the different fungal treatments did not utilize glycerol effectively resulting in a lack of correlation. However, in wheat bran mixed culture fermentation, glycerol was utilized rapidly and by day 5 there was no residual glycerol, resulting in a lack of correlation between glycerol and carotenoids. Except for defatted rice bran, where the highest yields of both carotenoids were on day 9 of fermentation, all other substrates showed highest yields on day 11.

Discussion

In this study, carotenoid enrichment of milo whole stillage, rice bran, soy flour, soy meal, soy hull, and wheat bran by fermentation of red yeasts was carried out as previously conceived for corn whole stillage, thus supporting hypothesis 4.1. Astaxanthin yields of *P. rhodozyma* and mixed culture fermentation varied depending on the substrate from 0 to 80 µg/g and 0 to 17 µg/g, respectively, and β-carotene yields of *P. rhodozyma*, *S. roseus*, and mixed culture varied from 34 to 162 µg/g, 0 to 837 µg/g, and 12 to 282 µg/g, respectively, confirming that the carbon source in the medium influences carotenoid production (Nghiem et al., 2009). Additionally, the fat content of the substrates also influences carotenoid production. Ciegler et al. (1959) have previously shown that addition of natural oils and fatty acids can stimulate β-carotene production in *Blakslea trispora*. They showed that oils and fats containing large amounts of oleic acid and linoleic acid particularly stimulated β-carotene production. Furthermore, the supplementation of oils and their concentrations in the media influence not only the carotenoid production but also their relative abundance in *B. trispora* (Mantzouridou and Tsimidou, 2007). Although the substrates were not evaluated for their fat content, nutritional profiles indicate that fat content of

these substrates is highly variable (Table 5.3). In this study, β-carotene production on the different substrates seems proportional to their fat content (Table 5.3): the best β-carotene production was on full fat soy flour, and the least on soy hull, with intermittent production on other substrates.

The results of the present study were compared with those from the unoptimized corn whole stillage medium on day 9 (Chapter 2) and found that the carotenoid yields in this study surpassed that from corn whole stillage. Overall, rice bran and full fat soy flour were the best substrates for astaxanthin and β-carotene production. Unlike carotenoid enrichment of corn whole stillage (Chapter 2), results of the present study support the hypothesis that mixed culture can produce higher carotenoid yields than the respective monocultures. For example, mixed culture fermentation of rice bran and milo whole stillage produced higher β-carotene yields than the respective monocultures.

Glycerol supplementation was carried out because (1) glycerol can act as a carbon source for astaxanthin production by *P. rhodozyma* (Kusdiyantini et al. 1998) and β-carotene production by *B. trispora* (Mantzouridou et al. 2008); (2) carotenoid production is increased by the balanced and increased formation of acetyl Co-A, pyruvate, and glyceraldehyde-3-phosphate, all of which can be produced by glycolysis of glycerol (Das et al. 2007); and (3) glycerol is a cheap and abundantly produced co-product of the biodiesel and soap industries and was evaluated as an effective supplement for β-carotene production by *B. trispora* (Mantzouridou et al. 2008). In this study, as in Kusdiyantini et al. (1998) and Mantzouridou et al. (2008), *P. rhodozyma* and *S. roseus* used glycerol as a carbon source for carotenoid production.

About 63 to 76 million tons of rice bran are produced annually worldwide (Kahlon, 2009); 7.5 million tons of wheat bran (Food navigator magazine, 2008;

www.foodnavigator.com) and 4.75 to 7.6 million tons of soy hull are produced annually in the United States alone (USDA, 2006: www.nass.usda.gov). Despite the abundant production of these products, they are used mainly as animal feed, as is corn whole stillage. Carotenoid enrichment provides an excellent opportunity for value-addition of these underutilized feed products. The value addition process described herein provides more than the prescribed dosage of carotenoids, which allows for production of feed blends. In addition to use in livestock and poultry production, these carotenoid-enriched feeds can also be used in the aquaculture industry, which relies heavily on carotenoids, especially astaxanthin (Venugopal, 2009). Process optimization for enhanced yield on these substrates can be carried out similarly to that of corn whole stillage (Ananda and Vadlani, 2010), along with the use of high-yielding strains.

Conclusions

This study confirms that carotenoid value addition of animal feeds, such as rice bran, wheat bran, milo whole stillage, and soy products, can be achieved by yeast fermentation similarly to that achieved for corn whole stillage, and can provide valuable carotenoids (i.e., astaxanthin and β-carotene) required in animal feed. Carotenoid enrichment allows these feeds which are traditionally used for livestock and poultry, to enter the aquaculture feed market, which has an inherent requirement for carotenoid pigments.

Table 5.1 Glycerol utilization and carotenoid production by red yeasts on different substrates

Substrate [a]	Fungus [b]	Compound [c]	Day 3	Day 5	Day 7	Day 9	Day 11
MWS	Mx	Glycerol	25.44 ± 0.71	25.23 ± 0.15	1.2 ± 0.79	0	0
		Astaxanthin	0.42 ± 0.09	2.0 ± 0.22	4.91 ± 0.19	6.48 ± 0.41	**6.61 ± 0.52**
		β–carotene	34.9 ± 2.68	62.66 ± 0.42	159.74 ± 4.42	169.49 ± 1.49	**254.82 ± 2.84**
	PR	Glycerol	35.06 ± 0.04	27.8 ± 0.34	18.92 ± 0.75	16.59 ± 1.26	0
		Astaxanthin	3.64 ± 0.44	7.21 ± 2.05	14.8 ± 0.36	22.12 ± 1.66	**28.2 ± 0.89**
		β–carotene	5.55 ± 0.38	43.62 ± 4.18	72.49 ± 0.51	85.21 ± 1.21	**138.03 ± 3.52**
	SR	Glycerol	26.87 ± 0.69	15.265 ± 0.685	0.27_0.1	0	0
		β–carotene	44.2 ± 1.08	95.95 ± 6.04	190.21 ± 2.0	196.39 ± 5.25	**199.96 ± 1.85**
FFRB	Mx	Glycerol	19.62 ± 0.29	6.45 ± 1.95	0	0	0
		Astaxanthin	0	1.69 ± 0.52	2.79 ± 0.31	2.97 ± 0.78	**3.25 ± 0.45**
		β–carotene	81.65 ± 1.93	124.81 ± 12.87	226.28 ± 9.2	232.24 ± 3.89	**282.43 ± 13.27**
	PR	Glycerol	22.41 ± 1.09	13.45 ± 0.77	0.47 ± 0.27	0.28 ± 0.12	0
		Astaxanthin	6.12 ± 0.6	15.69 ± 1.98	34.09 ± 1.93	52.47 ± 4.98	**80.42 ± 16.33**
		β–carotene	62.56 ± 0.5	58.94 ± 4.64	107.45 ± 6.19	149.61 ± 10.36	**149.53 ± 27.74**
	SR	Glycerol	19.79	10.2	2.1	0.09 ± 0.06	0
		Beta–carotene	0	68.4 ± 5.98	119.92 ± 14.25	128.92	**196.0 ± 11.47**
DRB	Mx	Glycerol	26.87 ± 0	4.37 ± 1.2	0.085 ± .025	0	0
		Astaxanthin	1.61 ± 0.67	2.45 ± 0.82	**11.77 ± 9.17**	3.09 ± 0.62	2.42 ± 0.28
		β–carotene	71.1 ± 0.34	104.36 ± 4.41	132.71 ± 15.87	**156.82 ± 11**	132.67 ± 25.83
	PR	Glycerol	34.16 ± 0	31.2 ± 0	28.41 ± 1.855	24.62 ± 1.62	23.4 ± 0.48
		Astaxanthin	2.0 ± 0.55	14.86 ± 1.29	16.67 ± 2.21	**20.84 ± 1.1**	16.94 ± 2.65
		β–carotene	0	29.0 ± 1.72	47.59 ± 3.09	**53.54 ± 2.11**	37.26 ± 1.11
	SR	Glycerol	25.24 ± 2.61	2.24 ± 0	0.89 ± 0.45	0.37 ± 0.2	0
		β–carotene	0	38.07 ± 7.12	66.21 ± 1.56	**236.145 ± 19.95**	80.46 ± 14.2
FFSF	Mx	Glycerol	18.65 ± 0.57	1.02 ± 0.24	0	0	0
		Astaxanthin	2.61 ± 0.01	3.04 ± 0.06	4.3 ± 0.3	**4.48 ± 0.19**	3.55 ± 0.035
		β–carotene	157.48 ± 0.4	422.59 ± 1.72	753.32 ± 15.29	**809.91 ± 4.69**	753.62 ± 0.89
	PR	Glycerol	33.45 ± 0.14	18.54 ± 0.12	8.08 ± 0.26	0.33 ± 0.12	0
		Astaxanthin	5.73 ± 0.01	12.84 ± 0.16	20.37 ± 0.03	38.14 ± 0.3	**46.0 ± 0.2**
		β–carotene	12.89 ± 1.2	68.72 ± 1.36	36.83 ± 3.6	118.99 ± 0.69	**126.01 ± 1.55**
	SR	Glycerol	23.60 ± 0.46	0.64 ± 0.07	0	0	0

		β–carotene	142.43 ± 2.22	377.31 ± 1.29	625.0 ± 3.79	727.31 ± 3.97	**836.55 ± 6.61**
DSF	Mx	Glycerol	15.21 ± 0.42	4.44 ± 0.75	0	0	0
		Astaxanthin	2.95 ± 0.15	6.66 ± 0.06	10.44 ± 0.06	**11.61 ± 0**	11.48 ± 0.18
		β–carotene	103.2 ± 2.58	165.35 ± 0.55	394.72 ± 3.33	**449.63 ± 0.73**	402.55 ± 1.76
	PR	Glycerol	34.98 ± 0.49	33.19 ± 0.19	28.25 ± 0.84	19.14 ± 0.9	6.15 ± 1.15
		Astaxanthin	5.08 ± 0.03	8.34 ± 0.09	17.53 ± 1.2	27.61 ± 0.23	**36.55 ± 0.35**
		β–carotene	0	72.0 ± 0.86	128.27 ± 2.28	144.01 ± 0.27	**161.64 ± 1.52**
	SR	Glycerol	17.12 ± 0.26	1.38 ± 0.11	0.32 ± 0.09	0	0
		β–carotene	108.53 ± 2.23	174.06 ± 0.34	372.46 ± 4.61	428.67 ± 1.2	**532.58 ± 4.21**
SM	Mx	Glycerol	15.46 ± 0.25	3.94 ± 0.71	0	0	0
		Astaxanthin	1.63 ± 0.09	2.84 ± 0.01	5.36 ± 0.12	5.81 ± 0.1	**8.58 ± 0.03**
		β–carotene	103.46 ± 1.29	139.94 ± 0.01	371.16 ± 1.37	392.4 ± 0.27	**433.9 ± 1.36**
	PR	Glycerol	38.41 ± 0.29	33.98 ± 0.04	21.96 ± 0.28	16.86 ± 0.14	4.32 ± 0.10
		Astaxanthin	5.9 ± 0.28	5.94 ± 0.03	15.73 ± 0.05	20.52 ± 0.07	**30.98 ± 0.19**
		β–carotene	0	68.12 ± 0.37	112.78 ± 0.62	123.32 ± 0.5	**135.62 ± 0.07**
	SR	Glycerol	22.85 ± 0.29	5.51 ± 0.18	0	0	0
		β–carotene	120.81 ± 0.69	158.94	289.51 ± 0.23	434.17 ± 6.75	**462.76 ± 1.39**
SH	Mx	Glycerol	23.33	18.77 ± 3.65	10.01	4.11 ± 3.61	1.01 ± 0.84
		Astaxanthin	0	0	0	0	0
		β–carotene	0	1.4 ± 0.9	4.8 ± 0.99	10.56 ± 1.22	**12.05 ± 1.25**
	PR	Glycerol	37.75 ± 7.73	35.09 ± 0.85	34.34 ± 1.29	31.8 ± 1.19	29.19 ± 2.83
		Astaxanthin	0	2.6 ± 0.25	2.78 ± 0.11	4.07 ± 1.16	**5.22 ± 0.36**
		β–carotene	0	18.29 ± 3.23	25.04 ± 10.34	27.92 ± 7.3	**34.46 ± 6.24**
	SR	Glycerol	18.5	15.72 ± 2.34	12.4 ± 0.59	4.0 ± 0.5	0.84 ± 0.43
		β–carotene	0	0	0	0	0
WH	Mx	Glycerol	13 ± 1.54	0	0	0	0
		Astaxanthin	1.88 ± 0.69	2.92 ± 1.23	3.6 ± 1.42	4.67	**7.42 ± 1.29**
		β–carotene	57.16 ± 7.64	87.97 ± 16.68	145.0 ± 6.83	140.29 ± 3.58	**159.58 ± 2.68**
	PR	Glycerol	34.55 ± 0.55	33.39 ± 4.44	23.71 ± 0.12	14.53 ± 0.04	0
		Astaxanthin	4.54 ± 0.76	9.4 ± 1.64	15.36 ± 1.95	25.31 ± 6.22	**66.75 ± 8.21**
		β–carotene	0	38.02 ± 7.8	43.04 ± 12.31	64.65 ± 4.2	**78.91 ± 6.45**
	SR	Glycerol	20.62 ± 1.54	0.765 ± 0.26	0.59 ± 0.5	0.38 ± 0.3	0
		β–carotene	0	70.46 ± 29.13	122.79 ± 9.12	143.94 ± 18.16	**198.39 ± 8.41**

[a] MWS-milo whole stillage, FFRB-full fat rice bran, DRB-defatted rice bran, FFSF-full fat soy flour, DSF-defatted soy flour, SM-soy meal, SH-soy hull, WB-wheat bran

[b] Mx-mixed culture, PR-*Phaffia rhodozyma*, SR-*Sporobolomyces roseus*

[c] Glycerol expressed as mg/g of media; Carotenoids expressed as μg/g of media; Means and standard error expressed; Highest yield per treatment is boldfaced.

Table 5.2 Correlation of residual glycerol and carotenoids produced

Substrate [a]	Fungus [b]	Astaxanthin [c]		β-carotene	
		R^2	*P*	R^2	*P*
MWS	Mx	–0.675	*0.0321*	–0.869	*0.011*
	PR	–0.96	*<0.0001*	–0.96	*<0.0001*
	SR	-	-	–0.675	*0.032*
FFRB	Mx	–0.909	*0.0017*	–0.876	*0.0019*
	PR	–0.912	*0.006*	–0.761	*0.0171*
	SR	-	-	–0.63	0.0689
DRB	Mx	–0.054	0.89	–0.588	*0.076*
	PR	–0.238	0.57	–0.588	0.219
	SR	-	-	–0.468	0.203
FFSF	Mx	–0.887	*0.0006*	–0.86	*0.0014*
	PR	–0.984	*<0.0001*	–0.829	*0.003*
	SR	-	-	–0.87	*0.0009*
DSF	Mx	–0.89	*0.0006*	–0.87	*0.0009*
	PR	–0.98	*<0.0001*	–0.98	*<0.0001*
	SR	-	-	–0.75	*0.0113*
SM	Mx	–0.88	*0.0006*	–0.88	*0.0006*
	PR	–0.951	*<0.0001*	–0.88	*0.0006*
	SR	-	-	–0.87	*0.0009*
SH	Mx	-	-	0.058	0.87
	PR	0.389	0.26	–0.276	0.438
	SR	-	-	-	-
WB	Mx	–0.547	0.126	–0.38	0.266
	PR	–0.936	*<0.0001*	–0.927	0.*0026*
	SR	-	-	–0.77	*0.0136*

- carotenoid not produced; Significant *P* (<0.05) is italicized

Table 5.3 Nutrient composition of various agricultural products

Substrates [a] **Components (%)**	**Corn DDGS** [b]	**Milo DDGS** [c]	**FFRB** [d]	**DRB** [e]	**FFSF** [d]	**DSF** [d]	**SM** [d]	**SH** [f]	**WB** [d]
Protein	27.77	32.0	13.35		34.54	47.0	44.95	9-12	15.55
Fiber, total dietary	5.39	25.0	21.0		9.6	17.5	-		42.8
Carbohydrates			49.69		35.19	38.37	40.14		64.51
Sugars, total			0.9		7.5	18.88			0.41
Total lipid (fat)	14.59	11.8-8.0	20.85	≤2.0	20.65	1.22	2.39		4.25
Fatty acids, total saturated (% of total fat)	2.46		4.171		2.987	0.136	0.268		0.63
Fatty acids, total MUFA	3.98		7.549		4.561	0.208	0.409		0.637
Fatty acids, total PUFA	8.02		7.459		11.657	0.533	1.045		2.212
18:2	7.64		7.143		10.28	0.47	0.921		2.039
18:3	0.217		0.316		1.378	0.063	0.123		0.167

[a] DDGS-distillers dried grains with solubles, FFRB-full fat rice bran, DRB-defatted rice bran, FFSF-full fat soy flour, DSF-defatted soy flour, SM-soy meal, SH-soy hull, WB-wheat bran

[b] DDGS sample from Abengoa Bioenergy (Colwich, KS) used in Ananda and Vadlaini (2010), nutritional analyses by AESL, University of Missouri, Columbia, MO

[c] Lodge et al. 1997

[d] USDA National Nutritional database for standard reference, 2009

[e] Nutracea, 2007

[f] Mullin and Xu, 2001

CHAPTER 6 - Effect of precursors on carotenoid yield from *Phaffia rhodozyma* fermentation of different substrates

Abstract

Stimulation of carotenogenesis in carotenoid producing red yeasts, algae or bacteria for enhanced carotenoid production has been achieved by mevalonic acid addition. Recently, carotenoid-enriched feed was produced by *Phaffia rhodozyma* fermentation of inexpensive animal feeds whole stillage, rice bran, wheat bran and other cereal products. Since mevalonic acid improved carotenoid yield of *P. rhodozyma* in synthetic medium, this study tested if a similar enhancement was possible on animal feed substrates. Four concentrations, 0, 0.02, 0.04 and 0.1% of mevalonic acid as a precursor of *P. rhodozyma* production of astaxanthin and β-carotene were evaluated in five substrates namely defatted rice bran, full fat rice bran, wheat bran, corn whole stillage and synthetic media. Additionally, apple pomace and tomato pomace were also evaluated as a precursor of carotenogenesis. Four concentrations, 0, 0.05, 0.1 and 0.5% of apple pomace and tomato pomace were evaluated in *P. rhodozyma* fermentation of whole stillage and rice bran. Mevalonic acid, tomato pomace and apple pomace enhanced carotenoid yields in all substrates in that order. However, the optimal concentration of precursor and the percent increase of carotenoid yield in each substrate were variable indicating that substrate influenced the carotenoid stimulation. Among animal feed substrates, mevalonic acid in whole stillage resulted in the best astaxanthin yield of 220 μg/g and β-carotene of 904 μg/g. Tomato pomace resulted in 29% astaxanthin and β-carotene enhancement in whole stillage and apple pomace increased β-carotene production by 26% in whole stillage. Even if mevalonic acid is expensive, it is offset by the quantity used and also by the inexpensive process of producing

carotenoid-enriched DDGS. Optimization of tomato or apple pomace addition may further enhance the carotenoid yields.

Introduction

Natural astaxanthin and β-carotene obtained from algae, yeasts or fungi are high value products requiring continuous exploration of ways to enhance their yields. Process optimization, use of high yielding strains or strain improvement by mutagenesis or genetic engineering are well researched and commonly employed for carotenoid yield improvement especially in *Phaffia rhodozyma* (see reviews by Frengova and Beshkova, 2009, and Lukács et al., 2006). Apart from these routinely used methods, yield enhancement has been achieved by co-culturing with other microbes (Chapter 1), or by the addition of simple nutrients (Chapter 3), precursors, chemicals or elicitors: many natural oils, fatty acids, surfactants and β -ionone (Ciegler et al., 1959), Span-20 a surfactant (Kim et al., 1997) and hydrogen peroxide (Jeong et al., 1999) have enhanced β-carotene production in *Blakslea trispora*; lycopene (Johnson and Lewis, 1979), β-ionone (Lewis et al., 1990), acetic acid (Meyer and du Preez, 1993), valine (Meyer et al., 1993), α-pinene (Meyer et al., 1994), ethanol (Gu et al., 1997), mevalonate (Calo et al., 1995), citrate (Flores-Cotera et al., 2001), n-hexadecane (Liu and Wu, 2006a) and hydrogen peroxide (Liu and Wu, 2006b) have enhanced astaxanthin or total carotenoid production in *P. rhodozyma*; organic acids of TCA cycle enhanced astaxanthin production in algae *Rhodopseudomonas sphetoides* (Higuchi and Kikuchi, 1963), *Rhodopseudomonas gelatinosa* (Noparatnaraporn et al., 1986), *Flavobacterium* sp. (Alcantara and Sanchez, 1999) and *Chlorella zofingiensis* (Chen et al, 2009), mevalonate and pyruvate enhanced carotenoid synthesis in *Haematococcus pluvialis* (Kakizono, 1991), and lycopene and β-carotene act as precursors for astaxanthin production in *H. pluvialis* (Harker and Young., 1995); addition of fungal elicitors like *Epicoccum nigrum* (Echavarri-

Erasum and Johnson, 2004), *Aspergillus* sp. (Margalith, 1993) and *Rhodotorula rubra*, *Rhodotorula glutinis*, *Panus conhatus*, *Coriolus versicolor*, *Mucor mucedo* and *Motieralla alpina* (Wang et al., 2006) enhanced carotenoid production in *P. rhodozyma*. It is important to note that all these compounds were evaluated in synthetic yeast extract based medium. For practical purposes, it is essential to understand if similar yield enhancements are possible in inexpensive substrates that can be used in large-scale production of carotenoids.

In Chapters 3 and 5, the development of a unique method to produce carotenoid-enriched animal feeds is outlined. The commonly used animal feeds like corn and milo distillers dried grains with solubles (DDGS), wheat bran, rice bran, soybean hull and soy meal with glycerol supplementation were fermented using red yeasts, *P. rhodozyma* and *Sporobolomyces roseus* to produce astaxanthin and/or β-carotene enriched feeds that could be used as animal feed or feed blends. Instead of investigating new compounds, effects of established precursors like mevalonic acid and lycopene can be evaluated in carotenoid fermentation of animal feed substrates (see Fig. 1.3). Lycopene is predominantly found in tomatoes. For economic viability, apple pomace and tomato pomace, both rich in carotenoids can be evaluated as potential precursors of astaxanthin and β-carotene production in *P. rhodozyma*.

Hypothesis 5.1: Mevalonic acid, apple pomace and tomato pomace when used as precursors can substantially improve astaxanthin and β-carotene yields in *P. rhodozyma* fermentation. The yield improvement is dependent on the concentration of the precursor, and is independent of the fermentation substrate.

The objectives of this study were to evaluate the following precursors for enhanced carotenoid yield from *P. rhodozyma* fermentation 1) mevalonic acid in whole stillage, full fat rice bran, defatted rice bran, wheat bran and synthetic medium, and 2) apple pomace and tomato

pomace in whole stillage and rice bran. Carotenoid yield enhancement in *S. roseus* upon mevalonic acid addition to synthetic and whole stillage media was also evaluated.

Materials and methods

Microbial culture and inoculum generation

Culture maintenance and inoculum generation of *P. rhodozyma* and *S. roseus* are outlined in chapter 2. A 10% (v/v) inoculum was used for fermentation.

Media preparation

For the mevalonic acid addition, optimized media composition of whole stillage was used as outlined in Chapter 3. Full fat rice bran, defatted rice bran and wheat bran fermentation media were prepared as outlined in Chapter 5. Synthetic yeast extract medium was prepared as outlined in Chapter 2. For the apple pomace and tomato pomace additions, unoptimized whole stillage medium outlined in Chapter 2 and full fat rice bran medium as outlined in Chapter 5 were used. Precursors were added to the media at respective concentrations and 50 ml of respective media in 250 ml flasks were sterilized by autoclaving at 121°C for 30min.

Percursors

Four concentrations 0, 0.2, 0.4 and 1.0 mg/ml of mevalonic acid (Sigma, MO, USA) were added to whole stillage, full fat rice bran, defatted rice bran, wheat bran and synthetic media. Apple pomace and tomato pomace (from Dr. Alavi, Grain Science and Industry, KSU) at concentrations 0, 0.05%, 0.1% and 0.5% were added to whole stillage and full fat rice bran media. Tomato pomace sample contained 62.67 µg/g of lycopene and 99.86 µg/g of β-carotene.

Fermentation conditions

Carotenoid production of *P. rhodozyma* monoculture was evaluated in five substrates (defatted rice bran, full fat rice bran, wheat bran, whole stillage and synthetic medium) amended with different concentrations of mevalonic acid, and two substrates (full fat rice bran and whole stillage) amended with different concentrations of apple pomace or tomato pomace. Carotenoid production of *S. roseus* monoculture was evaluated only in two substrates (whole stillage and synthetic medium) amended with mevalonic acid. Submerged fermentation was conducted in all cases. Flasks were inoculated and incubated at 18°C, 180 rpm for 11 days. Control flasks without precursors were maintained. Two replicates per treatment were employed. For the mevalonic acid experiment, samples were harvested only on day 11, while samples were harvested on days 3, 5, 7, 9 and 11 for the apple pomace and tomato pomace experiments. Harvested samples were centrifuged and supernatant discarded. Pellets were freeze dried for 24 h and stored at –80°C until further analyses.

Carotenoid extraction and analyses

High performance liquid chromatography (HPLC) was used for quantification of carotenoids, astaxanthin and β-carotene and is outlined in Chapter 2.

Results

Effect of mevalonic acid on carotenoid yield

Carotenoid yield enhancement seems to be influenced by the fermentation substrate and the concentration of mevalonic acid (Table 6.1). Mevalonic acid increased both astaxanthin and β-carotene yields of *P. rhodozyma* on all substrates except β-carotene yield on wheat bran at 0.02 %. The optimal concentration of mevalonic acid that enhanced astaxanthin yield in each substrate was variable. In defatted and full fat rice bran, 1 mg/ml resulted in best yield, 0.04 % in

wheat bran and 0.02 % in whole stillage and synthetic media respectively (Table 6.1). The percent increase in yield was also variable depending on the substrate. The best yield enhancement by 144% and 140% was seen in synthetic medium and whole stillage respectively. Among all the substrates, *P. rhodozyma* produced the highest astaxanthin yield of 220 µg/g on whole stillage. β-carotene yield was enhanced the most by 0.1% of mevalonic acid on all substrates (Table 6.1). The best yield enhancement by 945% was seen in synthetic medium. However, *P. rhodozyma* produced the highest yield of β-carotene (904 µg/g) on whole stillage.

In *S. roseus*, the best yield enhancement of β-carotene was seen in synthetic medium with 0.1% resulting in best yield enhancement of 233%. The maximum yield enhancement on whole stillage resulted from 0.04% mevalonic acid on whole stillage. The magnitude of carotenoid yield enhancement was not as substantial as that in *P. rhodozyma* fermentation. Mevalonic acid at 0.1% and 0.04% resulted in 233% and 190% β-carotene enhancement in YM and whole stillage respectively.

Effect of apple and tomato pomace on carotenoid yield

The effect of apple and tomato pomace on the production profile of carotenoids on rice bran and whole stillage are outlined in Figs. 6.1-6.4. Table 6.2 presents the highest carotenoid yield per treatment and the percent increase in carotenoid production upon precursor addition. Stimulation of carotenogenesis by the precursor seems to be influenced by both, precursor concentration and the substrate. Overall, apple pomace seems to have a negative influence on astaxanthin production on both substrates (except 0.1% in rice bran and 0.05% in whole stillage), negative influence on β-carotene production in rice bran (except 0.1%), and a positive influence on whole stillage (Table 6.2). Apple pomace at 0.1% yielded the best astaxanthin and β-carotene production on rice bran, while 0.05% yielded the best astaxanthin and 0.1% the best β-carotene

production on whole stillage. Tomato pomace had a positive influence on astaxanthin and β-carotene production on both substrates except 0.05% on whole stillage. Tomato pomace at 0.05% and 0.5% resulted in the best astaxanthin and β-carotene yields in rice bran, while 0.1% produced the best carotenoid yields in whole stillage.

Discussion

This study showed that mevalonic acid, tomato pomace and apple pomace can act as precursors of carotenoid production in *P. rhodozyma* fermentation of agricultural substrates supporting hypothesis 5.1. The precursor concentrations influenced the level of carotenoid enhancement further supporting hypothesis 5.1. However, the yield enhancement was not independent of the substrate as hypothesized. Overall, mevalonic acid resulted in the best yield enhancement, followed by tomato pomace, and apple pomace resulted in least enhancement.

Mevalonic acid was chosen as a precursor in this study because it is the first precursor in the terpenoid biosynthetic pathway (see Fig.1.4 and Frengova and Beshkova, 2009) and has been effectively used to enhance carotenoid production in *P. rhodozyma* (Calo et al., 1995), *H. pluvialis* (Harker and Young, 1995) and recombinant *E. coli* (Yoon et al., 2007). In this study, among all the substrates tested, 0.02% and 0.1% mevalonic acid resulted in the best astaxanthin and β-carotene yield enhancement, respectively, in whole stillage. In synthetic YM medium, Calo et al. (1995) reported astaxanthin and total carotenoids yield enhancement by 400%, accompanied by negligible β-carotene yield enhancement by the addition of 0.1% mevalonic acid. Surprisingly in this study, 0.1% mevalonic acid resulted in 945% yield enhancement of β-carotene along with 13% enhancement of astaxanthin. However, the best astaxanthin yield was promoted by 0.02% mevalonic acid. The use of different *P. rhodozyma* strains in both studies and their utilization of mevalonic acid seem to be the only plausible explanation for the observed

differences. Overall, in *S. roseus*, mevalonic acid resulted in better yield enhancement of β-carotene in synthetic medium than whole stillage. Although mevalonic acid is an excellent promoter of astaxanthin production, its high cost (1g=$125.00) makes it unattractive for use in large-scale production of carotenoids. However, if whole stillage or any other inexpensive animal feed substrate is used for production of carotenoid-enriched feed where the inherent cost of production is very low, use of mevalonic acid as a precursor at 0.02-0.1% makes it a commercially viable option.

Tomato and apple pomace were evaluated as potential precursors because 1) both are inexpensive products of tomato and apple processing industry, 2) lycopene is the first precursor of cyclic carotenoids in yeasts (see Fig. 1.3 and Frengova and Beshkova, 2009), lycopene and β-carotene are precusors of astaxanthin in *H. pluvialis* (Harker and Young, 1995) and tomato pomace contains lycopene and β-carotene (Mansoori et al., 2008), and 3) apple pomace contains at least seven different carotenoids (Molnár et al., 2010) and previously used as a substrate to produce carotenoids from *Micrococcus* sp. (Attri and Joshi, 2005). Whole stillage and rice bran amended with 0.1% tomato pomace resulted in the best β-carotene yield in both substrates and astaxanthin yield in whole stillage, while rice bran showed negligible improvement of astaxanthin yield. Apple pomace at 0.1% resulted in yield enhancements that were less than 10% for astaxanthin and about 26% for β-carotene. Overall, tomato pomace was a better precursor than apple pomace.

Conclusions

Precursors for carotenogenesis are usually evaluated in synthetic media to study their effect on carotenoid yield enhancement. However, it cannot be assumed that they would work equally well on all substrates. This study shows that yield enhancement is largely influenced by

the fermentation substrate and the concentration of the precursor. Mevalonic acid and tomato pomace can be used as precursors for carotenoid production on various animal feed substrates.

Table 6.1 Effect of mevalonic acid on carotenoid yield on different substrates

Fungus [a]	Carotenoid [b]	Substrate [c]	0 mg/ml	0.2 mg/ml	0.4 mg/ml	1 mg/ml
PR						
	Astaxanthin	DRB	49.49±0.1	56.33±0.15 (14%)	57.9±0.07 (17%)	62.41±0.04 **(26%)**
		FFRB	45.34±0.63	56.54±0.49 (25%)	58.0±0.2 (28%)	71.68±2.5 **(58%)**
		WB	53.03±0.02	65.25±0.03 (23%)	73.97±0.04 **(40%)**	71.48±0.57 (35%)
		WS	91.74±2.77	220.17±1.19 **(140%)**	213.99±1.85 (133%)	211.6±1.5 (131%)
		YM	71.78±1.45	175.42±4.5 **(144%)**	81.33±0.01 (13%)	81.3±1.71 (13%)
	β-carotene	DRB	117.93±0.74	152.25±1.63 (29%)	136.87±0.78 (16%)	172.99±3.22 **(47%)**
		FFRB	133.66±1.01	167.46±0.8 (25%)	187.5±1.25 (40%)	259.74±6.1 **(94%)**
		WB	102.47±0.54	94.44±1.02 (-8%)	129.96±0.95 (27%)	168.25±5.02 **(64%)**
		WS	269.18±2.04	721.5±19.5 (168%)	887.41±6.7 (230%)	904.4±1.79 **(236%)**
		YM	84.81±3.4	743.4±2 (777%)	754.34±5.2 (790%)	886.54±0.91 **(945%)**
SR						
	β-carotene	WS	283.79±0.21	579.02±9.94 (104%)	823.24±5.8 **(190%)**	764.39±1.38 (169.35%)
		YM	269.62±1.6	756.15±1.14 (180%)	878.44±12.8 (226%)	898.11±4.03 **(233%)**

[a] PR-*P. rhodozyma*, SR-*S. roseus*

[b] carotenoid yield µg/g of freeze dried sample except YM where yield is µg/g of yeast

[c] means and standard error are reported; Percent increase in yield compared to control in parentheses; Best % yield increase for each substrate is bold-faced

[d] DRB-defatted rice bran, FFRB-full fat rice bran, WB-wheat bran, WS- corn whole stillage, YM-yeast extract malt extract synthetic medium

Table 6.2 Best carotenoid yield and percent yield increase in *P. rhodozyma* fermentation of whole stillage and synthetic media amended with apple pomace or tomato pomace

Carotenoid [a]	Substrate [b]	Precursor [c]	0%	0.05%	0.1%	0.5%
Astaxanthin						
	FFRB	AP	66.36	31.41 [*] (–52.67)	72.90 **(9.86)**	59.53 [*] (–10.29)
		TP	66.36	71.71 **(8.06)**	69.04 (4.04)	69.52 (4.76)
	WS	AP	32.04	34.48 **(7.62)**	30.84 [*] (-3.75)	23.20 (–27.61)
		TP	32.04	31.25 (–2.48)	41.29 **(28.87)**	35.85 [**] (11.89)
β-carotene						
	FFRB	AP	198.29	156.57 [*] (–21.04)	225.63 **(13.79)**	182.80 [*] (–7.81)
		TP	198.29	212.71 (7.27)	203.75 (2.75)	255.00 **(28.60)**
	WS	AP	130.49	143.82 (10.21)	164.80 [*] **(26.28)**	139.58 (6.96)
		TP	130.49	109.53 (–16.07)	162.45 **(24.49)**	148.42 [**] (13.73)

[a] means and standard error are reported; Highest yield per treatment is noted irrespective of the day of fermentation; Percent increase in yield compared to control in parentheses; Best % yield increase for each substrate is bold-faced;

[b] FFRB-full fat rice bran, WS-whole stillage

[c] AP-apple pomace, TP-tomato pomace

[*] yield on day 9

[**] yield on day 7

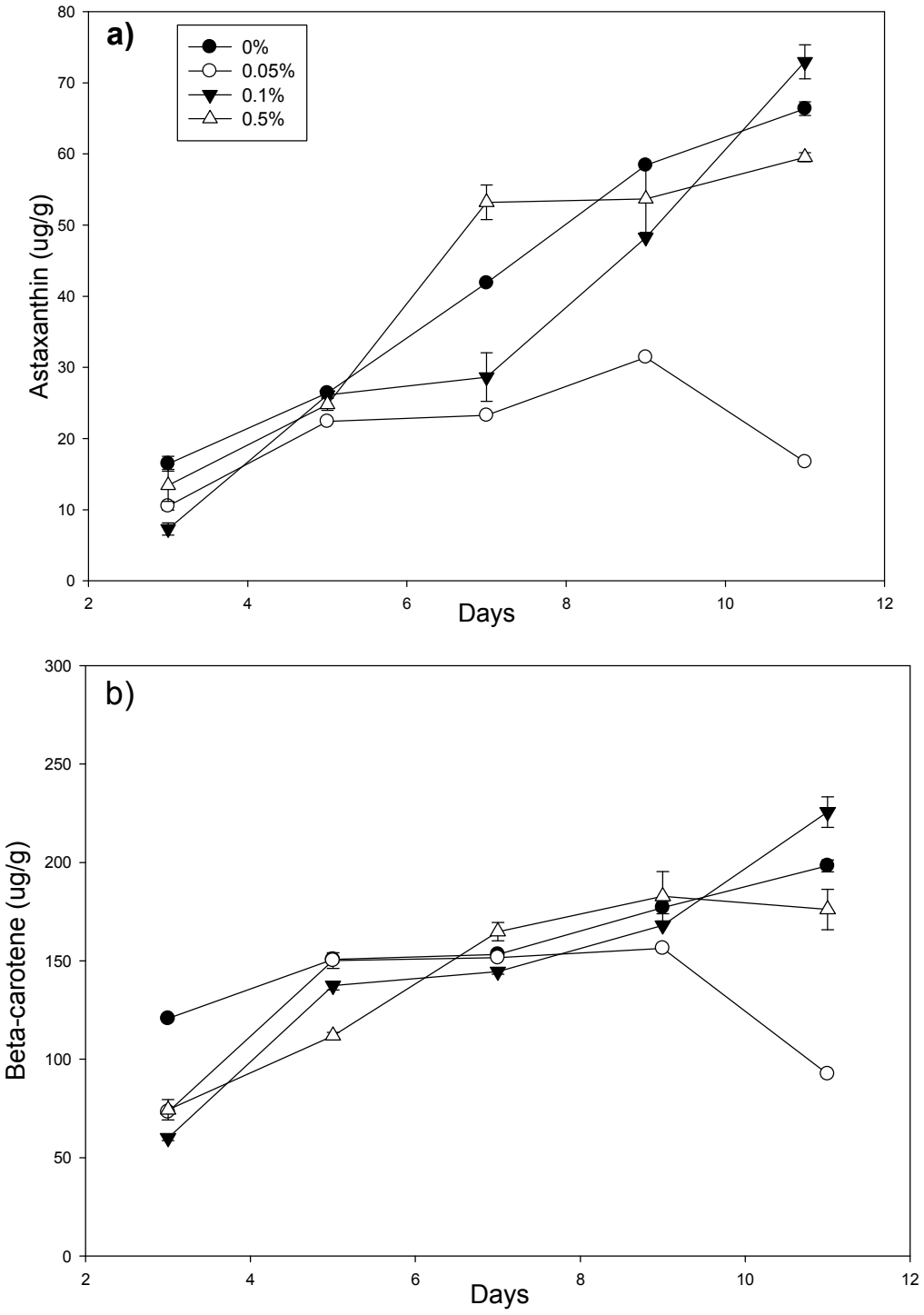

Figure 6.1 Carotenoid production in rice bran with apple pomace precursor.

a) astaxanthin b) beta-carotene.

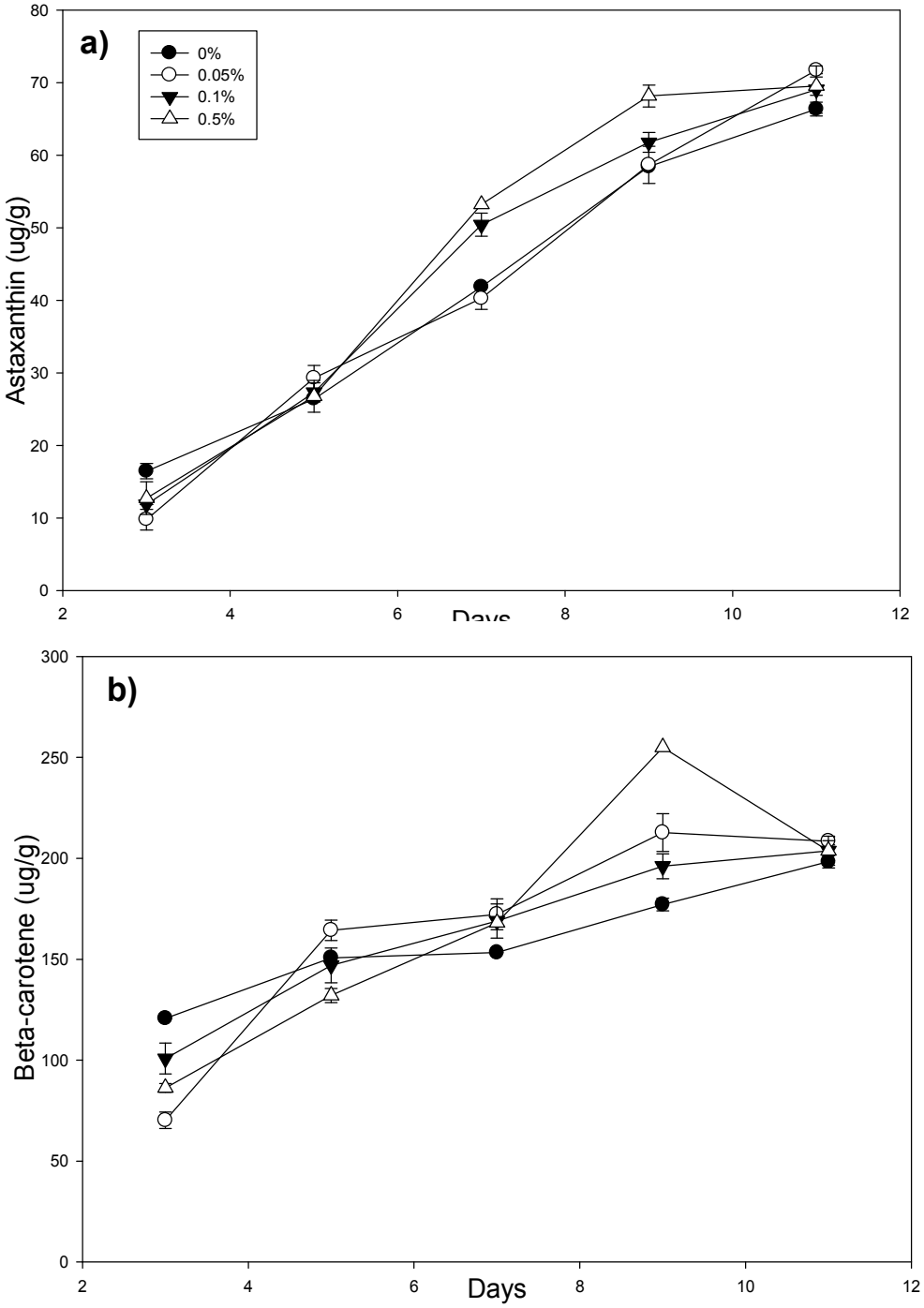

Figure 6.2 Carotenoid production in rice bran with tomato pomace precursor.

a) astaxanthin b) beta-carotene.

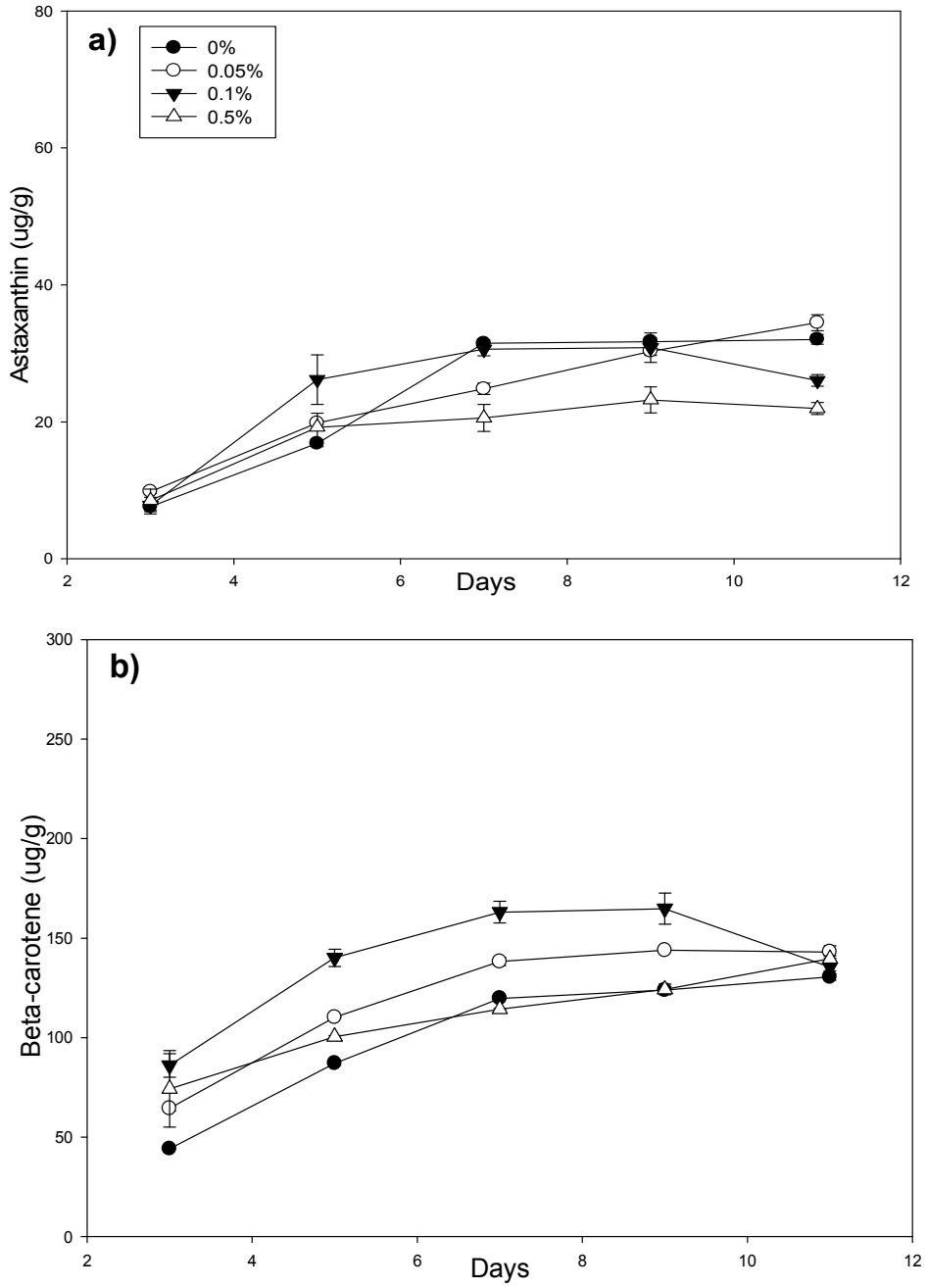

Figure 6.3 Carotenoid production in whole stillage with apple pomace precursor.
a) astaxanthin b) beta-carotene.

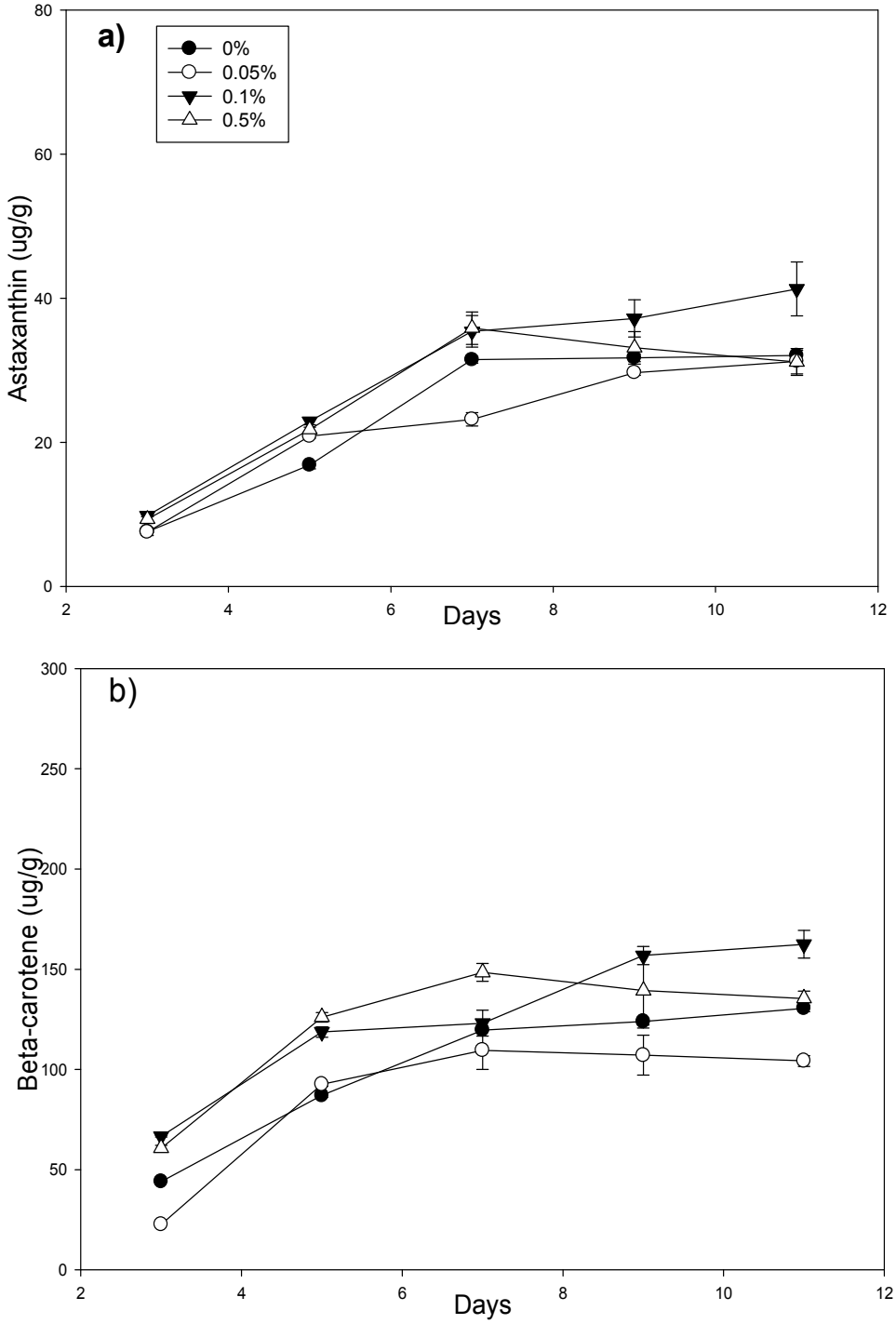

Figure 6.4 Carotenoid production in whole stillage with tomato pomace precursor.
a) astaxanthin b) beta-carotene.

CHAPTER 7 - Conclusions and future research

Carotenoids are expensive, yet essential animal feed additives. All the published reports till date have outlined the production of carotenoids from red yeasts or fungi on cheap substrates with the intention of extracting these carotenoids. However, in this study, a simple yet effective carotenoid value addition to corn whole stillage and other agricultural products is outlined, which directly provides carotenoid-enriched animal feed or feed blends.

This study establishes that 1) corn whole stillage upon secondary fermentation by red yeasts can yield carotenoids, astaxanthin and β-carotene required in animal nutrition, 2) supplementation of the whole stillage medium with co-products of biodiesel and corn wet-milling industry namely, glycerol and corn steep liquor provides additional nutrition, 3) media optimization and addition of precursors can enhance the carotenoid yields, 4) the value-added product not only provides carotenoids, but also increased fatty acids, reduced protein and fiber, all of which are highly desirable in animal feeds, and 5) the proof of concept developed for DDGS is also applicable for other cereal products used as animal feed.

Merits of carotenoid value addition to corn whole stillage

The carotenoid value addition outlined in this study has many advantages, 1) animal feeds are themselves used as substrates to produce carotenoid-enriched feeds or feed blends, 2) use of yeasts and secondary fermentation in established ethanol plants requires minimal operational changes, 3) does not require complete removal of ethanol from whole stillage; residual ethanol may in fact be useful for carotenoid production as ethanol is a known stimulator of carotenoid synthesis (Gu et al., 1997), 4) use of inexpensive nutrient supplements such as glycerol and corn steep liquor, both of which used at a commercial production capacity can help sustain the biodiesel and corn wet-milling industry, 5) does not require expensive, time

consuming down-stream processing as the product need not be extracted, 6) does not use corrosive chemicals at any stage, 7) product can be dried by any means convenient, 8) has good shelf life at room temperature (Chapter 3), 9) provides 'natural' carotenoids, and 10) provides more fat, less proteins and fiber allowing the capture of aquaculture and poultry feed industry as they depend on feed with less fiber.

The economics associated with Carotenoid value addition to DDGS can potentially benefit the biofuel industry. A conservative estimate is calculated based on DDGS price, cost of production of carotenoid-enriched DDGS and cost of commercial fish feed. DDGS costs about \$0.046/lb (\$102.25 per ton, USDA, Iowa Market, July 30, 2010). Back calculating from the price of ethanol, cost of production of carotenoid-enriched DDGS is estimated to be \$0.57/lb. Commercial fish feed varied from \$0.65 to 0.72/lb (Niewinski 2009). Based on these estimates, carotenoid-enriched DDGS as fish feed can cost \$0.65 to 0.72/lb, leading to a profit of \$0.08 to 0.13/lb. Biofuel plants can make profit of \$176 to 287 per ton. With commercialization of the process and further optimization, the profits are estimated to increase further.

Future directions

For practical application of the carotenoid value-added agricultural products including corn whole stillage as animal feed, further research in the following areas is required: 1) *Product extrusion*: both astaxanthin and β-carotene are stable at high temperatures, making it ideal for the use of extrusion technology to develop animal feed. High temperature, sheer and specific mechanical energy (SME) can fracture yeasts and release intra cellular contents; 2) *Scale-up studies*: in this study the process was scaled-up from shake flasks to 2-L bench-top fermenter. It can be further scaled-up to pilot scale in an ethanol plant for evaluation of the process on a large scale. Additionally, higher concentration of solids can be evaluated to ensure greater utilization

of whole stillage or agricultural products; 3) *Release of carotenoids*: while whole yeast cells can be consumed by fish (Jacobson et al., 2000), for effective utilization of carotenoids, the cells can be subject to fracturing by acid and mild pressure (An et al., 2006) followed by sterilization. Additionally, the fermented broth can be evaluated as liquid feed; 4) *Other yeast strains*: use high yielding strains to produce higher amounts of astaxanthin and β-carotene or use strains to produce other carotenoids like lutein which required in certain animal feeds; 5) *Animal feeding trials*: Before commercialization of the product, animal feeding trials are a must. The carotenoid-enriched products with different concentrations of carotenoids need to be evaluated as livestock feed, swine, poultry and aquaculture feed; 6) *Microbial toxins*: since *P. rhodozyma* whole cells (Jacobson et al., 2000) have been evaluated in animal feeding trials, it appears to be a safe product. However, the value-added products should be screened for mycotoxins and other microbial toxins. 7) *Further optimization*: of precursors and high-density fermentation.

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Appendix A - Copyright permission from Journal of Industrial Microbiology and Biotechnology

RE: Copyright Permission December 3, 2010 10:22 AM

From: “Elisabeth Elder” eelder@lsusa.edu

To: "Chris Lowe" <chris.lowe@simhq.org>; robert2753@comcast.net; ananda@k-state.edu

Dear Dr. Nanjunda:

Congratulations on completing your doctorate. Good luck as you pursue your career. Also, thank you for publishing your article in the *Journal of Industrial Microbiology and Biotechnology.*

Please accept this e-mail as permission to include the publication in your dissertation. In doing so, the original publication needs to be properly referenced.

Feel free to contact me if you have any questions.

Elisabeth D. Elder, PhD

Secretary, Society for Industrial Microbiology.

From: Chris Lowe [mailto:chris.lowe@simhq.org]
Sent: Friday, December 03, 2010 9:59 AM
To: robert2753@comcast.net
Cc: Elisabeth Elder
Subject: Re: Copyright Permission

Bob,

Betty has to give formal approval as Secretary. I'll forward to her.

Chris

On Dec 3, 2010, at 10:48 AM, robert2753@comcast.net wrote:

I have no problem granting this request.

Please confirm approval.

Thanks,

Bob

----- Original Message -----
From: "Ananda Nanjunda" <ananda@k-state.edu>
To: robert2753@comcast.net
Sent: Thursday, December 2, 2010 7:51:32 PM
Subject: Copyright Permission

Dear Dr. Waukegan,

I am a doctoral candidate with Department of Grain Science and Industry, Kansas State University. One of my original research articles was published in Journal of Industrial Microbiology and Biotechnology 2010 Nov; 37(11):1183-92. (Title: Production and optimization of carotenoid-enriched dried distillers grains with solubles by Phaffia rhodozyma and Sporobolomyces roseus fermentation of whole stillage.)

I am graduating in December 2010. I am writing to seek copyright permission to include the information published in the article as a part of my dissertation.

I look forward to hearing from you.

Thank you for your time and consideration.

Sincerely,

Ananda Nanjundaswamy

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