Production of Astaxanthin by *Xanthophyllomyces dendrorhous* DSMZ 5626 using Rapeseed Meal Hydrolysates as Substrate

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Specially dedicated to my pillars of strength;

My late parents;

Tuan Harith Tuan Mohamed
Tengku Zubaidah Tengku Yusoff

Husband;

Mohd Hasbullah Mohd Nawi

Children;

Ahmad Mohd Hasbullah
Aisyah Mohd Hasbullah

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Abstract

Astaxanthin is one of the most important carotenoids in humans and has vast applications in the food, nutraceutical and aquaculture sectors. Currently, astaxanthin is primarily produced through chemical synthesis, whereas the microbial fermentation method for producing astaxanthin is hindered by its high production costs as compared to the synthetic route. Valorisation of rapeseed meal, a by-product of rapeseed oil processing industry holds potential to serve as an alternative route for the sustainable production of microbial astaxanthin. The aim of this thesis was to investigate the feasibility of producing microbial astaxanthin from rapeseed meal using a yeast species, *Xanthophyllomyces dendrorhous* DSMZ 5626 and its extraction strategies.

The preliminary study of *X. dendrorhous* growth in semi-defined media revealed that the yeast can consume a wide range of carbon sources including glucose, fructose, xylose, cellobiose, galactose, arabinose and glycerol with biomass values ranging from 10.7 g/l up to 13.3 g/l when 30 g/l of each carbon source was used individually. Suppression of astaxanthin production was observed when high glucose concentrations (> 30 g/l) were used due to Crabtree effects. The findings served as preliminary data to understand the biochemical behaviour of the selected yeast species.

Proximate analysis of the rapeseed meal used in this study demonstrated that it contained high protein (25 %, w/w), lignin (18%, w/w) and total carbohydrate (34%, w/w) contents, with the latter consisting primarily of glucose (20%, w/w) and to lesser extent arabinose (6%, w/w), galactose (3%, w/w) and also uronic acids (3%, w/w). Four commercial enzymes, namely (i) Viscozyme L, (ii) Accellerase 1500, (iii) pectinase and (iv) cellulase (from *Aspergillus niger*) were tested at different concentrations (1 – 15 %, v/v) for the individual assessment of their ability to break down the cellulosic and hemicellulosic compounds of rapeseed meal into monomeric fermentable sugars. Specifically, Viscozyme L and Cellulase treatments exhibited the highest glucose recovery yields (47 – 52% yield for 15 % (v/v) of enzyme used) and rapeseed meal derived total sugar concentration (74-77 g/l). A thermal pre-treatment step (126 ^{o}C, 30 min) prior to enzyme hydrolysis by Accellerase 1500 was also evaluated and was found to improve the hydrolysis rate of rapeseed sugars by 25%. Rapeseed meal hydrolysates were tested as fermentation media for microbial astaxanthin production using separate hydrolysis and fermentation (SHF) approach in batch and fed-batch fermentation modes. Batch fermentation with pectinase derived rapeseed meal hydrolysates supported both biomass (42 g/l) and astaxanthin production (11 mg/l) as the presence of glycerol from the enzyme formulation acted as additional carbon source for yeast growth.

Intracellular astaxanthin was extracted by using three cell disruption methods including glass beads, enzymatic cell lysis and supercritical fluid extraction. Results showed that highest astaxanthin extractability (>100 %) was obtained when enzymatic cell lysis with Glucanex, accompanied with acetone extraction was used under optimised conditions (pH 4.6 at temperature of 30.8 °C). Overall, the findings of the study can serve as basis towards the commercialisation potential of microbial astaxanthin using cheap and renewable substrate as fermentation feedstock (rapeseed hydrolysates) and gave useful insights on the extraction strategies that can be applied in future scaling up processes.

Keywords: astaxanthin, *X. dendrorhous*, rapeseed meal, extraction

Declaration: I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

Zuharlida binti Tuan Harith

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1 INTRODUCTION

Carotenoids constitute a major class of natural pigments produced by plants, algae, fungi, bacteria and yeasts and can be classified into carotenes or xanthophylls depending on the presence of a hydroxyl group in their structure. Carotenes such as *β*-carotene, *α*-carotene and lycopene contain only carbon and hydrogen atoms in their structure whereas xanthophylls such as lutein, astaxanthin and zeaxanthin contain an additional keto or hydroxyl group (Sanchez et al., 2013). Carotenoids are important natural ingredients with a wide range of applications in various industries; for instance, *β*-carotene and astaxanthin have been used as natural food colourants. In addition, astaxanthin has been applied in the aquaculture industry where it is incorporated into feed formulations for salmon, shrimp and trout to produce a greater orange-pink pigmentation in the flesh of the fish (Xiao, Jiang, Ni, Yang & Cai, 2015).

In terms of producing carotenoids, humans and animals do not have the capability to metabolically synthesize them, and therefore these can only be sourced through their diet. In contrast, plants (vegetables and fruits) and microorganisms such as yeasts, microalgae, fungi and bacteria are capable of producing carotenoids. There are several species of red yeasts, including *Rhodotorula* sp. (Aksu and Eren, 2007), *Rhodosporidium* sp. (Enshaeieh, Abdoli, Nahvi, & Madani, 2013) and *Sporidiobolus* sp. (Valduga et al., 2014), that have been shown to produce a wide range of carotenoids such as astaxanthin, torulene and *β*-carotene. Focusing on astaxanthin, the yeast species *Xanthophyllomyces dendrorhous* (formerly known as *Phaffia rhodozyma*) is one of the major natural astaxanthin producers, with astaxanthin comprising about 90% of the total carotenoids produced.

The focus of this study is to develop a sustainable route for producing microbial astaxanthin via a biotechnology approach using the yeast species, *Xanthophyllomyces dendrorhous*. One of the obstacles to commercialising natural astaxanthin is its high price

compared to the synthetically produced astaxanthin that is produced via the Wittig reaction. In order for natural astaxanthin to be able to compete with synthetically produced astaxanthin in the market, an approach of utilising lignocellulosic waste from agricultural industries, such as wheat straw, sugarcane molasses and corn fibre, is one of the options available to produce microbial astaxanthin at reduced cost. In addition, oilseed meals such as rapeseed meal, sunflower meal and soybean meal might become potential sources of substrate for microbial astaxanthin production.

Rapeseed (*Brassica napus L.*) is an important crop that has many uses in the agriculture industry. The main product, rapeseed oil, is rich in omega-3 fatty acids, antioxidants and other phytonutrients that are beneficial for health. The increased demand for rapeseed oil has increased the production of rapeseed oil as well as the generation of by-products from the oil-processing industry. The principal by-product of the rapeseed oil production process is rapeseed meal (or cake), which is generated in large quantities. In 2013, almost 33.6 million tonnes of rapeseed meal were produced worldwide (Carré & Pouzet, 2014). Rapeseed meal contains substantial amounts of protein and other nutrients such as carbohydrates and phenolics (Wang et al., 2010). It is regarded as a low-value by-product and is primarily used as animal feed for pigs, chickens, broilers and lambs (Rymer & Short, 2003; Wang et al., 2010). However, this by-product has a lot of potential to be explored for other purposes, for example as a fermentation feedstock in microbial bioconversion into high value-added bioproducts. Therefore, utilising rapeseed meal as a medium for microbial astaxanthin production in yeast is a sustainable option and, most importantly, it does not compete with the food supply chain for humans.

1.1 Aim and Objectives

The overall aim of this study was to investigate the feasibility of utilising lignocellulosic by-product rapeseed meal for microbial conversion into natural astaxanthin by the yeast *Xanthophyllomyces dendrorhous* DSMZ 5626. The experimental work in this study was divided into two major parts: 1) investigation of the feasibility of using rapeseed meal for bioconversion into natural astaxanthin production by the yeast *X. dendrorhous* DSMZ 5626; and 2) the extraction of intracellularly produced astaxanthin pigment from the yeast.

To achieve the overall aim, the following specific objectives were designed:

1. To investigate the role of different carbon sources in semi-defined media for the microbial production of astaxanthin by *X. dendrorhous* in order to understand the biochemical behaviour of selected yeast species;
2. To evaluate the effects of chemical inducers, such as ethanol, citric acid and hydrogen peroxide, on astaxanthin production in *X. dendrorhous*;
3. To optimise the enzymatic hydrolysis of rapeseed meal using a variety of enzymes (e.g. Accellerase 1500, Viscozyme L and pectinase) to produce a nutrient-rich hydrolysate;
4. To investigate the effects of thermal pretreatment as a pretreatment step on the efficiency of the subsequent rapeseed meal hydrolysis step;
5. To investigate the effects of rapeseed meal hydrolysate as a growth medium for *X. dendrorhous* in flask fermentation;
6. To scale up microbial astaxanthin production using selected rapeseed meal hydrolysates from shake flasks to a 2-litre stirred tank bioreactor;
7. To develop a scalable and efficient method for extracting astaxanthin from yeast cells using different techniques, namely glass bead extraction, enzymatic cell lysis and carbon dioxide supercritical fluid extraction (CO_2-SCFE).

1.2 Scope and Structure of the Thesis

This thesis covers the production of natural astaxanthin from rapeseed meal as a fermentation feedstock. Chapter 2 presents a literature review in the area of interest, which includes topics such as carotenoids, rapeseed and its production, fermentation and downstream processing (extraction).

Chapter 3 covers the experimental research for objectives 1 and 2. In this chapter, the growth of *X. dendrorhous* cultivated using different types of carbon sources is investigated using semi-defined medium in flasks fermentation. Also, the addition of chemical inducers such as ethanol, citric acid and hydrogen peroxide to improve astaxanthin pigmentation is evaluated in this chapter. The results in this chapter give an overview of yeast behaviour and show that subsequent rapeseed meal hydrolysis can be tailored to yeast preference.

Chapter 4 presents the experimental research for objectives 3, 4 and 5. The chapter reports that enzymatic hydrolysis of rapeseed meal was conducted using different types of commercial enzymes, i.e. Viscozyme L, Accellerase 1500, pectinase and cellulase from the fungus *Aspergillus niger*. The effects of thermal pretreatment to improve enzymatic hydrolysis were also investigated. The produced hydrolysates were then tested for their ability to support yeast growth and astaxanthin production in shake flasks.

Chapter 5 presents the results for objective 6. In this chapter, astaxanthin production was scaled up in a 2-litre stirred tank reactor. In the first part, a semi-defined medium was used to investigate the physical parameters (pH and agitation) of bioreactor fermentation that were optimum for yeast growth. The optimised parameters were then applied for fermentation using rapeseed meal hydrolysates as fermentation medium. Different approaches, including separate

hydrolysis and fermentation (SHF) and partial simultaneous saccharification and fermentation (pSSF), were investigated.

Chapter 6 explores the different extraction strategies for intracellular astaxanthin extraction from *X. dendrorhous*. Methods including glass beads followed by solvent extraction with a range of solvents (e.g. ethanol, methanol), enzymatic cell lysis followed by acetone extraction and CO_2-SCFE with ethanol as co-solvent were tested. A design of experiment (DoE) approach was applied in this study. Finally, Chapter 7 provides a general discussion and presents the conclusions drawn from the study. Suggestions for further studies are also included in this chapter.

2 Literature Review

2.1 Carotenoids

Carotenoids are tetraterpenoids organic pigments produced by various sources, such as plants, algae, fungi, yeasts and bacteria (Aksu & Eren, 2007; Sanchez, Ruiz, Nacional, & México, 2013), and numerous conjugated carbon double bonds present in their structure (Stahl and Sies, 2003). Although more than 600 different carotenoids have been identified, there are only a few carotenoids which are considered highly beneficial and have attracted extensive interest for their positive roles in plants, animals and humans. Among these carotenoids, β-carotene, lutein, astaxanthin, zeaxanthin, capsanthin and lycopene are those which have been widely studied and characterised for their functions. Carotenoids are divided into two classes, namely carotenes and xanthophylls, according to their chemical structure. Carotenes (e.g., lycopene) contain only carbon and hydrogen atoms in their molecules. In comparison, xanthophylls have a more complex chemical structure than carotenes that includes an additional keto or hydroxyl group in their chain, such as astaxanthin and β-carotene (Berman et al., 2014).

Plants, in particular, tomatoes, carrots, red capsicums and all the leafy green plants, are the main sources of carotenoids (Omayma & Abdel Nasser, 2013). Carotenoids are responsible for providing their host with red, yellow, purple and orange pigmentation (Ribeiro, Barreto and Coelho, 2011; Valduga et al., 2014), and act as photosynthetic pigments in plants (Gu et al., 2008), oxygen scavengers in photosynthetic tissue and as structural determinants in plastid pigments (Bartley and Scolnik, 1995). Meanwhile, in non-photosynthesis tissues, they contribute to the colour of the plants.

In humans and animals, carotenoids play essential roles as antioxidants and vitamin A precursors and potentially offer protection against cardiovascular diseases (Fassett & Coombes,

2011). Among these carotenoids, lycopene, lutein, zeaxanthin, astaxanthin, β-carotene and canthaxanthin are commercially important due to their specific applications in the food, pharmaceutical and cosmetics industries. In the food industry, they are primarily used as natural food colourants, mostly from plant extracts, and are responsible for a wide range of colours, including orange, red and yellow. Humans fulfil their intake of the major carotenoids via dietary consumption of fruits and vegetables that contain a high carotenoid content, such as tomatoes, carrots, oranges and corn (Ishida & Chapman, 2009). Carotenoids (β-carotene, astaxanthin, lutein, zeaxanthin) have also been commercialised as human supplements, due to their potential to increase human health. Meanwhile, in the cosmetic industries, carotenoids have been applied to formulate skin care products, aftershave lotions, bath products, hair conditioners and shampoos (Sanchez, Ruiz, Nacional & México, 2013).

Due to the extensive use of carotenoids in various products, there is huge demand of these compounds. BBC Research (2011) reported that the market demand for carotenoids was estimated at US$ 1.2 billion in 2010, with the possibility of reaching $1.8 billion, in 2018. Nowadays, the supply of these carotenoids is dominated by synthetic production, due to limitations in scaling up the production of naturally produced carotenoids. The worldwide growing demand for carotenoids has triggered research focused on finding alternative ways of production, such as extraction from natural resources, synthesis from microorganisms, as well as proving the effectiveness of these carotenoids in various applications. Currently, the extraction of carotenoids from plants is limited, as a result of the low carotenoids yield, instability of the pigments when exposed to air and light, as well as the use of harsh solvents, such as acetone, in the recovery process (Thomas, Diebler & Barmore, 1998).

The commercialisation of natural carotenoids produced from microbial technology has not been able to compete with the synthetic production, due to marketing and technical reasons,

such as the complexity of the fermentation process which is reflected by high production costs (Schmidt et al., 2011). Natural carotenoids are more expensive than their synthetic counterparts, driving the competition between the sources for market demand. For carotenoids derived naturally via the microbial technology route, a very limited number of products have penetrated the market and obtained a generally regarded as safe (GRAS) status, including astaxanthin sourced from algae (*Haematococcus pluvialis*) and yeast (*Xanthophyllomyces dendrorhous*) (Bhosale & Bernstein, 2005). The sections below provide more information on the different types of carotenoids which are β-carotene, lycopene, lutein, zeaxanthin and astaxanthin.

2.1.1 β-Carotene

β-carotene is a tetraterpene ($C_{40}H_{56}$) with a β-ring structure at both ends of the molecule (Sanchez, Ruiz, Nacional & México, 2013). It is the main source of pro-vitamin A, which can be converted to vitamin A, and is believed to be the most important carotenoid in human nutrition, due to its health promotion capabilities, including helping the vision, reproductive efficiency and epithelial tissue maintenance (Ribeiro, Barreto & Coelho, 2011). β-Carotene has also been applied in the food industry as a food colourant, ranging from yellow to orange (Figure 2-1).

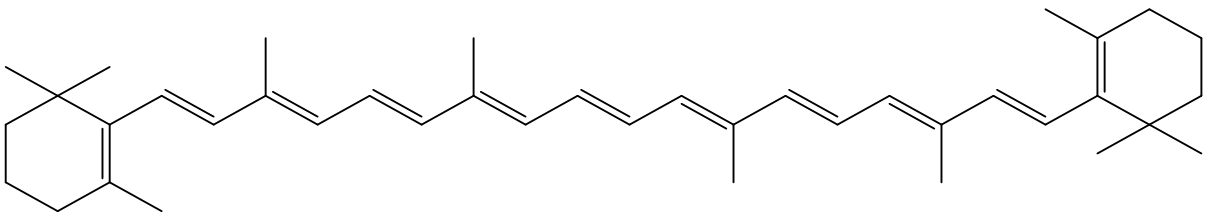

Figure 2-1: Chemical structure of β-carotene

The market value of β-carotene was estimated at US$ 250 million in 2007; it was further increased to US$ 261 million in 2010 and is expected to grow to US$ 334 million by 2018 (BBC Research, 2011). The supply of β-carotene is obtained by chemical synthesis, mostly via the Wittig reaction (Ernst, 2013). Among the natural sources rich in β-carotene are carrots, sweet potatoes, oil palm (*Elaeis guineensis*) and buriti (*Mauritia vinifera*) (Ribeiro, Barreto and Coelho, 2011).

2.1.2 Lycopene

Lycopene has the chemical formula $C_{40}H_{56}$, and a molecular weight of 536.85 Da (Figure 2-2). It is a lipophilic compound and is insoluble in water but is an effective oxygen scavenger and has good antioxidant activity. Lycopene is capable of quenching singlet oxygen (O_2^-) and trapping peroxide radicals (ROO-) at a higher rate than β-carotene. This attribute is associated with the presence of 11 conjugated double bonds, as well as the opening of the β-ionic rings in its structure that increase its quenching ability towards singlet oxygen compared to other carotenoids (Stahl & Sies, 2003; Stajčić et al., 2015).

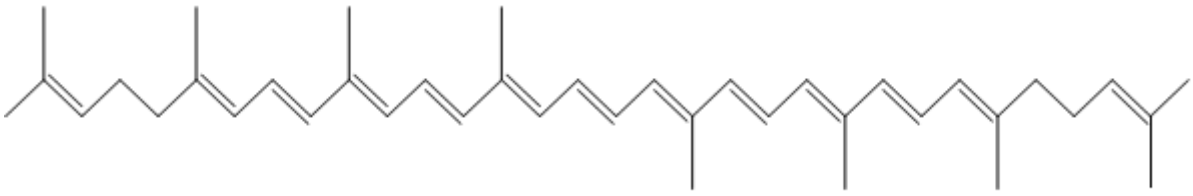

Figure 2-2 : Chemical structure of lycopene

Lycopene is present in fruits, vegetables and green plants, as part of photosynthetic mechanisms, which acts to absorb light and provide protection against photosensitisation in plant tissue. The main source of lycopene is tomatoes, although apricots, guava, watermelon, papaya and pink grapefruit also have high levels of this carotenoid. Lycopene is found to be responsible for the yellow, red and orange pigmentation of fruits and vegetables (Rao et al., 2006).

In the European Union (EU), lycopene (E160) is defined as a food additive (Directive 94/36/EC) and can be produced either synthetically via the Wittig condensation reaction or extracted from red tomatoes (Ernst, 2013; Stajčić et al., 2015). Synthetic lycopene is not approved as a food colourant in the EU but is considered as GRAS in the US (GRAS notice No GRN 000119) (Bresson et al., 2008). Besides that, the fungi *Blakeslea trispora* and *Phycomyces blakesleeanus* have been investigated for the production of natural lycopene (Berman et al., 2014; Chandi & Singh Gill, 2011). Generally, commercial lycopene can be found in the form of suspensions in edible oils or as water-dispersible powders where it is formulated to contain an antioxidant for stabilisation purposes. Lycopene has been applied mainly in food products, such as soups, pasta, sauces and spreads (Chandi & Gill, 2011).

2.1.3 Lutein and Zeaxanthin

Lutein (Figure 2-3) and zeaxanthin (Figure 2-4) are xanthophylls consisting of 40 carbon atoms with nine conjugated double bonds in a polyene structure; both have the same chemical formula, i.e., $C_{40}H_{56}$. Zeaxanthin is a stereoisomer of lutein; the main difference is the position of the conjugated double bond in one of the hydroxyl groups. Zeaxanthin has a double bond at the 5',6' position, whereas, in lutein, the double bond migrates to position 4',5' (Bone et al., 1997). Although their structures are very similar to that of β-carotene, they do not possess the pro-vitamin A activity due to the presence of an oxygenated group in the terminal ionone

rings that results in the inability of a specific enzyme to cleave the 15–15' bonds in the structure (Ma & Lin, 2010). However, the presence of a hydroxyl group on both ends of these two molecules makes them much more polar compared to other carotenoids (Krinsky & Johnson, 2005).

The intake of lutein and zeaxanthin by humans and animals relies directly upon dietary intakes. Good sources of these compounds include leafy vegetables such as spinach and cabbage as well as egg yolk (Krinsky & Johnson, 2005). Although lutein and zeaxanthin are not essential carotenoids for human health, they play a vital role in preventing certain serious eyes diseases such as cataracts (Ma & Lin, 2010) and exhibit protection against age-related macular degeneration (Perry, Rasmussen, & Johnson, 2009). These two carotenoids are the major pigments present in the human macula and retina (Sommerburg et al., 1998) where they function as blue light filters and as antioxidants, by protecting the eye tissue against free radicals (Ma & Lin, 2010).

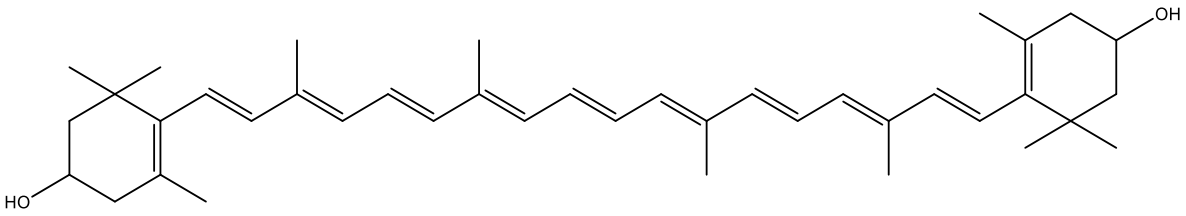

Figure 2-3: Chemical structure of zeaxanthin

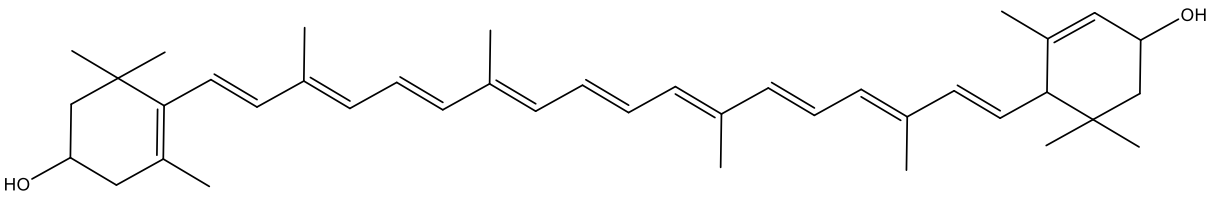

Figure 2-4**:** Chemical structure of lutein

2.1.4 Astaxanthin

Astaxanthin (3,3'-dihydroxy-β, β-carotene-4,4'-dione) (Figure 2-5) is a keto-carotenoid pigment that has been used in the nutraceutical, cosmetic and feed industries, among others. The chemical formula of astaxanthin is $C_{40}H_{52}O_4$, and it has a molecular mass of 596.84 Da. Astaxanthin contains two terminal rings which are joined by a polyene chain; it has two asymmetric carbons located at the 3,3' positions of the β-ionone ring with a hydroxyl group (-OH) on either end of the molecule (Ambati et al., 2014).

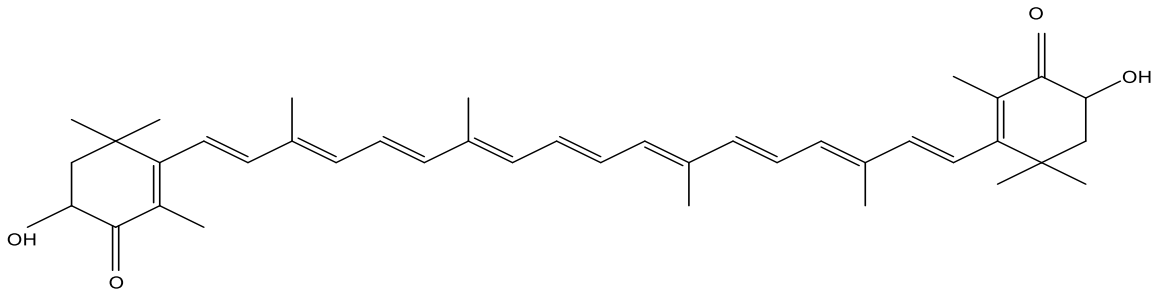

Figure 2-5: Chemical structure of astaxanthin

In aquaculture, astaxanthin is used as a feed additive as it contributes to the attractive colouration of the bodies of aquatic animals, like salmon, shrimps and crustaceans, as well as maintains their growth and survival (Ip, Wong, & Chen, 2004). One of the distinguishing features of salmon is the orange–pink flesh, representing the quality of the fish produce. This appealing look is attributed to astaxanthin that is also responsible for producing the luxurious appearance to the salmon (Baker & Günther, 2004).

Besides its contribution to colour, astaxanthin acts as an antioxidant by inhibiting the oxidation of low-density lipoproteins (Berman et al., 2014). It also possesses other bioactivities, such as anti-inflammatory and could potentially act as a therapeutic agent against

cardiovascular diseases (Choi, Youn, & Shin, 2011; Fassett & Coombes, 2011; Visioli & Artaria, 2017).

Since humans and animals lack the biochemical pathways that are responsible for the production of carotenoids, the supply of astaxanthin is fulfilled via the diet (Frengova & Beshkova, 2009). In the aquatic environment, the supply of astaxanthin is fulfilled by consuming the carotenoids producing microalgae, *Haematococcus pluvialis*, which is later being consumed by crustaceans, zooplankton and insects. As for farmed aquatic animals (fish and crustaceans), the access to natural source of astaxanthin is limited, hence the astaxanthin intake must be derived from their feed (Shah, Liang, Cheng, & Daroch, 2016)

Currently, the high demand for astaxanthin is fulfilled by both synthetic and natural supplies. The value of market for synthetically produced astaxanthin is more than USD 200 million per year and correspond to 130 metric tonnes of product (Shah et al., 2016). Nowadays, there are growing trends on using natural ingredients in food and nutraceuticals markets, due to increasing concern for consumer safety and regulatory issues over the introduction of synthetic chemicals into human food chain. Therefore, the market for natural astaxanthin derived from microbial sources has been increased owing to consumer awareness of its benefits. The value to for natural astaxanthin is high with about USD 2500–7000 /kg, depending on the purity of the substance as compared to the synthetically-produced astaxanthin at USD 2000 /kg. (Guerin, Huntley, & Olaizola, 2003; Panis & Carreon, 2016). Furthermore, natural astaxanthin is three to four times more valuable than the synthetic alternative in the nutraceutical and pharmaceutical industries (Shah et al., 2016). The high value of the natural astaxanthin compound has gain interest among researchers to investigate the possible way to improve its microbial production at reduced cost which is further discussed in section 2.1.6.

Astaxanthin originates from yeast, *Xanthophyllomyces dendrorhous* dominates the market for natural astaxanthin supply in aquaculture. As for human consumption, microalgae derived astaxanthin has gain high demand in the market. Astaxanthin was first regulated in 1998 by Directive 87/552/EC for use in salmon and trout feed at a maximum concentration of 100 mg/kg. As for synthetically produced astaxanthin, the market for supply in aquaculture industry is dominated by BASF and Hoffman-La Roche. Hoffman-La Roche's main astaxanthin product is named CAROPYLL® Pink and is specifically formulated for aquaculture feed formulation in the form of an encapsulated powder in maize starch. Currently, BASF commercialises Lucantin® Pink that contains 10% synthetic astaxanthin, aiming to expand into the aquaculture industry as well (Berman et al., 2014). However, synthetic astaxanthin is not approved for human consumption due to the difference in the molecular structure relative to the natural product.

The United States Food and Drug Administration (US FDA) (60 FR 18738, 13 April 1995) has approved the use of astaxanthin (E161) as a food colourant in animal and fish feed, and the European Commission (EC) also approved the use of natural astaxanthin as a food dye in 2003 (Panel, 2014).

2.1.5 Microbial Production of Astaxanthin

The microbial production of astaxanthin for human and animal uses has primarily been carried out commercially by the green microalgae *H. pluvialis* and yeasts (Panis & Carreon, 2016; Shah et al., 2016). The process was first commercialised in the early 1990s by the AstaReal group (Fuji Chemical Industries Co., Ltd., Japan) using astaxanthin sourced from *H. pluvialis* for human consumption (Guerin, Huntley & Olaizola, 2003). Since then, a number of products have appeared in the market, all naturally produced through microbial technology with *H. pluvialis*, including AstaReal®, AstaTROL® (Fuji Chemical Industries Co., Ltd.),

Astaxanthin (Parry Nutraceuticals, India), and BioAstin™, JointAstin™ and EyeAstin™ (Pure Healing Foods, CA, USA).

H. pluvialis can be cultured via several techniques, including closed systems, such as photobioreactors and open pond techniques (Olaizola, 2003). Domínguez-Bocanegra et al. (2007) investigated the effect of different environmental factors, such as light intensity, aeration and media composition on the growth of *H. pluvialis* and identified that using unaerated complex media with continuous illumination (345 µmol photon m^{-2} s^{-1}) resulted in the highest astaxanthin production (98 mg/g) with 1 g/L sodium acetate. Ambati et al. (2014) reviewed astaxanthin production in *H. pluvialis* in the range of 2.7–3.8% on a dry weight basis discovered that depending on the strain, and reported that depending on the strain, astaxanthin production on a dry weight basis is in the range of 2.7–3.8%, higher than that produced by the yeast *X. dendrorhous* (0.5% of dry weight). However, the cultivation period of *H. pluvialis* is about 13 days which is generally longer than *X. dendrorhous*, which is 5 days (Domínguez-Bocanegra, Ponce-Noyola & Torres-Muñoz, 2007). Besides *H. pluvialis*, *Dunaliella salina* and *Chlorella vulgaris* are among the microalgae species that have been exploited for the production of other carotenoids, including β-carotene and lutein (Guedes, Amaro & Malcata, 2011).

In addition to microalgae, several species of red yeasts have also been found to produce a wide range of carotenoids. These include *Rhodotorula* sp. (Aksu & Eren, 2007), *Rhodosporidium* sp. (Enshaeieh et al., 2013), *Sporidiobolus* sp. (Valduga et al., 2014) and *Sporobolomyces* sp. (Ramírez et al., 2001; Yang et al., 2011), as well as *Xanthophyllomyces dendrorhous*, previously known as *Phaffia rhodozyma*, which has been reported to produce high concentrations of astaxanthin. Specifically, the astaxanthin in *X. dendrorhous* accounts for almost 80–90 % of the total carotenoids that are accumulated within the yeast cells (Tinoi, Rakariyatham & Deming, 2005). *Rhodotorula* sp., such as *R. glutinis*, *R. rubra* and *R.*

mucilaginosa, produce a wide range of carotenoids, such as β-carotene or astaxanthin (Aksu & Eren, 2005; Bhosale & Gadre, 2001; Buzzini & Martini, 1999; Frengova, Simova, & Beshkova, 2004).

2.1.5.1 Biochemical pathway for astaxanthin synthesis

The biochemical synthesis of carotenoids occurs in the plastids of the cells via two different routes that both lead to the production of isopentyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). Both IPP and DMAPP are required as the precursors for carotenoid production (Miao et al., 2011; Sanchez et al., 2013). The pathways that are responsible for the production of these compounds are the mevalonate (MVA) pathway and 2C-methyl-D-erythritol 4-phosphate (MEP) pathway (Shumskaya & Wurtzel, 2013) (Figure 2-6). Yeasts and bacteria generally follow the MVA pathway, whereas plants can use both the MVA and MEP pathways.

In *X. dendrorhous*, carotenoids are produced via the MVA pathway, where three acetyl-coenzyme A (CoA) molecules which also serve as substrates for fatty acid synthesis, undergo condensation, yielding acetoacetyl-CoA. Subsequently, hydroxymethylglutaryl-CoA (HMG-CoA) is produced which is then condensed to form MVA before transforming to IPP (Figure 2-6). The condensation of IPP molecules leads to the production of geranylgeranyl pyrophosphate (GGPP). The first carotenoid, phytoene, is produced when two GGPP molecules are condensed. Subsequent dehydrogenation steps and one cyclisation step lead to the formation of β-carotene. Astaxanthin is derived from β-carotene and is the end-product of the synthesis pathway (Schmidt et al., 2011). Figure 2-7depicts the biosynthetic pathway of astaxanthin production in yeasts.

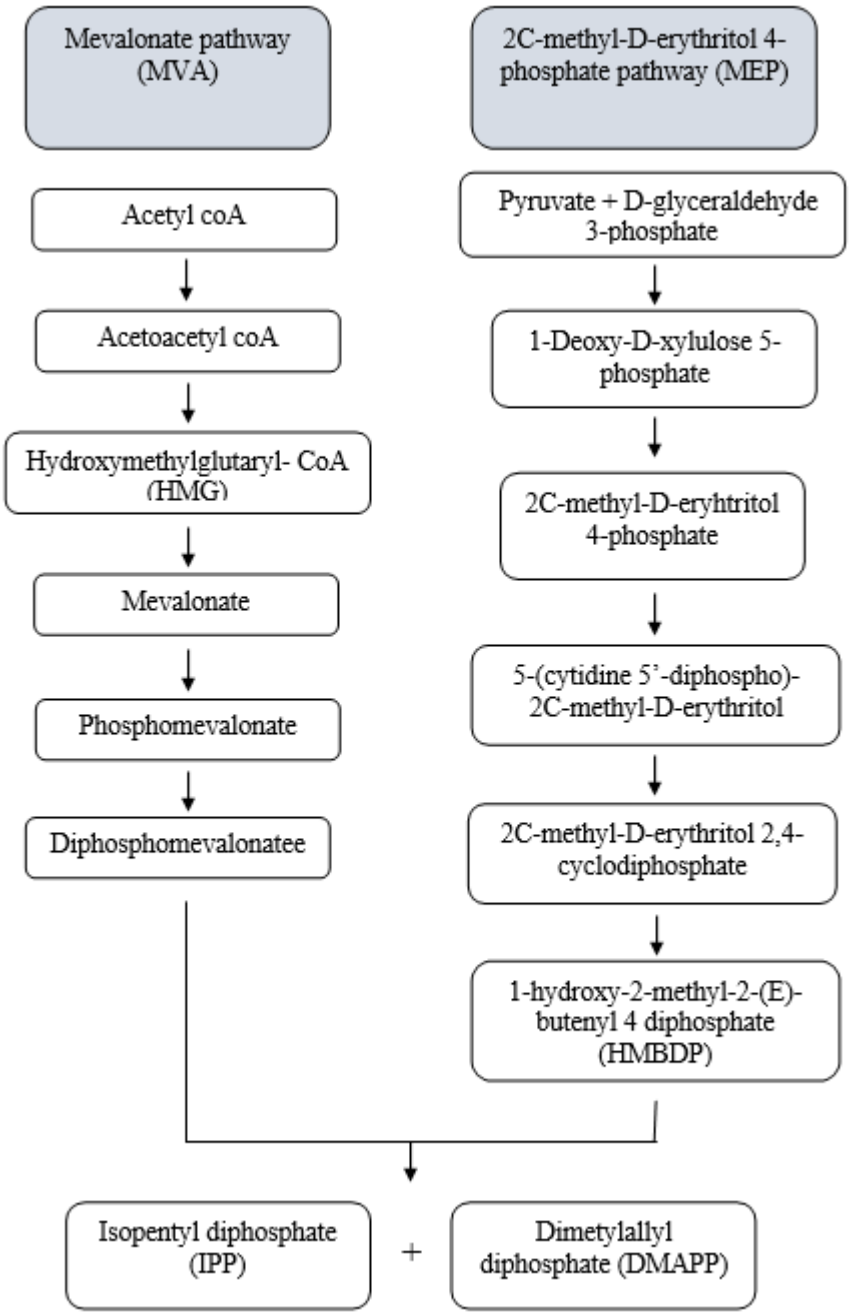

Figure 2-6: Mevalonate (MVP) and 2C-methyl-D-erythritol 4 phosphate pathway (MEP) pathways used for synthesis of IPP and DMAPP, which are precursors for carotenoids synthesis (Adapted from Sanchez et al., 2013).

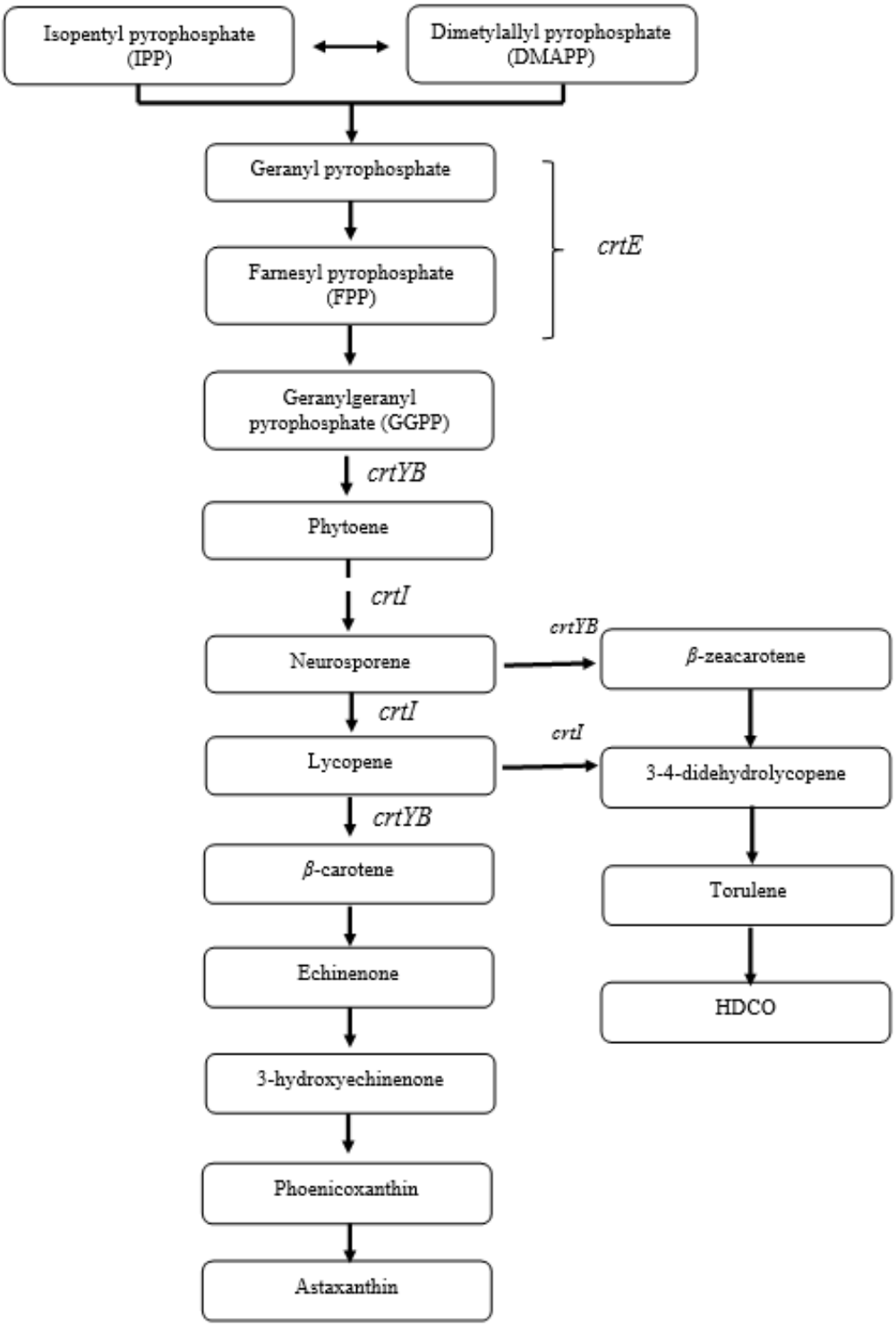

Figure 2-7: Biosynthetic pathway of astaxanthin synthesis in *X. dendrorhous*. Abbreviations: crtI (phytoene desaturase), crtYB (phytoene-β-carotene synthase, crtE (geranylgeranyl pyrophosphatase), crtS (astaxanthin synthase), crtR (cytochrome P450 synthase) and HDCO (3-hydroxy-3',4'-didehydro-β-φ-carotene-4-one) (adapted from Contreras et al., 2013)

2.1.6 Research Trends in Carotenoids Production

The production of carotenoids via microbial technology seems to be a suitable alternative to compete with chemically synthesised carotenoids due to its ability to produce a wide range of carotenoids at relatively high productivity (Frengova & Beshkova, 2009). However, as previously mentioned, until today, the production of natural carotenoids via microbial routes has not been developed sufficiently enough to compete with the synthetic route primarily due to technical issues (e.g., low production yields and productivities) and high production costs (Schmidt et al., 2011). To overcome these constraints, a significant amount of research has been undertaken on genetic modification of production strains, utilisation of low-cost agricultural by-products (e.g., lignocellulosic residues) as fermentation feedstocks and implementation of effective extraction processes.

Recombinant DNA technology has been applied to carotenoid-producing microorganisms, such as *X. dendrorhous*, to introduce novel or modify existing specific biochemical reactions responsible for carotenoid production (Contreras et al., 2013; Fang & Wang, 2002; Mata-Gómez, Montañez, Méndez-Zavala, & Aguilar, 2014). Various low-value agricultural by-products, including cane molasses, *Eucalyptus* woods, spent coffee ground and whey, have been investigated for their feasibility to support carotenoid production in various microorganisms, including yeast and microalgae (Liu, Huang, Jiang, & Chen, 2012; Mata-Gómez et al., 2014; Petrik, Obruča, Benešová, & Márová, 2014; Vázquez, Santos, & Parajó, 1998). Extraction of carotenoids from microbial sources is not a direct process since the pigments are located within the cells. Various cell disruption strategies are often applied, including glass beads, ultrasonication and enzymatic cell lysis, before solvent extraction (Machado et al., 2016; Michelon et al., 2012). Alternatively, technologies including

supercritical fluid extraction (SFE) and ionic liquids, show high potential to be explored for carotenoid extraction (Saini & Keum, 2018; da Silva, Rocha-Santos & Duarte, 2016).

2.2 Rapeseed

Rapeseed (*Brassica napus* L.) (Figure 2-8) is an important oil crop that has been planted across the world, with the major producing countries being the EU, Canada, China and India (Figure 2-9). In 2017, about 73 million tonnes of rapeseed was produced which is about 12% of the world's total oilseed production (Krautgartner et al., 2017). There are mainly two types of rapeseed of rapeseed being grown, which are known as winter and summer rapeseed, respectively. In the EU and Asia, rapeseed is cultivated as a winter or semi-winter variety. The winter varieties have longer vegetation periods and produce a higher yield than the summer ones, but can only be grown in areas with a mild winter climate. Summer rapeseed is cultivated in comparatively cooler continental climates, in China, northern European countries, Canada and Australia (Szydłowska-Czerniak, 2013). The world production of rapeseed has not increased since 2013 due to bad weather and planting reduction in the EU (Figure 2-10).

Figure 2-8: Rapeseed flower

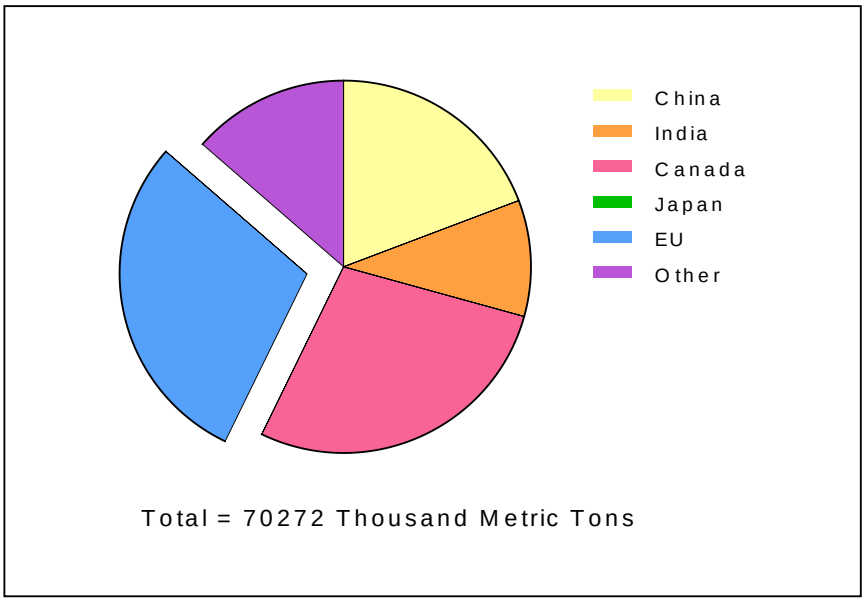

Figure 2-9: World production of rapeseed worldwide in 2016/17. Source: (Krautgartner et al., 2017).

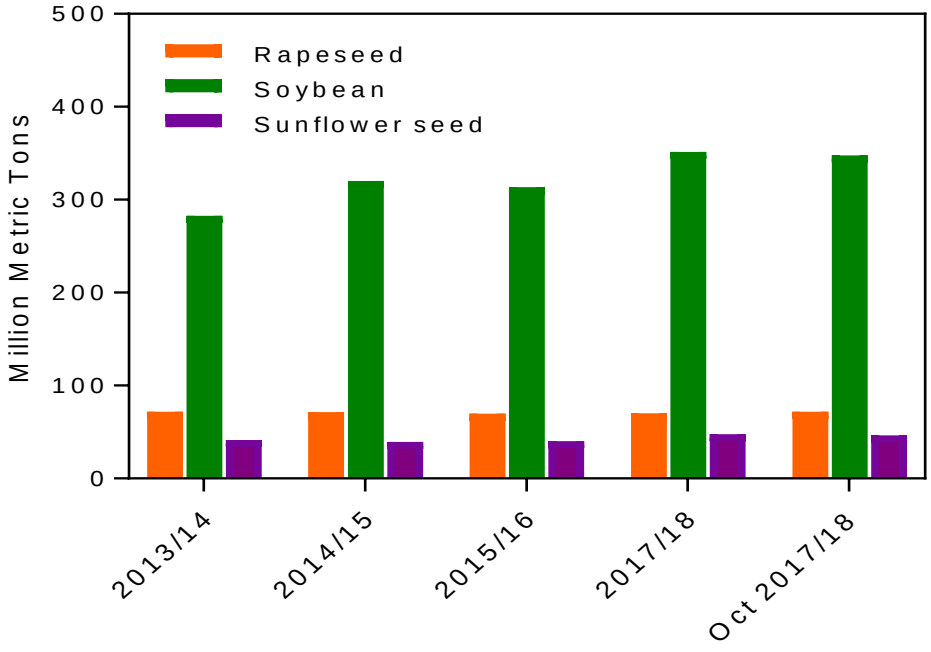

Figure 2-10: World production of major oilseeds. Source: (Krautgartner et al., 2017)

Rapeseed has been mainly cultivated for its high oil content (40–45%, w/w) since it has high nutritional value. Rapeseed oil contains a low amount of saturated fatty acids, which account for 5–7% (w/w), but is high in polyunsaturated fatty acids, containing about 7–10% α-linolenic acid and 17–21% linoleic acid (Kramer, 2012). Several different varieties of rapeseed are cultivated for distinct purposes. For example, varieties that contain high levels of erucic acid (40–50%, w/w) and glucosinolates (> 30 µmol/g) are mainly used for non-edible purposes, such as for the production of biodiesel and engine lubricant as they are toxic to humans after prolonged exposure (Mintec, 2012). The oil extracted from these varieties also has a bitter taste, making it less favoured by consumers (Kramer, 2012). Due to all of the constraints, selective plant breeding approaches have been carried out leading to the generation of improved rapeseed cultivars that contain small amounts of glucosinolates (<30 µmol/g) and less than 2% erucic acid. This variety is found to be more suited for food purposes and ensured for its safety for consumers. In Canada, this edible variety is known as canola (Szydłowska-Czerniak, 2013).

Nowadays, rapeseed oil is nutritionally desirable as a cooking ingredient, as it contains the lowest levels of saturated fatty acids, among the major commodity oils, such as soybean and sunflower oil. Rapeseed oil can be used as a salad dressing, for margarine production, as well as for frying purposes. The fatty acids composition also attracts interest of utilising the rapeseed oil as source of lubricant and chemical industries (Bockey, 2013).

2.3 Rapeseed Structure

Rapeseed which is the reproductive organ in rape, consists of the hull (seed coat), the endosperm and a large embryo (consisting of a radicle and 2 conduplicate cotyledons). The hull acts as a protective barrier for seeds during dormancy. It consists of an epidermal layer, a subepidermal layer, a thick palisade layer and a pigment layer. As for the endosperm, it consists

of an aleurone cell layer and a hyaline layer of crushed parenchyma cells (Yiu, Altosaar, & Fulcher, 1983). The structure of rapeseed is given in Figure 2-11.

According to the requirements of different physiological process, nutrients and other metabolites are distributed and deposited in various seed organs. The seed coat is mainly composed of fibre (18%) with a high proportion of lignified material and lower amount of oil compared to the endosperm. In contrast, the endosperm that adheres to the inner of seed coat contains high proportions of oil bodies and protein bodies that make it rich in oil and protein content (Fang et al., 2012). In rapeseed, the total oil content can reach approximately 50% (w/w) (Fang et al., 2012). Besides that, tannins are found rich in the rapeseed hull as compared to in the embryo, whereas glucosinolates and phytate content are lower in the hull (Lammerskotter et al., 2017).

The structural organization of rapeseed is mechanically disrupted during pressing process. Normally, the rapeseed meal that is produced as the by-product of de-oiling process is composed of both the seed coat part (brown in colour) and the oil cake, part which is yellow in colour, reflecting the yellow colour of the cotyledon. In some processing plants, the hull part of the rapeseed is separated from the seed part and is subjected to oil extraction, that is normally used as biofuel (Lammerskotter et al., 2017). Theoretically, the dehulling of oilseed improves the quality of the meal produced. However, this technique is rarely applied at industrial scale due to loss of oil in the hull, low oil extraction yield and high possibility of glucosinolates to accumulate in the dehulled meal (Carré, Citeau, Robin, & Estorges, 2016).

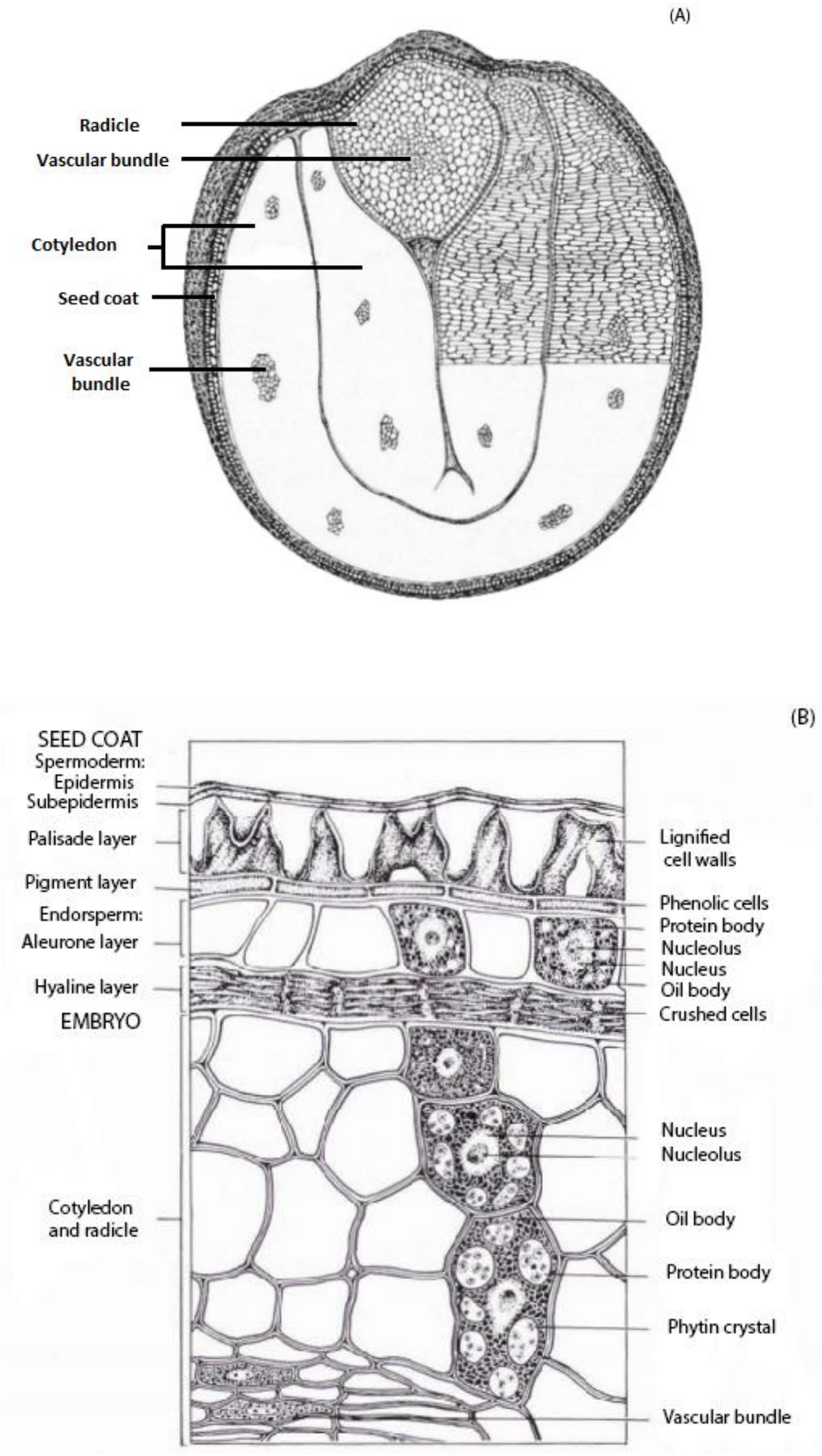

Figure 2-11: The structure of rapeseed. (A) The structural organisation of whole rapeseed. (B) Detail structural and microchemical organization of rapeseed. Adapted from Yiu et al. (1983).

2.4 Rapeseed Processing and By-Products

The processing steps for the extraction of oil from rapeseed can vary, according to the practices of the individual manufacturer or the end use of the oil. Generally, the process starts with seed cleaning, followed by seed flaking where the cell wall is ruptured. Afterwards, the flakes are passed through steam or cookers, aiming to 1) denature hydrolytic enzymes, 2) adjust the moisture of the flakes, and 3) increase the oil viscosity to ease the oil collection process. This cooking process takes about 15–20 min at 85–105 °C. Once cooked, the flakes enter the pressing stage where the seed is pressed for the oil. At this stage of the process, the oil extraction yield is around 40% of the total oil initially present in rapeseed (Mintec, 2012).

Depending on the purpose of the produced oil, the rapeseed meal (the residue after pressing) may undergo further solvent extraction process with hexane, to extract the remaining oil and provide an oil-free rapeseed meal (less than 1% w/w oil). Usually, if the extracted oil is targeted for biofuel purposes, this additional step of solvent extraction is therefore necessary to obtain high oil yields. However, the additional solvent extraction step has led to several problems, such as air pollution (Kumar et al., 2017). Due to differences in the extraction methods used, variations in the oil yield and nutritional quality often occur, mainly in terms of the fatty acid composition and total tocopherols and phenolics content (Ghazani, García-Llatas & Marangoni, 2014). The resultant oil is refined to remove compounds like phospholipids, free fatty acids and colour pigments and the meal is heated to strip off the solvent before being pelleted for further applications. Currently, rapeseed meal is being used as animal feed. A general schematic of the oil extraction process for rapeseed is depicted in Figure 2-12.

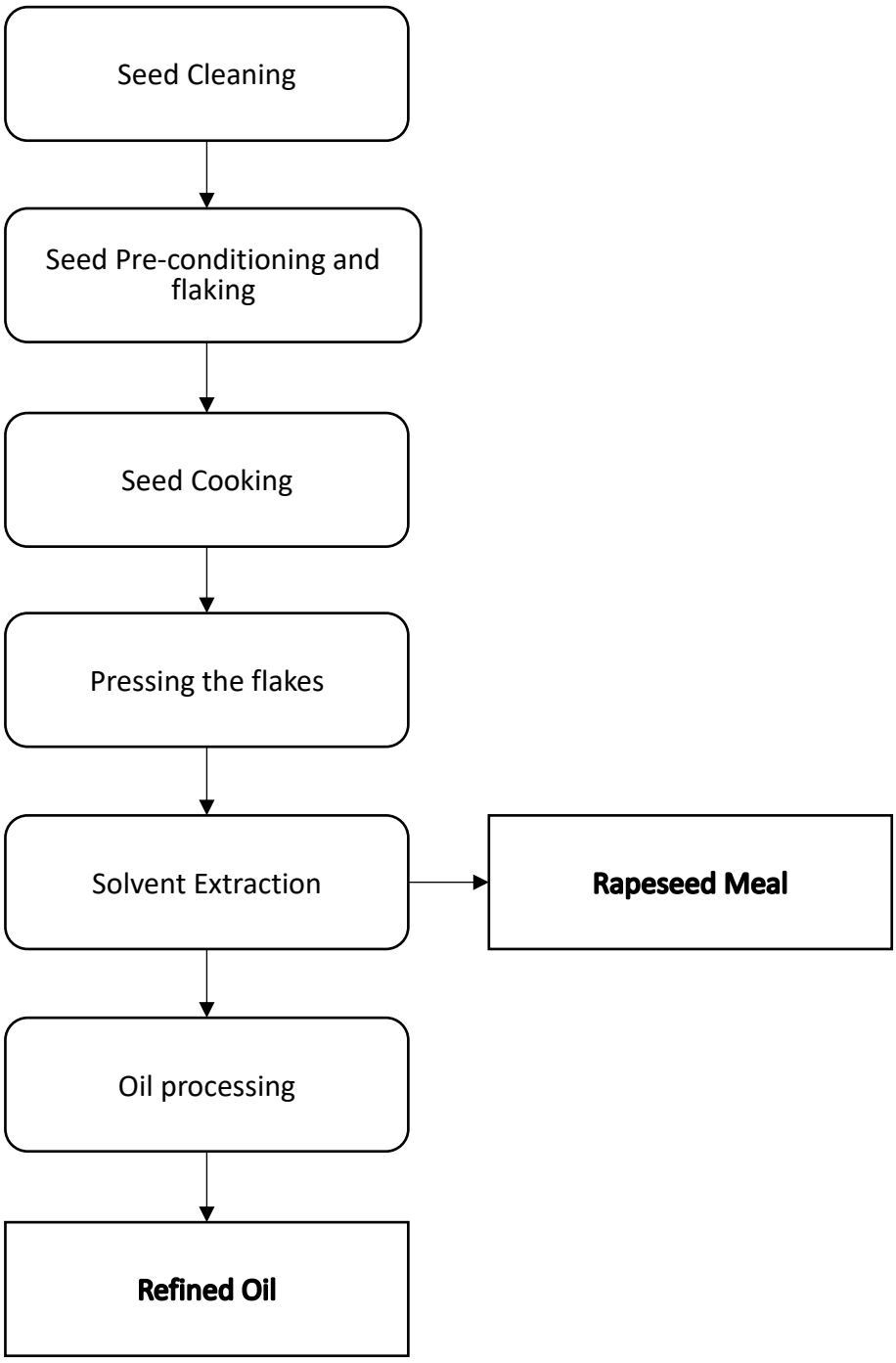

Figure 2-12 Common solvent extraction process for rapeseed oil (Source: Canola Council of Canada, 2014)

Another oil extraction method used is the cold pressing method. Generally, it is used to produce oil of high nutritional quality and is known as extra virgin or cold-pressed rapeseed oil, which is primarily used for cooking or dressing purposes. In this procedure, the seed is not exposed to high temperatures or chemicals, and the extraction is customarily carried out below 40 °C, as at a higher temperature, the flavour and nutritional quality of extracted oil are compromised. Under these mild conditions, the nutritional quality of the extra virgin rapeseed oil is preserved. The extracted oil may retain its high content of tocopherols and phytosterols, which also act as antioxidants, improving the stability of the oil (Hoed et al., 2010). This procedure is usually used by small-to-medium sized companies targeting food applications, as the oil extraction yield is not as high as compared to the heat treatment extraction process.

Pressed rapeseed meal contains substantial amounts of residual oil, usually over 19%. However, further solvent extraction, particularly by the biodiesel industry can potentially result in a meal with less than 2% residual oil (Wang et al., 2010). The rapeseed meal generated is rich in proteins and polysaccharides such as pectins, hemicellulose and cellulose (Wang et al., 2010). Hemicellulose in rapeseed meal mainly consists of xyloglucans (Pustjens et al., 2013). The composition of the rapeseed meal varies according to genetic factors, climate, soil and the applied oil extraction process (Lomascolo et al., 2012).

In 2016/17, about 39.5 million tonnes metric of rapeseed were generated as a result of increasing rapeseed production (Figure 2-13). The global production of rapeseed meal has been increasing since 2005, reaching a stable output since 2013 with a value of almost 39.5 million tonnes in 2016/17 (USDA, 2018). This increase is due to high amount of rapeseed grown to meet the increasing demand for vegetable oils. The rising trend of rapeseed meal suggests that considerable opportunities exist for developing alternative uses beyond animal feed for this readily available material (Lomascolo et al., 2012).

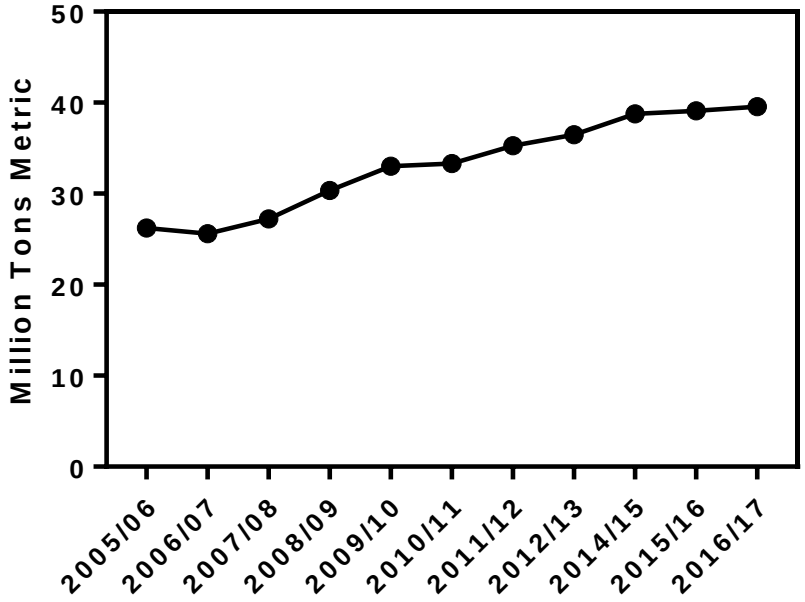

Figure 2-13: Worldwide production of rapeseed meal from 2005 to 2016 (USDA, 2017)

Oil-free rapeseed meal typically contains 30% (w/w) protein, ~36% (w/w) total carbohydrates and 24% (w/w) lignin (Pustjens et al., 2013). Rapeseed meal has been traditionally utilised as a fertiliser and also as animal feed (as mentioned earlier in this section) as it contains a healthy balance of amino acids (El-Beltagi & Mohamed, 2010; Wang et al., 2010). However, the utilisation of rapeseed meal as feed is limited compared to other oilseed meals such as soy and sunflower due to the presence of anti-nutritive components, such as phytic acid and erucic acid, and the presence of glucosinolates and phenols, which act as precursors for the formation of toxic compounds (Uçkun Kiran et al., 2012). Moreover, rapeseed meal protein is more suited for ruminants as it is not as easily digested by monogastric animals due to high content of cell wall polysaccharides (Pustjens et al., 2013) when compared to fish meal or soybean meal (Uçkun Kiran et al., 2012). Since the composition of rapeseed meal is rich in non-starch polysaccharides, utilisation of rapeseed meal as feed for monogastric animals is regarded not suitable as they lack the enzymes required to break down the cell wall polysaccharides of rapeseed meal. An additional step, such as enzyme pre-treatment is

necessary to improve the digestibility of rapeseed meal by monogastric animals such as pigs (Pustjens et al., 2012).

The protein in rapeseed meal can be potentially isolated and used as an ingredient in high-value food products, such as food supplements and sports drinks (Chabanon et al., 2007). Moreover, due to its high carbohydrate content, rapeseed meal can potentially be used as a fermentation feedstock, namely, as a carbon and nitrogen source, for microbial bioconversions. However, most microorganisms cannot directly assimilate the complex carbohydrates present in rapeseed meal as they lack the necessary enzymes needed to break down the cellulosic and hemicellulosic components (Wang et al., 2010). Therefore, to be used for such application, rapeseed meal needs to be hydrolysed into its primary components, i.e., sugars, peptides, amino acids, before being utilised for microbial bioconversion purposes (Chatzifragkou et al., 2014).

To design a multi-step process for the hydrolysis of lignocellulosic materials such as rapeseed meal and their subsequent utilisation for the production of a range of chemicals, it is important to have a good understanding of their proximal chemical composition. Figure 2-14 depicts a general process scheme for the hydrolysis of the key macromolecules present in lignocellulosic materials. It is evident that the hydrolysis strategy should be designed with consideration of the composition of the lignocellulosic biomass as well as the capabilities of the microorganisms used for subsequent bioconversions. The hydrolysis strategy can be performed either by physical, chemical (e.g., acid, alkali) or enzymatic hydrolysis (Chatzifragkou et al., 2014; Sun & Cheng, 2002; Taherzadeh & Karimi, 2007).

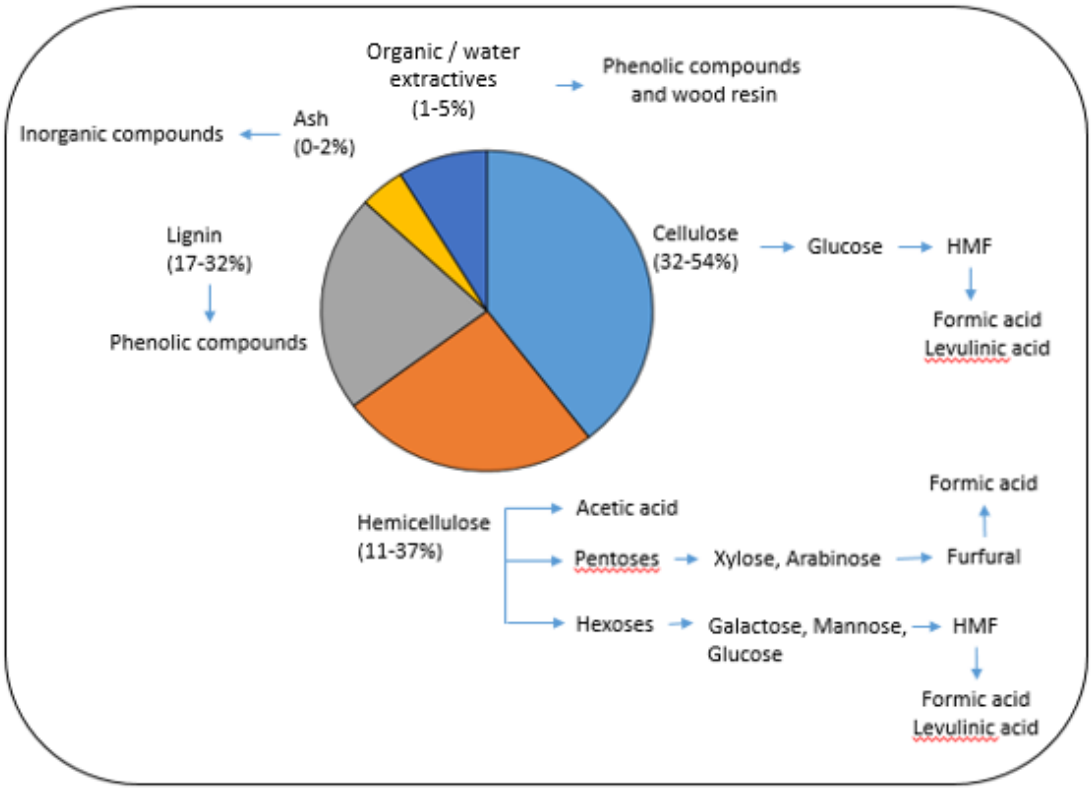

Figure 2-14: Composition of lignocellulosic biomass and potential hydrolysis products (Adapted Sarip, Hossain, Azemi & Allaf , 2016).

2.5 Pre-Treatment of Lignocellulosic Materials

The complexity of lignocellulosic biomass hinders the efficient enzymatic hydrolysis of the macromolecules (polysaccharides and proteins) into their monomeric compounds (sugars and amino acids). Therefore, as mentioned in section 2.3, to increase the enzymatic hydrolysis of rapeseed meal, a pre-treatment step is necessary, with the primary aim to separate out the lignin and hemicellulose components from the biomass. This step will also reduce the cellulose crystallinity, as well as increase the porosity of the materials, which in turn facilitates the accessibility of the enzymes to cellulose (Taherzadeh & Karimi, 2007). A successful pre-treatment step should be cost-effective to ensure that the fractionation/separation of the biomass has been effective and thus avoid the formation of sugar degradation products, such as furans

(hydroxymethylfurfural (HMF) and furfural), as these are inhibitory towards enzymes and microbes (Öhgren et al., 2007; Petrik, Kádár & Márová, 2013; Sun & Cheng, 2002).

To achieve this, several processes including physical, chemical and biological processes have been applied as pre-treatment strategies, notably hydrothermal, chemical and enzymatic treatments (Sánchez and Cardona, 2008). Such a pre-treatment step will generally be followed by enzymatic treatment, targeting the hydrolysis of cellulose into its monomers. Figure 2-15 depicts the changes in the biomass structure after different pre-treatments, while the subsequent sections discuss each of these pre-treatments in more detail.

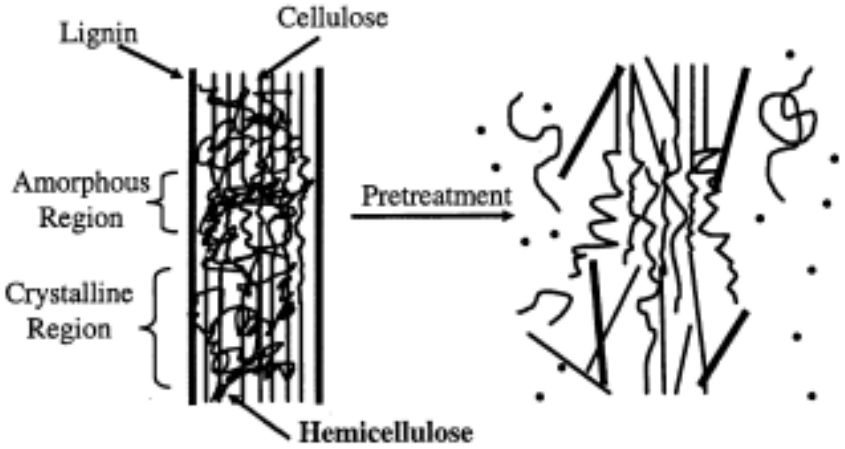

Figure 2-15: Schematic diagram depicting the changes in the biomass structure before and after pre-treatment (adapted from Mosier, 2005)

2.5.1 Chemical pre-treatments

Acid pre-treatment has been applied to treat lignocellulosic material and improve subsequent enzymatic hydrolysis of biomass to release fermentable sugars (Kumar, Barrett, Delwiche & Stroeve, 2009). Strong acids, such as sulphuric acid (H_2SO_4) and hydrochloric acid (HCl), at mild concentrations (<4% v/v) under high temperatures (>120 °C), have been applied to pre-treat rapeseed by-products (straw and meal) in various studies (Castro et al., 2011; Chen, Zhang, Miao, Wei & Chen, 2011; Jeong et al., 2013; López-Linares et al., 2014). Dilute H_2SO_4 (<4% v/v) pre-treatment on lignocellulosic materials results in a high reaction rate and improves cellulose hydrolysis, as well as effectively hydrolyses hemicellulose into dissolved sugars, with almost 100% hemicellulose hydrolysis yield. Once hemicellulose is removed, the digestibility of the cellulose in the residual solids is increased (Kumar et al., 2009).

Even though concentrated acids are powerful agents for cellulose and hemicellulose pre-treatment, they are toxic, corrosives and can be hazardous. Specialised reactors are also required that resist corrosion against acid such as glass-based reactors (Sivers & Zacchi, 1995). Moreover, strong acid hydrolysis results in the formation of compounds, such as furfural from xylan degradation, which can be inhibitory to microbial growth (Jung, Yu, Eom & Hong, 2013; Kumar et al., 2009). Jeong et al., (2013) compared several strategies for pectin extraction from rapeseed meal hydrolysis, including mild acid treatment (1% HCl), enzymatic hydrolysis and solvent extraction using alcohol–benzene mixtures. Results showed that samples pre-treated with mild acid yielded less pectin when compared to enzyme hydrolysis and solvent extraction. It suggests that mild acid treatment was too harsh on pectin and caused hydrolysis of galacturonic acid. Therefore, it can be said that the suitability of mild acid pre-treatment depends on the aims of the study.

Apart from acid pre-treatment, alkali can be used to pre-treat lignocellulosic materials which acts by removing the lignin and a part of the hemicellulose that lowers the accessibility of enzymes to the cellulose structure (Silverstein et al., 2006). The commonly used alkali reagents are sodium hydroxide (NaOH), calcium hydroxide, potassium hydroxide and ammonium salts (Taherzadeh & Karimi, 2008). Alkali pre-treatment is usually performed at ambient temperature and pressure unlike acid hydrolysis (Kumar & Sharma, 2017). Dilute alkali pre-treatment results in degradation of side-chain esters and glycosides, leading to structural modification of lignin and swelling of the cellulose in the lignocellulosic material. The swelling of cellulose further leads to an increase in the internal surface area, a decrease in the degree of polymerisation and cellulose crystallinity as well as the disruption of the lignocellulosic structure (Kumar & Sharma, 2017; Sun & Cheng, 2002; Taherzadeh & Karimi, 2008).

Han et al., (2012) investigated the use of dilute NaOH as a pre-treatment strategy prior to enzymatic hydrolysis of wheat straw under various conditions, including NaOH amount (0.2–1.5 % w/v) and reaction time (0.5–2.5 h) at fixed substrate concentration. The authors found that under optimised conditions (1% NaOH for 1.5 h) the cellulose content of wheat straw increased by 44% while the contents of hemicellulose and lignin decreased by 44 and 43%, respectively. Significant changes in the microscopic structure of wheat straw after alkali pre-treatment suggests that cellulose was more accessible to cellulase. Even though alkali is considered a good pre-treatment strategy, it requires an additional neutralisation step to remove salts from the biomass before being used in further stages, including enzymatic hydrolysis of cellulose and the subsequent fermentation process (Kumar & Sharma, 2017; Sun & Cheng, 2002). Furthermore, this strategy is found to be more effective towards agricultural residues and herbaceous crops than wood materials (Silverstein et al., 2006).

2.5.2 Hydrothermal pre-treatment

Hydrothermal pre-treatment, also known as cooking of lignocellulosic materials under high temperature (>150 °C) in liquid hot water and pressure (up to 5 MPa), is considered the most common pre-treatment strategy to treat biomass (Taherzadeh & Karimi, 2008). During this process, autohydrolysis occurs, where acids are formed by solubilisation of acidic compounds and de-esterification of ester groups (acetate) in hemicellulose occurs, producing weak acids, such as formic acid, acetic acid and glucuronic acids. The acids produced then act on the hemicellulose structure, resulting in the solubilisation and hydrolysis of hemicellulose to its monomeric sugars (e.g., xylose, arabinose, galactose) (Kumar & Sharma, 2017). During the hydrothermal process, no chemicals are added to the reaction; hence, the pre-treatment relies on the autohydrolysis that occurs. This process increases the surface accessibility for enzymatic hydrolysis, promotes cellulose decrystallisation and reduces the content of recalcitrant lignin (Sarip et al., 2016). It has been shown that the cellulose exposed by hydrothermal treatment of the biomass is markedly more susceptible to enzymatic activity (Yoon, 1998).

Hydrothermal pre-treatment can be performed in three different ways, based on the direction of flow between water and biomass. In the first strategy, both biomass and water are heated to the required temperature and held for a set period before being cooled. This method is known as concurrent pre-treatment. Another technique, known as counter-current pre-treatment, involves pumping hot water against the biomass in a controlled manner. Finally, in the flow-through pre-treatment, the biomass acts as a stationary bed, hot water flows through the biomass, and the hydrolysed fraction is carried out of the reactor (Kumar & Sharma, 2017; Mosier et al., 2004).

Pińkowska, Wolak & Oliveros (2013) investigated the effects of hydrothermal pre-treatment on rapeseed straw for the generation of xylose- and glucose-rich hydrolysates using an experimental design approach. They proposed a 3-stage hydrothermal treatment involving an initial stage performed at a relatively low temperature (195–212 °C) with short exposure times (9.5–16.5 min), in which solubilisation and acid hydrolysis of hemicellulose occur, but with most of the cellulose remaining in the residue. In the second stage, relatively higher temperature (245–267 °C) at shorter treatment time (11-6 min, respectively) is applied to hydrolyse the cellulose. Furfural and HMF are also formed in this step. Finally, lignin is decomposed by applying heat treatment at 330 °C for 60 min. In a different study, a 1-step hydrothermal pre-treatment of sunflower stalks was undertaken at an optimised temperature (180 °C), favouring the extraction of both hemicellulosic and cellulosic derived sugars while limiting the HMF formation (Jung, Yu & Eon, 2013). Data showed that the pre-treatment prior to enzymatic hydrolysis resulted in a higher hemicellulosic sugars recovery (xylose, mannose and galactose, 75%) compared to glucose (67%) (Jung et al., 2013). These studies suggested that hydrothermal pre-treatment can be tailored to suit the final applications.

2.6 Enzymatic Hydrolysis

Enzymes can also be used for the hydrolysis of lignocellulosic biomass and assist its conversion into a hydrolysate that could then serve as a generic feedstock for microbial bioconversions (Kiran et al., 2012). Such enzymatic treatments are advantageous as they are more environmentally-friendly compared to chemical hydrolysis (e.g., acid hydrolysis). Moreover, they are usually carried out under mild reaction conditions (e.g., ~30–50 °C), minimising the formation of degradation products such as furfural and HMF (Rodrigues, Carvalho & Rocha, 2014).

Cellulose is a linear polymer of glucose monomers linked together by β-1,4-glycosidic bonds. Saccharification of this material can be achieved using cellulase enzyme for the production of glucose. Commercial cellulase enzymes are usually mixtures of enzymes that have endoglucanase, exoglucanase and β-glucosidase activities and they work synergistically to ultimately break down the cellulose into glucose (Taherzadeh & Karimi, 2007). Endoglucanase attacks the low crystallinity region of cellulose producing a sugar-free chain end. Exoglucanase then works from the non-reducing end to degrade the sugar chain, releasing cellobiose which is an intermediate of cellulose depolymerisation. β-Glucosidase then acts to cleave the cellobiose unit into two molecules of glucose. The enzymatic hydrolysis of cellulosic compounds into its monomers in 5 steps: 1) the enzymes are transferred from the aqueous phase to the surface of the cellulose particles; 2) the enzymes are absorbed to the particles and form an enzyme–substrate complex; 3) cellulose is hydrolysed; 4) the products of hydrolysis, including cellodextrins, glucose and cellobiose are transferred to the aqueous phase; and 5) cellobiose is further hydrolysed by β-glucosidase, to produce glucose from the non-reducing end of the oligosaccharides (Sajith, Priji, Sreedevi & Benjamin, 2016; Taherzadeh & Karimi, 2007). The schematic diagram for the hydrolysis of cellulose is given in Figure 2-16.

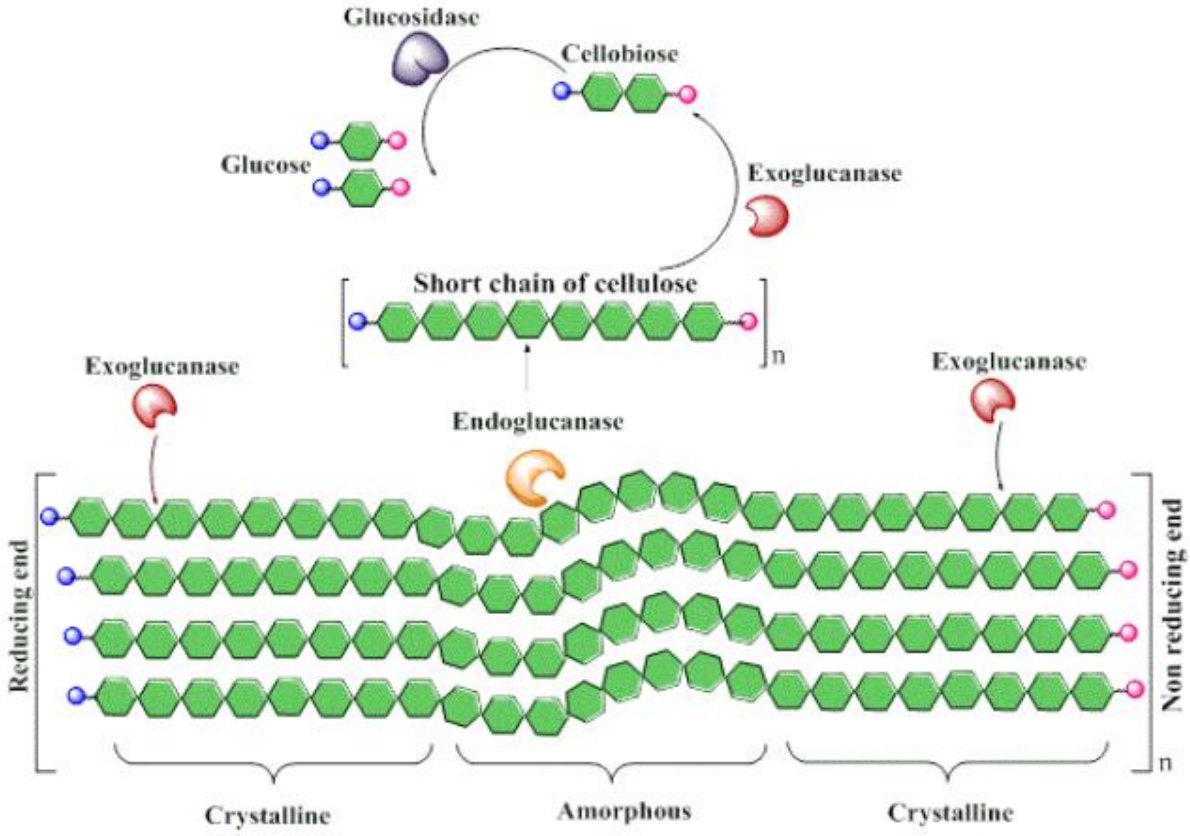

Figure 2-16: Schematic diagram of lignocellulosic biomass hydrolysis by cellulase mixtures (adapted by Sajith, Priji, Sreedevi & Benjamin, 2016).

The enzymatic conversion of rapeseed meal can be accomplished either via solid-state fermentations which involve fungal strains, such as *Aspergillus oryzae* or by adding particular enzymes such as cellulases and proteases (Jeong et al., 2014). *A. oryzae* is a well-known producer of proteolytic enzymes, as well as phytase, cellulase, xylanase and amylolytic enzymes (Chatzifragkou et al., 2014). Some commercial or developmental enzymes can be potentially used in this approach. Rodrigues et al., (2014) evaluated three commercial carbohydrases (Viscozyme L, Cellucast and Pectinex Ultra SP-L), independently, for their efficiency of rapeseed meal hydrolysis and was shown that Viscozyme L could produce protein-rich hydrolysates, containing more than 68% total protein and 80% total reducing sugars. Sari et al., (2013) evaluated the feasibility of using commercial proteases to extract protein from

several oilseed meals, including soybean, rapeseed and microalgae meal. They obtained almost 50–80% protein yield in rapeseed meal when combining alkali treatment with the different types of proteases. Overall, the hydrolysis of meals was influenced by pH, and the types of biomass and enzymes used.

Enzymatic hydrolysis offers several advantages compared to chemical and thermal methods, eliminating the use of chemicals and resulting in a more environmentally-friendly process. It is also done under comparatively milder conditions, which minimises the formation of sugar degradation products (HMF and furfural) (Rodrigues, Carvalho & Rocha, 2014). However, the enzymatic hydrolysis of lignocellulosic biomass is relatively slow (>24 h), and without any pre-treatment of the biomass, it is unlikely to result in high yields. The reason is most likely due to the dense structure of the biomass and the fact that linkages between the lignin, cellulose and hemicellulose cannot be accessed by enzymes which are used to hydrolyse the polysaccharides. Therefore, the application of alternative pre-treatment methods such as the chemical (section 2.4.1) and physical (section 2.4.2) methods are necessary to enhance the susceptibility of lignocellulosic biomass to hydrolytic enzymes (Brodeur et al., 2011).

2.7 Production of Astaxanthin by Fermentation Using *Xanthophyllomyces dendrorhous*

As mentioned in section 2.1.5, *X. dendrorhous* is a promising microorganism for the production of astaxanthin by fermentation. In designing such fermentation process, several factors need to be considered, including the strain used, the fermentation substrate and the effects of pH, agitation, aeration and inducers. These factors are discussed in more detail in the following sections.

2.7.1 Xanthophyllomyces dendrorhous

X. dendrorhous (asexual stage, *P. rhodozyma*) was first isolated from wounds of birch trees by Herman Phaff in the late 1960s, in Japan and Alaska. *Phaffia* species can synthesize carotenoids, and this biotechnological process attracts considerable scientific interest. The species belong to the Basidiomycetes class and has no sexual cycle (Roy, Chatterjee & Sen, 2008). *X dendrorhous* is the teleomorphic state of *P. rhodozyma* (Globulev, 1995). The sexual stage of *X. dendrorhous* is unique as it involves a holobasidia with terminal basidiospores formed after mother–daughter cell conjugation (Roy, Chatterjee & Sen, 2008). Table 2-1 shows the classification of *X. dendrorhous*.

Table 2-1: Classification of *Xanthophyllomyces dendrorhous*

Division	Basidiomycota
Subdivision	Agaricomycotina
Class	Tremellomycetes
Subclass	Tremellomycetidae
Order	Cystofilobasidiales
Family	Cystofilobasidiaceae
Genus	Xanthophyllomyces
Anamorph	*Phaffia rhodozyma*

The main pigment produced by *X. dendrorhous* is astaxanthin that accounts for about 83–87% of the total carotenoids produced intracellularly. The astaxanthin production in *X. dendrorhous* is well established, and it follows the MVA pathway, described in section 2.1.5.1 (Schmidt et al., 2011). *X. dendrorhous* is an aerobic organism; its primary metabolism is dependent on oxygen supply in the culture system. However, it can undergo fermentative catabolism of glucose, leading to ethanol and acetic acid production, even under aerobic conditions; this is called the Crabtree effect and takes place particularly when the concentration of hexose sugars present in the media is above its threshold values (Liu, Wu & Ho, 2006; Rodríguez-Sáiz, Luis De La Fuente & Barredo, 2010). This yeast has an advantage since it is capable of fermenting a wide range of sugars, including glucose, xylose and arabinose. The optimal growth conditions for *X. dendrorhous* are between pH 5–6 at 15–22 °C (Roy, Chatterjee and Sen, 2008). Figure 2-17 presents the macro- and microscopic images of *X. dendrorhous*. The colonies of *X. dendrorhous* can be observed as orange–pink pigments with a smooth spherical shape when cultured on yeast malt agar.

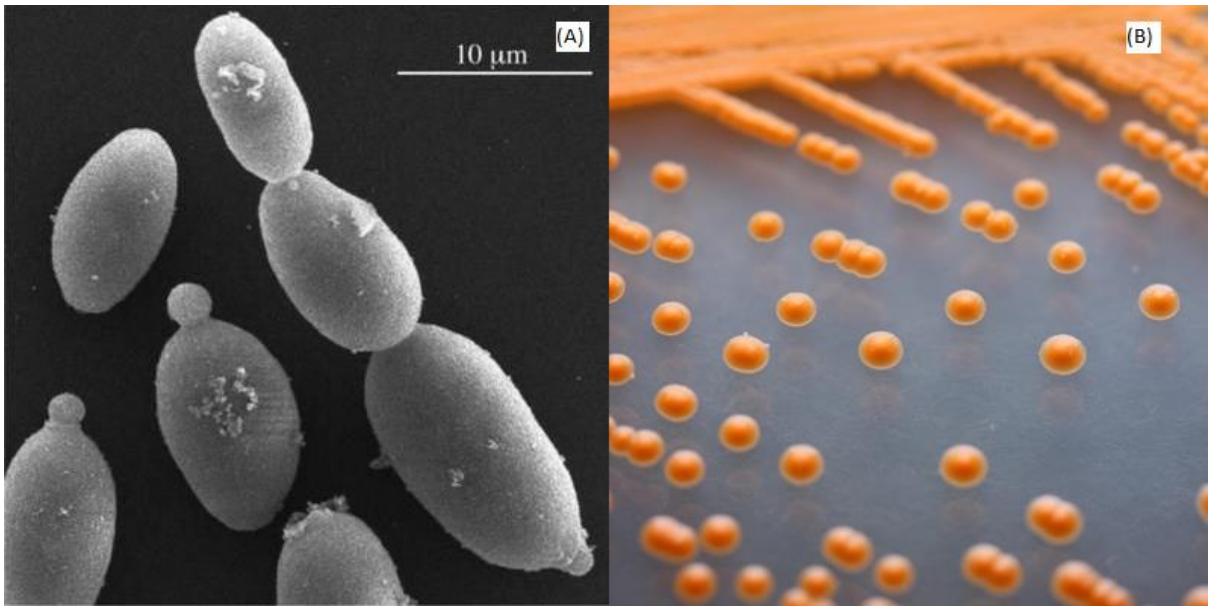

Figure 2-17: Images of *X. dendrorhous*: scanning electron microscopy image (A) and colonies on yeast and malt agar (YM agar) (adapted from Lin et al., (2012) and JCM catalog; http://jcm.brc.riken.jp/en/)

The wild strain of *X. dendrorhous* DSMZ 5626 was first isolated from *Fagus crenata* tree in 1989. Generally, wild-type *X. dendrorhous* is known to produce low quantities of astaxanthin (200–500 µg/g dry yeast), whereas biotechnologically improved strains result in higher values of 6000–15000 µg/g dry yeast (Lin et al., 2012).

2.7.2 Fermentation Substrates

The production of carotenoids, astaxanthin and β-carotene using a diverse range of substrates has been extensively studied. As mentioned in section 2.6.1, *X. dendrorhous* supports astaxanthin production and can utilise a wide range of carbon sources including hexoses such as glucose but particularly pentoses, such as xylose and arabinose, as well as disaccharides such as cellobiose (Kusdiyantini et al., 1998; Montanti, Nghiem & Johnston, 2011; Parajo, 1998). Apart from refined sugars, glycerol has also been used as a carbon source for several yeast strains, mainly from the genus *Rhodotorula* and *Xanthophyllomyces*, for converting glycerol into many different types of valuable metabolites (Aksu and Eren, 2005; Kusdiyantini et al., 1998). Table 2-2 summarises the previous literature research on refined sugars as the carbon source for microbial astaxanthin production. The cultivation of *X. dendrorhous* has been tested on a small-scale (e.g., flasks), benchtop-scale (e.g., 2-5 L fermenters and batch, fed-batch mode) and large-scale (>20 L bioreactors), and proved to support astaxanthin production in the cells.

As previously mentioned, agri-food wastes and by-products, as well as lignocellulosic biomass, can be potentially hydrolysed and used as fermentation feedstocks for the production of a variety of chemicals, including carotenoids. Extensive research has been undertaken using various feedstocks, including dried distillers grains with soluble (DDGS), palm oil waste, molasses, cereal straws and woody residues to produce chemicals such as biofuels, bioplastics, platform chemicals (e.g., succinic acid) and carotenoids (Aksu & Eren, 2005; Cui et al., 2014; Petrik et al., 2014). However, a limited number of studies (Table 2-3) have focused on

astaxanthin production by *X. dendrorhous* using agricultural wastes such as date juice and lignocellulosic biomass like wood hydrolysates. It must be noted that for lignocellulosic biomass, the polysaccharides present in the matrix need to be hydrolysed first into simple sugars, such as glucose, xylose, galactose and arabinose (refer to section 2.4) which can support good cell growth of *X. dendrorhous*. The results from the studies presented in Table 2-3 demonstrate that lignocellulosic hydrolysates are potentially good feedstocks for astaxanthin production. In particular, the astaxanthin yields which range from 0.38–0.99 mg/g of dry yeast depending on the yeast strain and cultivation method, are similar to those obtained using semi-defined media (Table 2-2) (Jirasripongpun, Pewlong, Natsathmonthra & Suthiyaporn, 2007; Vázquez et al., 1998). The lignocellulosic hydrolysates may also contain some other sugars, such as galactose and sucrose, depending on the type of lignocellulosic material used. Overall, an important aim during the production process is to ensure that the pre-treatment and hydrolysis steps release the fermentable sugars at high yields so as to improve the efficiency of the overall process.

Table 2-2: Summary of research works investigating the production of astaxanthin by fermentation of *X. dendrorhous* in semi-defined media containing refined sugars as the carbon source

Species	Carbon source	Process	Yield (mg/g of dry weight)	Reference
X. dendrorhous AS 2.1557	Glucose	Batch -flask	0.72	(Wu & Yu, 2013)
	Sucrose		0.69	
	Xylose		0.37	
	Glucose + soy bean oil		0.84	
P. rhodozyma UCD-FST- 67-385	Glucose + ethanol	Batch -flask	0.52	(Gu, An & Johnson, 1997)
P. rhodozyma UCD 67-210	Glucose	Batch -flask	0.42	(Lewis, 1979)
	Cellobiose		0.65	
	L-arabinose		0.38	
X. dendrorhous ZJUT 46	Glucose	Fed-batch – 200 L bioreactor	0.49	(Hu et al., 2007)
X. dendrorhous ZJUT 003	Glucose	Batch -50L bioreactor	0.53	(Zheng et al., 2006)
P. rhodozyma	Glycerol	Batch – 2L bioreactor	13.1	(Kusdiyantini et al., 1998)

Table 2-3: Summary of research works investigating the production of astaxanthin by fermentation of *X. dendrorhous* in low cost agri-food substrates.

Species	**Carbon sources**	**Cultivation mode**	**Yield (mg/g of dry weight)**	**Reference**
***P. rhodozyma* ATCC 24202**	Sugar cane juice	Fed-batch-continuous feeding	0.38	(Moriel et al., 2005)
		Fed-batch- pulse feeding	0.30	
***X. dendrorhous* 25-2**	*Yucca fillifera*, date juice	Fed-batch	0.60	(Ramírez, Obledo, Arellano & Herrera, 2006)
***P. rhozozyma* NRRL Y-17268**	*Eucalyptus* wood hydrolysates	Fed-batch (continuous feeding)	0.78	(M Vázquez, Santos & Parajó, 1998)
		Fed-batch (intermittent feeding)	0.99	
X. dendrorhous GM807	Molasses	Batch -flasks	0.45	(Jirasripongpun et al., 2007)

2.7.3 Effects of Agitation and Aeration

The oxygen transfer rate (K_La) is a major factor in determining the success of aerobic microbial growth, including astaxanthin production by *X. dendrorhous*. To achieve a good oxygen transfer, the agitation speed and the aeration rate play an important role. In shake flask fermentation, oxygen is supplied via adequate mixing and shaking with the culture volume also influencing the process. Usually, high shaking speed and low culture volume will enhance the agitation, thereby improves the oxygen transfer (Liu, Wu & Ho, 2006). Additionally, the agitation provides uniform mixing of the medium components within flasks/vessels, allowing increased dispersion of the cells and nutrients. It also facilitates the removal of gasses from the microenvironment of the cells (Rodmui, Kongkiattikajorn & Dandusitapun, 2008) which, in the case of *X. dendrorhous*, facilitates a high oxygen transfer from the microenvironment to the cells and removal of carbon dioxide from the vessel.

In stirred tank bioreactors, oxygen is supplied by sparging the air into the vessel. The agitation is provided by impellers, to allow homogenous distribution of the oxygen throughout the culture medium as well as ensure good mass and heat transfer. However, agitation will create shear forces that might affect the morphology and variation in growth as the product forms, so studies in optimising the agitation feed applied are important in bioreactor fermentation (Rodmui, Kongkiattikajorn & Dandusitapun, 2008).

Research has shown that high levels of oxygen supply are advantageous for the growth of *X. dendrorhous* and astaxanthin production. Wang & Yu (2009) demonstrated that at a high initial volumetric coefficient (K_La) of 148.5 h^{-1}, the maximal yeast growth was observed (19.4 g/L) and 18.1 mg/L of astaxanthin was produced. These outcomes were reduced to 4.5 g/L of yeast and 2.5 mg/L of astaxanthin when the K_La value was lowered to 21.5 h^{-1}, suggesting that high oxygen is required to support both yeast growth and astaxanthin production. Even though

lack of oxygen supply is known to affect cell growth, cellular morphology and metabolite intake, to date, few works have examined the influence of oxygen supply on the growth of *X. dendrorhous*, highlighting this area as a topic of interest to be explored (Yamane et al., 1997). Since *X. dendrorhous* is an aerobic yeast, investigating oxygen requirement in each strain is one of the critical parameters that affects yeast growth and subsequently, astaxanthin production.

2.7.4 Effects of Inducers

Addition of inducers or molecules that can act to stimulate carotenoid synthesis can be applied to enhance carotenoid production in yeasts, including *X. dendrorhous*. Several such molecules have promoted the carotenogenesis in yeast, including acetic acid, ethanol (Gu et al., 1997) and mevalonic acid (Calo et al., 1995). The mechanisms for each chemical to induce carotenogenesis in *X. dendrorhous* are different. For ethanol, the induction of carotenogenesis might be related to alteration of the microorganism's respiratory metabolism and formation of reactive oxygen species, as well as the possibility to induce specific enzymes involved in the MVA pathway such as the P-450 systems and oxidases (Gu et al., 1997). Induction of carotenogenesis by acetate occurs due to inhibition of glycolate bypass, resulting in an increased level of acetyl-CoA which is the starting material for the MVA pathway. Similar mechanisms arise when mevalonate acid is added to the system, such as an increase in MVA concentration and subsequently, an increase in key intermediate precursors in the MVA pathway (Calo et al., 1995). It was shown that the yield of astaxanthin increased up to 400% when 0.1% mevalonate acid was added to the media as compared with the control (Calo et al., 1995). However, the use of this precursor is not applicable to large-scale production due to the high cost.

Liu & Wu (2006) tested several hydrocarbon liquids such as *n*-hexane, toluene, *n*-decane and *n*-dodecane, aiming to improve the oxygen uptake rate by the yeast. The

experiments proved that adding 9% (v/v) *n*-hexadecane to the culture media significantly enhanced the production of astaxanthin by 58% and the oxygen uptake rate by 90%. The addition of oxygen vectors such as *n*-hexadecane, increases the oxygen levels present in the culture media and thereby increases the oxygen uptake rate (K_La) by the yeast. Thus, it represents an alternative to applying high aeration and high agitation rate, which might become limitations when scaling up.

2.7.5 Effect of pH

The pH of the culture media plays an important role in the production of products and growth of microorganisms. Typically, the pH influences the cell growth and product accumulation, but the effects vary according to differences in the strain, medium composition and fermentation condition. In *X. dendrorhous*, the yeast growth and astaxanthin pigmentation occur at pH in the range of 3.8–7.5, depending on the strain used (Johnson, Villa & Lewis, 1980). Hu, Zheng, Wang & Shen (2006) identified that the optimum pH for yeast growth and for astaxanthin pigmentation differed in *X. dendrorhous* strain 46. More specifically, in shake flask fermentations, the optimal pH for yeast growth occurred at pH 6 (17.21 g/L) while astaxanthin production was highest at pH 5 (20.5 mg/L). The same authors further demonstrated that pH control is more significant in bioreactor studies, as it was shown that the specific growth rates increased from 0.033 h^{-1} at pH 4 to 0.049 h^{-1} at pH 6. This result is supported by Dias et al., (2015) who reported a similar observation in the yeast species *Rhodosporidium toruloides* NCYC 921, whereby the optimum pH for carotenoid production (pH 4) differed from that for biomass yield (pH 5).

These findings led to an improved approach to controlling the pH, to suit the optimum condition for both yeast growth and product accumulation. The pH-shift strategy was applied by varying the pH control during the initial fermentation period and product formation stage

(Dias et al., 2015). Since carotenoids are produced during the late exponential growth phase of yeast kinetics, the pH was then shifted to the optimum pH required for carotenoids production. This pH-shift does not affect the yeast growth as they are already in the late exponential growth phase. This strategy was implemented during batch fermentation of *X. dendrorhous*, where astaxanthin production was maximised by controlling the fermentation at pH 6 for 80 h and then shifting it to pH 4. An increase of 24.1% astaxanthin production, i.e. to 2.7 mg/L, was achieved as compared to a constant pH control strategy, in the bioreactor (Hu et al., 2006). To date, there are not many reports available that focus on the pH control during fermentation of *X. dendrorhous*. Hence, further studies in this area are important to maximise astaxanthin production in yeast, as the control of the pH is an important target in achieving the maximum yield for the product of interest.

2.8 Process Strategies for the Hydrolysis and Fermentation of Lignocellulosic Materials

The bioconversion of lignocellulosic materials into value-added products can be accomplished using various approaches including simultaneous saccharification and fermentation (SSF), separate hydrolysis and fermentation (SHF), and pre-hydrolysis and simultaneous saccharification and fermentation (pSSF) (Taherzadeh & Karimi, 2008; Verardi, Bari, Ricca & Calabrò, 2012). These types of strategies could be potentially applied for the production of astaxanthin from *X. dendrorhous* and are discussed in more detail in the following sections.

2.8.1 Separate Hydrolysis and Fermentation (SHF)

SHF is one of the most common methods used for the bioconversion of lignocellulosic material. It involves two sequential steps in which the enzymatic hydrolysis of the lignocellulosic material (either with or without pre-treatment) is performed in a separate vessel

before the fermentation process. After that, the hydrolysates are separated from the biomass by centrifugation or filtration and then sterilised to be used as a fermentation substrate (Figure 2-18). The main advantage of this process is that the enzymatic hydrolysis step is conducted at its optimum temperature which is typically between 45–55 °C for cellulase hydrolysis but this also depends on the enzyme used (Taherzadeh & Karimi, 2007). In contrast to the high temperature during enzymatic hydrolysis, *X. dendrorhous* has a lower optimum temperature for growth (15–22 °C) (Roy, Chatterjee and Sen, 2008).

However, the main drawback of this process is that cellulase activity might be inhibited due to product inhibitions, i.e. by glucose and cellobiose (Taherzadeh & Karimi, 2008). This phenomenon was demonstrated by Xiao, Zhang, Gregg and Saddler (2004) who studied the degree of inhibitory effect of glucose on cellulase activity by supplying additional glucose at concentrations ranging from 0–100 g/L. A significant reduction of cellulase activity was noted even with 20% glucose supplementation. Another drawback is the high risk of microbial contamination during the hydrolysis step of the SHF process due to the long hydrolysis period and the moderately high temperature (45–55 °C). In practice, it is generally difficult to filter sterilise large quantities of enzymes and autoclaving is not an option due to enzyme deactivation at high temperatures (Taherzadeh & Karimi, 2007).

Currently, there is a limited number of studies which involve the use of lignocellulosic materials as substrates for microbial astaxanthin production by the yeast *X. dendrorhous*. Parajó, Santos, Vázquez and Cruz, (1997) applied the SHF approach for *X. dendrorhous* growth, utilising *Eucalyptus* wood hydrolysates as the fermentation substrate for astaxanthin production. Moreover, peat hydrolysate was used as the fermentation substrate for microbial astaxanthin production in *P. rhodozyma* ATCC 24202 (Vázquez & Martin, 1998). In that study, instead of enzymatic hydrolysis, 1.5% H_2SO_4 was used to hydrolyse peat at a ratio of 20 g

peat/100 g acid solution, followed by autoclaving at 121 °C for 2 h. The peat hydrolysates produced were then filtered prior to the fermentation process (Vázquez & Martin, 1998). This approach is commonly applied for the production of other bioproducts from lignocellulosic material. For instance, in SHF experiments, rapeseed straw was converted into ethanol by *Saccharomyces cerevisiae*, generating up to 39.9 g/L of ethanol (López-Linares et al., 2014).

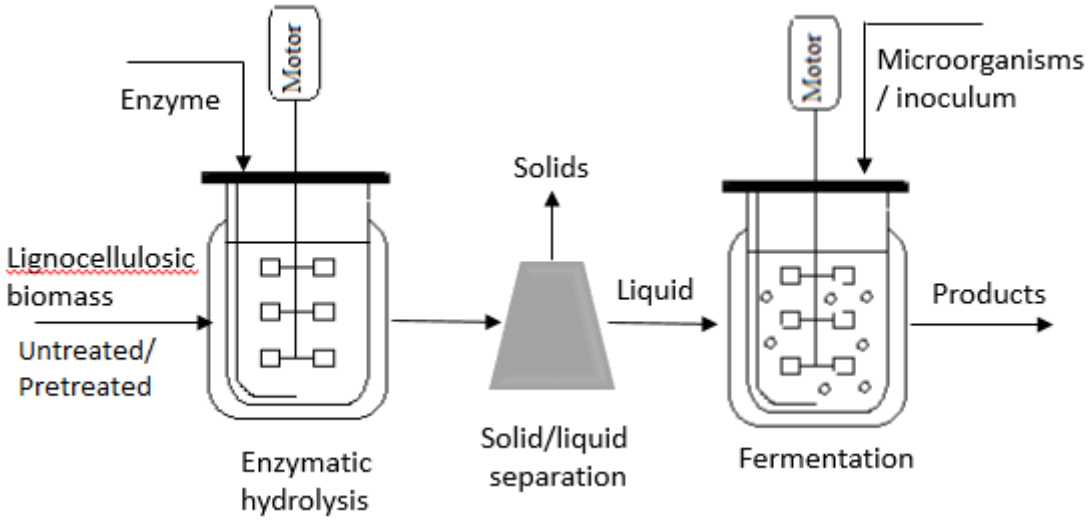

Figure 2-18: Schematic diagram for separate hydrolysis and fermentation process (SHF).

2.8.2 Simultaneous Saccharification and Fermentation (SSF)

The SSF method is commonly applied in biomass utilisation. In this procedure, both the biomass hydrolysis and microbial fermentation occur simultaneously in the same bioreactor. The schematic diagram for this process is given in Figure 2-19. This process overcomes the cellulase inhibition seen in SHF because the enzymatic hydrolysis products (glucose and xylose) are consumed simultaneously by the microorganism during the process and are

therefore maintained at low concentrations in the bioreactor (Kádár, Szengyel & Réczey, 2004; Öhgren et al., 2007; Taherzadeh & Karimi, 2008). This strategy may also reduce the requirement for high enzyme doses and thus lower the production costs (Kádár, Szengyel & Réczey, 2004). SHF has been successfully used for bioethanol production from lignocellulosic biomasses such as corn stover (Chu et al., 2012), pine (von Sivers & Zacchi, 1995) and wheat straw (Petrik, Kádár & Márová, 2013).

Nonetheless, there are several drawbacks to this strategy, including the different optima of temperature for the enzymatic hydrolysis of lignocellulosic biomass and microbial fermentation. Cellulase enzymes commonly have an optimum temperature between 45–55 °C, depending on the producer. In contrast, most carotenoid-producing microorganisms require a lower operating temperature, as exemplified by *X. dendrorhous*, which has an optimal operating temperature of 15–22 °C. Due to the substantial temperature difference between the two, it might not be a suitable strategy, in this instance. In some cases, this problem could be overcome by utilising a highly thermostable microorganism that can withstand the high temperatures during the hydrolysis step. In a study by Kádár et al. (2004), thermotolerant yeast strains were used for ethanol production using the SSF strategy. The temperature was set at 40 °C to achieve both the hydrolysis of the industrial waste as well as ethanol production in yeast and resulted in 55–60% cellulose conversion.

Another problem associated with this strategy is the presence of biomass solids in the bioreactor which can complicate the downstream processing, especially when the target product is located intracellularly as in the case of astaxanthin produced from *X. dendrorhous*. For this reason, SHF is preferred to SSF for intracellularly-produced products. However, for extracellularly-secreted products, such as bioethanol, SSF is preferred.

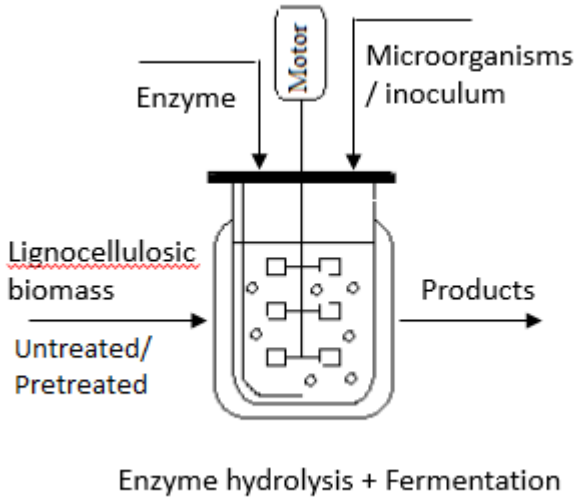

Figure 2-19: Schematic diagram of simultaneous saccharification and fermentation process (SSF).

2.8.3 Pre-Hydrolysis and Simultaneous Saccharification and Fermentation (pSSF)

The pSSF method has been designed to overcome the problems encountered in the SSF strategy. This process involves two steps that are both carried out in the bioreactor vessel. Initial enzymatic hydrolysis is done at its optimum temperature, allowing for high enzyme activity and thus a high sugar conversion. The second stage involves reducing the temperature to the optimum values required for the microbial fermentation process to enable high biomass and product formation. By following this strategy, the viscosity of the mixture is reduced as the lignocellulosic biomass is hydrolysed during the first stage (López-Linares et al., 2014). Reducing the viscosity of the mixture allows for mixing and hence, improves the mass and heat transfer inside the vessel. The method is schematically illustrated in Figure 2-20.

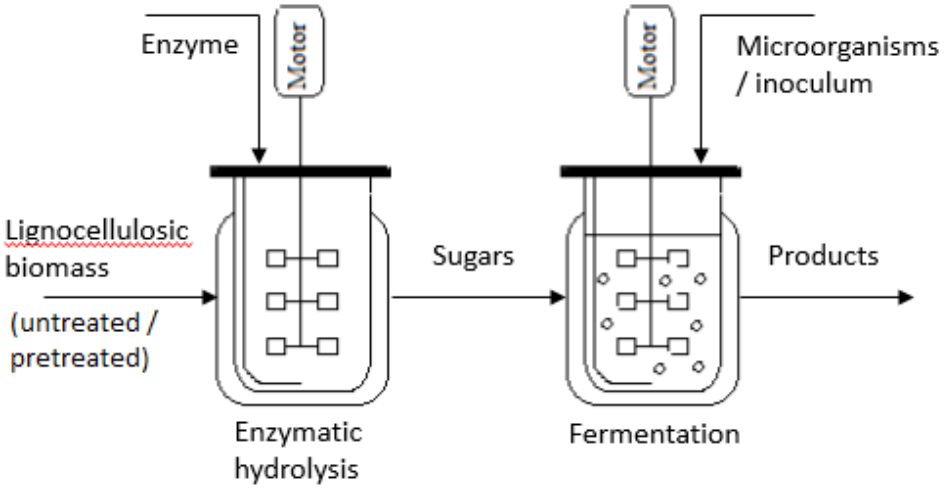

Figure 2-20: Schematic diagram of pre-hydrolysis and simultaneous saccharification and fermentation (pSSF).

The pSSF method is commonly employed for the bioconversion of lignocellulosic material into ethanol. As an example, Öhgren et al. (2007) applied pSSF to steam pre-treated corn stover, yielding 33.8 g/L ethanol, corresponding to 80.2% of the theoretical total glucose conversion. In this instance, the initial enzymatic hydrolysis involved using commercial enzymes from Novozymes before yeast inoculation (*S. cerevisiae*) (Öhgren et al., 2007). To our knowledge, this method has not been evaluated for the production of astaxanthin in *X. dendrorhous* using lignocellulosic materials.

López-Linares et al. (2014) compared the three different approaches (SHF, SSF and pSSF) for the valorisation of rapeseed straw to ethanol production by fermentation using *S. cerevisiae*. They revealed that SHF is the best strategy to obtain high ethanol production (39.9 g/L) compared to SSF and pSSF. As for microbial astaxanthin production using lignocellulosic materials, the strategy of using SHF has been applied with materials including wood hydrolysates and cassava residues (Parajó et al., 1997; Yang et al., 2011).

2.9 Downstream Processing: Extraction and Separation of Astaxanthin

Downstream processing is an important part in the production of carotenoids from microbial cells. Carotenoids are mostly lipophilic compounds that are insoluble in aqueous media, except in certain cases where highly polar functional groups are present in their structure (Oberholster, 2012). Norbixin, a carotenoid with a dicarbonyl acid structure, is one of the soluble carotenoids present in nature (Perera & Yen, 2007). The instability of carotenoids to light, heat and high oxygen environments also means that additional steps are needed during the extraction process to ensure that the carotenoids do not degrade (Saini & Keum, 2018). In this context, the extraction process needs to be performed as quickly as possible, to ensure a high extraction yield, as well as the stability of the product. Given that carotenoids are produced intracellularly within microbial cells, the main extraction methods that could be potentially used for the extraction of astaxanthin from *X. dendrorhous* are solvent extraction, either on its own or in combination with a physical or biological treatment, such as bead milling or enzyme treatment, as well as supercritical fluid extraction (SFE). These methods are discussed in more detail in the subsequent sections, after a detailed discussion on the yeast cell wall composition and structure.

2.9.1 Architecture of Yeast Cell Wall

The knowledge of the yeast cell structure helps in determining the efficacy of the extraction strategy. Yeast cells exhibit a great diversity of cell size, shape, cell wall structure and composition and colour. These attributes are affected by the species, the fermentation conditions, including cultivation temperature, pH, and oxygen, as well as the composition of the growth medium (Liu, Ding, Sun, Boussetta & Vorobiev, 2016). Different species of yeast will potentially exhibit differences in cell wall structure and cell composition. However, in this

section, the yeast structure of *Saccharomyces cerevisiae*, a well-studied yeast species is used as a yeast model to represent the general composition of the yeast cell wall (Figure 2-21).

The yeast cell wall consists mainly of mannoprotein and fibrous β-1,3-glucans, with some branches of β-1,6-glucans. These glucans are responsible for providing strength and elasticity to the cell wall. The β-1-3-glucan–chitin complex is the major component in the inner cell wall where it forms a fibrous scaffold. The mannoproteins present on the outer surface of the cell wall are densely packed, limiting the permeability of solutes (Salazar, 2008). β-1-6-Glucan action is to link the inner and outer cell wall components. Mannans are a polymer of mannose residues with α-(1-6) linkages and present short oligosaccharide side-chains. All these compounds are covalently linked to form a macromolecular complex. Chitin is a polymer of *N*-acetylglucosamine present in small quantities (2–4% of dry weight) in the cell wall and is mainly located in bud scars. Proteins are present in the inner part of the cell wall and provide the cell shape. Other components present in variable quantities are lipids and inorganic phosphate. The plasma membrane (~7 nm thickness) consists of a thin semi-permeable lipid bilayer, formed mainly by proteins and lipids that protect the integrity of the cell and separate the cytoplasm from the extracellular environment. The periplasmic space in yeast is a thin region between the plasma membrane and the cell wall that consists of large molecules of mannoproteins (Liu et al., 2016). Astaxanthin can be found in the cytoplasmic region of the yeast cell wall (Johnson & Schroeder, 1996). The complex structure of the yeast cell wall hinders the implementation of simple processes for the extraction of intracellular pigments from yeast cells. The rigid cell wall of yeast is remarkably thick (100–200 nm) and represents about 20–25% of the total dry weight of yeast (Liu et al., 2016). Overall, understanding the complexity of the yeast cell wall is important for selecting strategies for astaxanthin extraction from yeast cells (Duarte et al., 2017).

Since the complexity of the yeast cell wall limits the extraction of astaxanthin extraction, an initial treatment step is required to break the cell wall structure and render the intracellular components, such as astaxanthin, available for extraction. The key methods (i.e., physical, chemical and enzymatic) are usually carried out in combination, as described below for the extraction of astaxanthin from yeast cells.

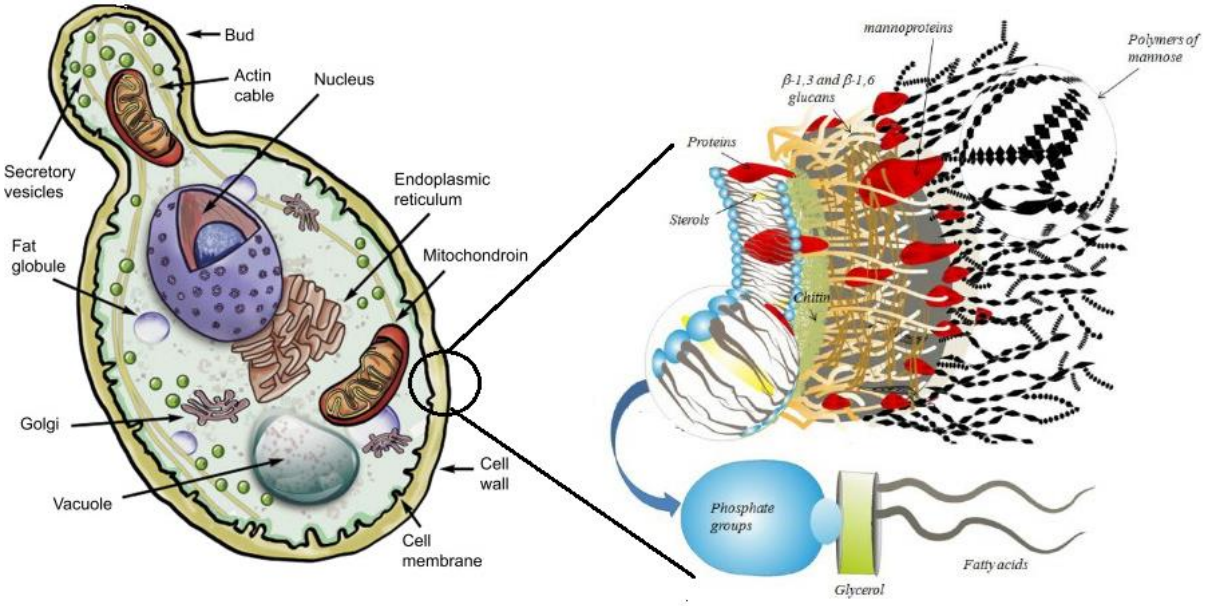

Figure 2-21: Structure of yeast cell wall [adapted from Speers & Forbes (2015) and Talavera et al. (2013)].

2.9.2 Solvent Extraction

Organic solvent extraction is commonly used for recovering carotenoids from microbes and plant materials. The use of organic solvents for carotenoid extraction from microbial cells, such as yeast cells, is a slow process that requires large amounts of chemical solvents. Depending on the solvent used, it can potentially be harsh and relatively toxic, as well as contribute significantly to the overall cost of the extraction process (Saini & Keum, 2018). Moreover, after the recovery of the carotenoids from either microbial or plant sources, an additional liquid–liquid extraction step is necessary to remove the organic solvent from the carotenoid-rich extract. The selection of the organic solvent used for the extraction of carotenoids depends on the characteristics of the carotenoids source (e.g., yeast cells, plants), as well as the properties of the target carotenoid, particularly its solubility. Normally, acetone is commonly used, due to its polarity and the ability to maintain pigment stability. Other suitable solvents are methanol, ethanol, diethyl ether, hexane, either individually or in combination (Monks et al., 2012; Saini & Keum, 2018).

The mechanism of organic solvent extraction of non-polar pigments, such as astaxanthin, from microbial cells is suggested to occur in 5 steps, which are depicted in Figure 2-22. When cells are exposed to a non-polar organic solvent, the solvent penetrates through the cell wall into the cytoplasm (step 1) and interacts with the neutral lipids located in the cytoplasm using van der Waals forces (step 2) to form an organic solvent–pigments complex (step 3). Driven by a concentration gradient, this complex then diffuses out of the cell wall (step 4) and through the static organic solvent film surrounding the cells (step 5) into the bulk organic solvent. A static organic solvent film is formed around the yeast wall, due to the interaction between the organic solvent and cell wall that remains intact even under solvent flow and

agitation. As a result, the pigments are extracted out of the cells and remain dissolved in the non-polar organic solvent (Halim, Danquah & Webley, 2012).

As mentioned in section 2.8.1, the extraction of astaxanthin from yeast cells, such as *X. dendrorhous*, is challenging due to the presence of a thick cell wall that renders the cell resistant to penetration by many solvents (Liu et al., 2016). For this reason, additional steps, involving either mechanical or chemical pre-treatments are required to degrade the cell wall prior to the solvent extraction process. Sedmak and Weerasinghe (1990) proposed the use of pre-heated dimethyl sulfoxide (DMSO) to rupture the cell wall of *P. rhodozyma* before solvent extraction using hexane:ethyl acetate (1:1) mixtures. Moreover, Valduga et al. (2014) used liquid nitrogen to freeze the yeast cell before maceration with a pestle and mortar and then added DMSO to break the cell wall of *Sporidiobolus pararoseus* for carotenoid extraction, which gave promising results.

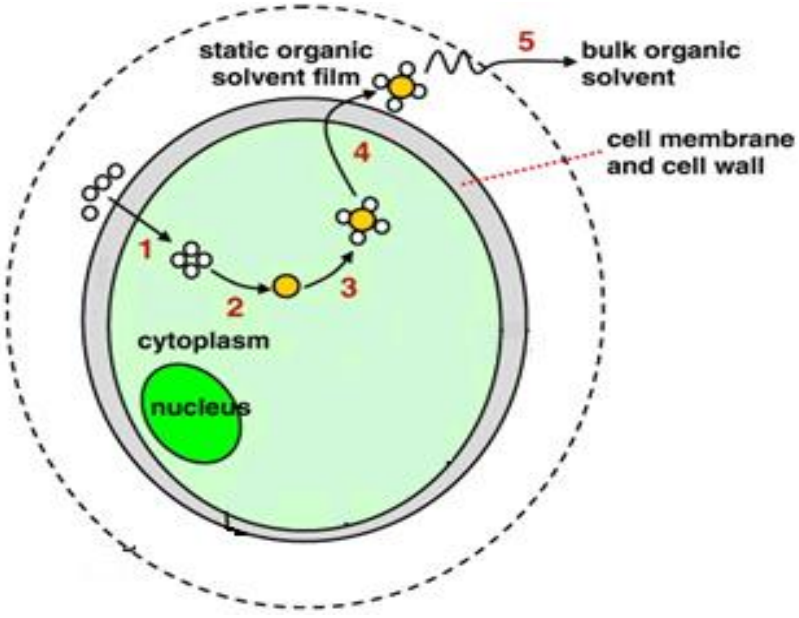

Figure 2-22: Schematic diagram depicting the steps taking place for the extraction of pigments from yeast cells using organic solvents (adapted from Halim et al., 2012).

2.9.3 Bead Milling in Combination With Solvent Extraction

The use of mechanical abrasion to break the yeast cells, coupled with solvent extraction has been investigated on a small-scale using glass beads (size 0.25 mm) in acetone, by vigorous shaking of *P. rhodozyma* cell suspensions (Fonseca et al., 2013; Johnson, Villa & Lewis, 1980). Glass beads extraction acts by disrupting the beads collision zones by compaction or shear force with energy transfer from the beads to the cells. Utilising small beads enhanced the disruption efficiency of yeast, due to increasing the bead–bead collisions, resulting in an increased cell disruption rate (Liu et al., 2016). It is considered as a feasible method for scaling up purposes and usually gives high extraction yield for carotenoids, at least in small-scale operations, i.e., 45–48% astaxanthin extractability in *P. rhodozyma* NRRL Y-17268 (Michelon et al., 2012). It needs to be noted that such a process is non-selective, as all polar/non-polar compounds present in both the cell wall and cytoplasm are extracted due to almost complete cell disruption, depending on the solvent used. Therefore, an effective purification process needs to be designed for the subsequent purification of the target carotenoid from the mixture.

2.9.4 Enzyme Hydrolysis in Combination With Solvent Extraction

The enzyme-assisted approach for degrading the yeast cell wall for carotenoid extraction is not yet well-reported in the literature. On the other hand, this approach is commonly used for the production of yeast extract and lipid extraction from yeast species, like *S. cerevisiae* (Milic, Rakin and Siler-Marinkovic, 2007) and *R. toruloides* (Jin et al., 2012). In this approach, the synergistic effects of different enzymes, including β-1,3-glucanase, mannanase, protease, β-1,6-glucanase and chitinase, is exploited to achieve yeast cell wall lysis (Salazar, 2008). These enzymes attack the mannoprotein complex and the glucan backbones of the yeast cell wall, without destroying the integrity of the cell wall (Liu et al., 2016). The mechanism of yeast cell lysis can occur in three steps, starting with the binding of the lytic

protease to the outer mannoprotein of the cell wall that results in the opening of the protein structure. In this step, the cell wall proteins and mannans are released, and glucan surface is exposed (step 1). Next, glucanase attacks the inner wall and solubilises the glucans (step 2). Finally, under the osmotic pressure difference, the cell bursts and releases its periplasmic contents into the media (step 3) (Liu et al., 2016; Prokopakis & Liu, 1997; Salazar, 2008). Organic solvents are used for the extraction of carotenoids, in step 3, as the opening up of the structure of the yeast cell wall facilitates the penetration of solvents into the cells (Michelon et al., 2012).

A few enzyme formulations are commercially available for yeast cell lysis. Most of these enzymes are used to retrieve yeast extract (usually containing mixtures of intracellular components, including amino acids, peptides and carbohydrates, which are commonly used as a component of fermentation media). Commercial enzymes include Zymolase, Lysozyme and Glucanex. Michelon et al. (2012) tested various strategies for cell lysis before extraction of carotenoids from *P. rhodozyma* NRRL Y-17268. They reported that by utilising a commercial enzyme formulation (Glucanex) for cell lysis, followed by solvent extraction with acetone, ~101% carotenoid extractability was achieved compared to a control, involving acetone extraction without the enzymatic cell lysis step. Furthermore, the combination of glass beads treatment with Glucanex treatment increased the extractability to 122 %, indicating that this particular approach was better than the standard chemical extraction method. Although the enzymatic lysis approach seems to provide high yields of carotenoids and is a more selective approach than mechanical disruption of the yeast cells, the high cost of the lytic enzymes currently limits its large-scale application (Liu et al., 2016).

2.9.5 Supercritical Fluid Extraction (SFE)

SFE is an emerging technology that has been applied in the food industry as an alternative to the traditional solvent extraction of natural products from a variety of materials. A fluid is termed as 'supercritical' when the temperature and pressure of the fluid are above their critical values (Halim et al., 2011). At this point, the fluid has distinctive properties as compared to liquid and gas, including high compressibility, high diffusivity, low viscosity and low surface tension. Due to these properties, supercritical fluid has greater ability to diffuse into matrices than the conventional fluid, improving the extraction yield of the desired products from their sources (Lim et al., 2002). The pressure and temperature phase diagram illustrating the supercritical fluid region of carbon dioxide (CO_2) is presented in Figure 2-23.

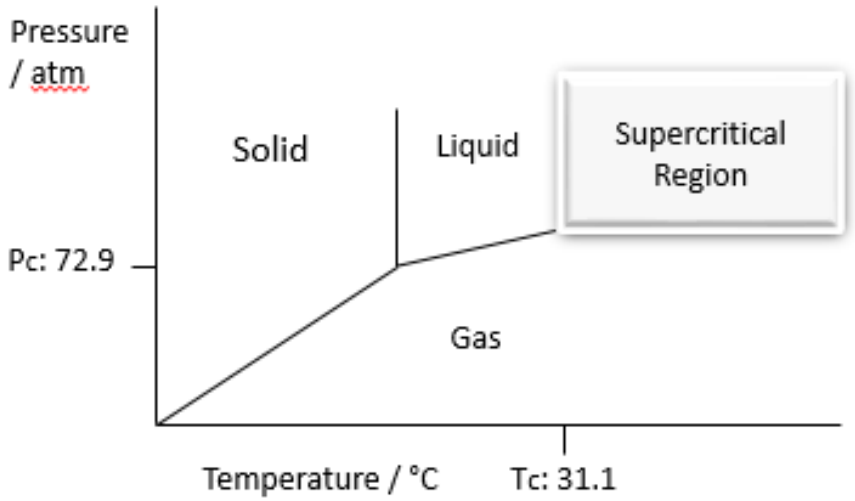

Figure 2-23: Pressure and temperature phase diagram for CO_2, showing the supercritical region (adapted from Halim et al, (2012)).

Several fluids have been used as supercritical fluids, including CO_2, ethane, ethene, methanol, *n*-butene and water (da Silva, Rocha-Santos & Duarte, 2016). However, SFE with CO_2 as the solvent offers considerable advantages over other fluids, as it is a non-toxic gas that is readily available, low-cost and considered as GRAS (Sharif et al., 2014). Besides, CO_2 has a low critical point, low flammability and low reactivity, making it suitable for the extraction of sensitive compounds, like carotenoids (Careri et al., 2001; Halim, Danquah & Webley, 2012). However, due to the non-polar nature of CO_2, this method is not suitable for the extraction of polar compounds, due to its poor solvating power of the fluid and insufficient interaction between SFE-CO_2 and the matrix (Careri et al., 2001). Therefore, the use of co-solvents as modifiers, such as methanol, ethanol or toluene, may help in extracting polar compounds, by enhancing the CO_2 affinity towards polar compounds (Oberholster, 2012). A major drawback of this method is that the cost of capital investment required is considerably high compared to other methods (e.g., solvent extraction), which hinders the scaling up of this process, despite the low price of CO_2 (Yen et al., 2015).

The extraction of carotenoids from yeast cells with high yields using SFE-CO_2, relies on the optimisation of various factors, including pressure, temperature, modifier addition, as well as the CO_2 flow rate. SFE-CO_2 has been shown to extract a wide range of natural compounds, such as essential oils, lipids and pigments, from various sources, including plants, yeast and organic waste (Halim et al., 2011; Mushtaq et al., 2015; Shilpi, Shivhare, and Basu, 2013; Sovová et al., 2010). Lim et al. (2002) used SFE-CO_2 to extract astaxanthin from *P. rhodozyma*, initially disrupted by bead milling and spray drying. Under optimised conditions (50 g CO_2, 40 °C and 500 bar), about 90% astaxanthin was extracted from the yeast cells. Moreover, ethanol (15% v/v) was found to enhance astaxanthin extraction by 24% at 60 °C and 500 bar (Lim et al., 2002).

Careri et al. (2001) implemented an experimental design procedure to investigate the extraction of carotenoids (β-carotene, zeaxanthin and β-cryptoxanthin) from *Spirulina platensis*. The experimental variables assessed were temperature, pressure, extraction time and ethanol as the modifier. The study revealed the individual carotenoids had different optimal conditions for extraction, suggesting that SFE-CO_2 has different selectivity for different target product. It was also reported that the conventional solvent extraction process yielded more β-carotene (120 mg/100 g) as compared to SFE-CO_2 (118 mg/100 g) (Careri et al., 2001). Although published works on astaxanthin extraction from *X. dendrorhous* yeast are scarce, SFE-CO_2 could be a promising method for such a purpose.

3 Investigation of Different Carbon Sources and Inducers on Microbial Astaxanthin Production by *Xanthophyllmyces dendrorhous* DSMZ 5626

Abstract

Microbial astaxanthin production by the yeast, *Xanthophyllomyces dendrorhous*, has tremendous potential for commercialisation and fulfilment of the demand for natural astaxanthin. In this study, the influence of different carbon sources (glucose, xylose, galactose, arabinose, cellobiose and fructose) and inducers (citric acid, hydrogen peroxide and ethanol) on *Xanthophyllomyces dendrorhous* DSMZ 5626 growth and astaxanthin production using semi-defined media in flask fermentations was investigated. The utilisation of glucose (10-70 g/l) as a carbon source was found capable of supporting yeast growth (6.8 – 12 g/l). However, the suppression of astaxanthin yield on biomass ($Y_{p/x}$) was clearly observed as the values were reduced from 255 µg/g when 30 g/l of glucose was used to 156 µg/g or lower when high glucose concentrations were applied. *X. dendrorhous* DSMZ 5626 was found to undergo Crabtree effects when high initial glucose concentration was present in the media as ethanol was produced during fermentation in these cases. However, it was observed that low concentrations of ethanol were consumed by the yeast as a carbon source when available sugars were nearly exhausted in the media. Glycerol was found to be able to support yeast growth and astaxanthin production in *X. dendrorhous*. The highest astaxanthin yield on biomass was observed when 10 g/l of glycerol was used (402 µg/g). Other refined sugars including fructose, xylose, cellobiose, galactose and arabinose (30 g/l) highly supported yeast growth with values ranging from 10.7 g/l to 13.3 g/l. High astaxanthin production (*P*) was observed in media containing cellobiose (4.24 mg/l), xylose (3.76 mg/l) and fructose (3.5 mg/l) with no significant different in yield ($Y_{p/x}$) values (294 - 319 µg/g). The addition of chemicals (ethanol, citrate and hydrogen peroxide) induced astaxanthin pigmentation in *X. dendrorhous* at optimised doses and feeding times. Overall, this study provided preliminary data to understand the biochemical behaviour

of the yeast, *Xanthophyllomyces dendrorhous* in semi-defined media, targeting astaxanthin as the primary metabolic product.

Keywords: *Xanthophyllomyces dendrorhous*, carbon source, inducers, astaxanthin, flask fermentation

3.1 Introduction

Astaxanthin (3,3'-dihydroxy-β, β-carotene – 4,4'-dione) is a keto carotenoid pigment that falls into the xanthophylls group and is one of the most important carotenoids in humans and animals along with β-carotene, lutein, zeaxanthin and torulene. It has been widely applied as a feed additive in aquaculture as it contributes to the attractive colouration in salmon, shrimps and crustaceans as well as to maintain their growth and survival. However, aquatic animals lack the biochemical pathway that enables the synthesis of carotenoids. Therefore, they need to obtain astaxanthin through what they consume, by means of natural consumption of algae or through the inclusion of carotenoids in the feed formulation.

Currently, the commercial demand for astaxanthin is fulfilled by its synthetic production (petroleum derived) via the Wittig reaction and Grignard condensation strategies (EFSA FEEDAP Panel, 2014). However, natural astaxanthin has been reported to have higher stability due to its esterified nature that prevents oxidation and contributes to the prolonged shelf life of astaxanthin (Bhatt, Ahmad, & Panda, 2012). Natural sources of astaxanthin constitute marine microalgae, such as *Haematococcus pluvialis* and yeasts, such as *Xanthophyllomyces dendrorhous*. These natural astaxanthin producers face a competitive market price compared to chemically derived astaxanthin due to the complexity of the production process that includes long cultivation time which directly relates to the high cost and low productivity of astaxanthin, particularly in wild strains (Gu et al., 1997).

Xanthophyllomyces dendrorhous, one of the best candidates available, could potentially fulfil the demand for natural astaxanthin production. Astaxanthin is the major pigment produced intracellularly in *X. dendrorhous*, accounting for more than 90% of the total carotenoids. In terms of yeast cultivation, it does not require light for growth and pigmentation. In addition, it is able to utilise a wide range of carbon sources including monosaccharides, disaccharides and

alcohols (Schmidt et al., 2011). Besides that, the shorter growth cycle (5 days), as compared to microalgae (2 weeks), provides additional advantages for the yeast (Yamane et al., 1997). Astaxanthin biosynthesis in *X. dendrorhous* is carried out via the mevalonate pathway as a secondary metabolite and its production is affected by other primary and secondary metabolites including ethanol, proteins and fatty acids, which may significantly impact cell growth as well as astaxanthin production in the yeast. Several yeast species, including *X. dendrorhous*, have been reported to undergo alcoholic fermentation; a phenomenon wherein yeast produces ethanol even in aerobic conditions (Crabtree effect) (Yamane et al., 1997). This phenomenon could affect both biomass and astaxanthin production and can occur when the concentration of hexoses during fermentation is above a threshold value, which is usually strain-dependent.

There are several obstacles that hinder the commercialisation of microbial astaxanthin by *X. dendrorhous* such as high production costs and low astaxanthin productivity in wild strains (Gu et al., 1997). Several attempts have been made to overcome these limitations, including the addition of inducers to stimulate pigmentation such as fungal elicitors (Wang et al., 2006), n-hexadecane (Liu & Wu, 2006), α-ketoglutarate, mevalonic acid (Calo et al., 1995) and acetic acid. Additionally, attempts have been made to reduce the production costs of astaxanthin via media optimisation and cultivation in low-cost substrates such as agricultural wastes and by-products as a way to avoid the utilisation of refined sugars (Cruz & Parajo, 1998).

The overall aim of this thesis was to develop a culture medium derived from lignocellulosic material, focusing on rapeseed meal as the starting material with a view to render the astaxanthin production economically more favourable. However, the produced rapeseed meal hydrolysates are composed of several sugars (glucose, galactose, xylose and arabinose). Hence, a detailed investigation on astaxanthin production by *Xanthophyllomyces dendrorhous* DSMZ 5626 using semi-defined media in shake flasks through the evaluation of various sugars

types and concentrations is key to understand the biochemical behaviour of the strain. In the end, the pre-treatment process of lignocellulosic material (rapeseed meal) can be tailored accordingly to optimise astaxanthin production in *X. dendrorhous* according to the results generated in this study.

3.2 Materials and Methods

3.2.1 Microorganism and Culture Conditions

The yeast strain *Xanthophyllomyces dendrorhous* DSMZ 5626 that was used in this study was purchased from the Leibniz Institute DSMZ. The strain was proliferated and maintained in a Yeast and Mold (YM) media containing (in g/l): yeast extract, (3.0); malt extract, (3.0); peptone from soybean, (5.0); glucose, (10.0) and agar (15). The stock of yeast cultures was stored at -80 °C until further use.

For the preparation of the inoculum, a loopful of yeast stock cultures were inoculated on the commercial YM agar (Sigma Aldrich, UK) and incubated at 20 °C for 5 days. After that, a single colony of yeast *X. dendrorhous* was transferred into 30 ml of YM medium containing a similar composition to the one mentioned above for cell proliferation and was incubated for 3 days prior to inoculation in semi-defined media to a final optical density (OD) of 0.1, measured using a Biomate 3 UV/VIS Spectrophotomer (Thermo Spectronic, NY). These yeast cultures acted as inoculum for all experiments that were conducted in this chapter.

3.2.2 Fermentation With Semi-Defined Media

The cultivation of *X. dendrorhous* was performed in 250ml shake flasks filled with 50 ml of semi-defined media. The composition of semi-defined media was as follows (in g/l): yeast

extract (2.0), malt extract (2.0), KH_2PO_4 (7.0), $(NH_4)_2SO_4$ (1.0), $MgSO_4.7H_2O$ (1.5), $FeCl_3.6H_20$ (0.15), $ZnSO_4.7H_2O$ (0.02), $MnSO_4.H2O$ (0.06), $CaCl_2.2H_2O$ (0.15). The types and concentration of carbon sources used in this study are as follows; glucose (10, 20, 30, 40, 60 70 g/l), xylose (30 g/l), galactose (30 g/l), arabinose (30 g/l) and glycerol (10, 30, 60, 75 g/l).

Shake flasks fermentation were performed on an orbital shaker (GFL 3015, SciQuip, UK) controlled at 250 rpm and temperature at 20 °C for 5 days. In the case of yeast fermentation, samples of 2 mL were withdrawn at several times in intervals of up to 5 days of the fermentation period.

3.2.3 Addition of Inducers

Three chemicals with two different concentrations were tested for their ability to induce microbial pigmentation in *X. dendrorhous* in shake flasks. Ethanol (0.5 and 1.5 g/l), citric acid (10 and 30 mM) and hydrogen peroxide (10 and 20 mM) were added to the shake flask cultures at two different time points (0 and 24 h), respectively, corresponding to the initial growth phase and early exponential growth phase. The fermentation conditions were similar to those described in section 3.2.2.

3.2.4 Analytical Methods

2 ml of the samples were withdrawn from the shake flasks at regular intervals. From there, an aliquot of 1 ml of sample was added into a pre-dried tube (24 h in a drying oven at 100°C) and centrifuged at 10845 x *g* for 10 min. The supernatant was collected for sugar composition and ethanol analysis. The pellet was washed twice using distilled water. The tubes were then kept frozen at -20°C prior to the freeze-drying process for 2 days (Virtis, UK). When dried, the tubes were then reweighed for its dried weight. The dry weight of yeast biomass was

calculated as the difference between the weight of the tubes before and after use. The freeze-drying method that was used in this study aimed to preserve the astaxanthin against degradation under high temperatures with normal drying techniques.

Sugars (glucose, xylose, galactose and arabinose), glycerol and ethanol were analysed by high-performance liquid chromatography (HPLC) using an Agilent Infinity 1260 system (Agilent Technologies, USA) with Aminex HPX-87H column (Bio-rad, CA) coupled to a differential refractometer and a DAD detector. Operating conditions were as follows: Sample volume: 20µl; Mobile phase: 0.5 mM H_2SO_4; Flow rate: 0.6 ml/min; Column temperature: 65°C. The quantification of each chromatogram was achieved on the basis of the standard curve, which was conducted using standard solutions.

Astaxanthin extraction was carried out using the following method for total carotenoids extraction by Sedmak et al (1990). 1 mL of dimethyl sulfoxide (DMSO) was briefly preheated at 55 °C and added to the freeze-dried biomass. This was followed by vortexing for 30-40 sec. Subsequently, 0.2 mL of 20 % sodium chloride (NaCl) and 1.0 mL of acetone were added to the mixture to extract the intracellular carotenoids. The aqueous and organic phases were separated by centrifugation at 5423 *x* g for 5 min. The extraction process was repeated until a colourless biomass was obtained. The organic phases were pooled together, and their absorbance was measured at 480 nm in a Biomate 3 UV/VIS Spectrophotomer (Thermo Spectronic, NY). The equation for total carotenoids concentration is given in the equation below:

$$Carotenoids\ content\ (\mu g/g) = \frac{A\ \times V\ (mL) \times\ 10^4}{A_{1cm}^{1\%}\ \times P\ (g)} \qquad \text{Eq 3-1}$$

Where A is the absorbance at 480 nm, V is the volume, $A_{1cm}^{1\%}$ is the coefficient (2150) and *P* is the weight of biomass.

3.3 Results and Discussion

3.3.1 Glucose-Based Semi-Synthetic Media

Perious studies demonstrated that several yeast species including *X. dendrorhous* are prone to alcoholic fermentation even in aerobic conditions in the presence of high initial glucose ceoncentrations in the media; this is often called the Crabtree effects (Reynders, Rawlings, & Harrision, 1997; Xiao et al., 2015; Yamane et al., 1997). Therefore, this preliminary investigation was performed to determine the threshold of glucose concentration upon which alcoholic fermentation (Crabtree effect) could be initiated by the particular yeast strain. The proliferation of *X. dendrorhous* in batch cultures using semi synthetic media was carried out in order to investigate the effect of initial glucose concentrations on yeast growth and astaxanthin production.

Figure 3-1 and Figure 3-2 depict the growth profile of *X. dendrorhous* cultivated at different glucose concentration ranging from 10 to 70 g/l of glucose, in semi-defined media. Table 3-1 summarizes the main fermentation data for the above experiments. Results showed that an increase in initial glucose concentration from 10 to 40 g/l of glucose, enhanced cell biomass production from 7 g/l to 12.6 g/l, respectively. Furthermore, an increase in the initial glucose concentration resulted in slight cell growth suppression, indicating that it may impose suppression effects on yeast growth. This finding was also supported by Yamane et al. (1997) who reported that in batch fermentations with glucose concentration from 10 – 30 g/l, *X. dendrorhous* exhibited increasing cell biomass production under aerobic conditions. However, when the glucose concentration was increased to 70 g/l, biomass concentration was suppressed.

There is a possibility that this occurs due to the Crabtree effect that was initiated when high initial glucose concentrations were present. Under these metabolic conditions *X. dendrorhous* tends to change its metabolism towards alcoholic fermentation and produce ethanol even under adequate oxygen supply. Several by-products apart from ethanol are also produced including acetic acid and carbon dioxide. It was observed that about 1.5 g/l of ethanol were present at the 24^{th} hour of fermentation which was later consumed once glucose was depleted from the media. The Crabtree effect was clearly observed in media containing > 40 g/l of initial glucose concentration. About 8 g/l of ethanol were detected and later consumed by yeast. However, not all of the ethanol was consumed leaving an excess of ethanol at the end of fermentation. Likewise, 13 g/l of ethanol were detected at 120 h of fermentation when 70 g/l of glucose was used. Significant implications of the Crabtree effect were observed in media containing 60 and 70 g/l of glucose, where biomass yield on glucose ($Y_{x/s}$) was significantly reduced from 0.72 g/g in 10 g/l of glucose to < 0.19 g/g when 60 g/l of glucose were used. However, in media with less than 30 g/l of glucose, all the produced ethanol was consumed by the cells contributing towards both biomass and astaxanthin production in *X. dendrorhous*. According to Liu & Wu (2008), ethanol was found to increase yeast growth but resulted in adverse effects on astaxanthin biosynthesis in *X. dendrorhous* ENM5.

Based on these results, it is evident that for this particular strain, the threshold of the initial glucose concentration to prevent the Crabtree effect is lower than 10 g/l. This is in agreement with Yamane et al., (1997) who reported that the Crabtree effect can occur in *X. dendrorhous* strains when cultivated in synthetic media containing 10 g/l of glucose.

As depicted in Table 3-1, astaxanthin production (*P*) and astaxanthin yield on biomass ($Y_{p/x}$) were obviously influenced by the initial glucose concentration. Both the *P* and $Y_{p/x}$ values decreased when cells were cultivated in media containing > 40 g/l of glucose. This further

suggested that the Crabtree effect did not only affect biomass production but astaxanthin accumulation as well. Similarly, biomass yield on glucose consumed ($Y_{p/s}$) showed a reduction when glucose concentrations were increased. As for $Y_{p/x}$, the highest values were observed to be at 30 g/l of glucose with 255 µg/g. However, at 70 g/l of glucose, 12 g/l of biomass were produced but the intracellular astaxanthin production was low ($Y_{p/x}$ = 105 µg/g).

In the growth experiments with different glucose concentrations, even though *X. dendrorhous* produced ethanol when cultivated in glucose-based media, it is interesting to note that astaxanthin continued to accumulate with time. It was observed that astaxanthin production in *X. dendrorhous* is a partially growth associated product, with its production being initiated at the exponential growth phase and continuing to accumulate during stationary phase. One possible explanation is that once glucose was depleted from the media (after 40 h), the yeast started to consume ethanol as a carbon source, indicating the presence of active alcohol dehydrogenase, which is responsible for ethanol conversion into acetate. In the presence of energy (ATP), acetate can be converted into acetyl CoA, which is the major precursor for both the tricarboxylic acid (TCA) cycle and the mevalonate pathway, which is responsible for carotenoids production (Thomson et al., 2005). However, a high concentration of ethanol has reverse effects on biomass production as it can suppress yeast growth as well as subsequent astaxanthin production.

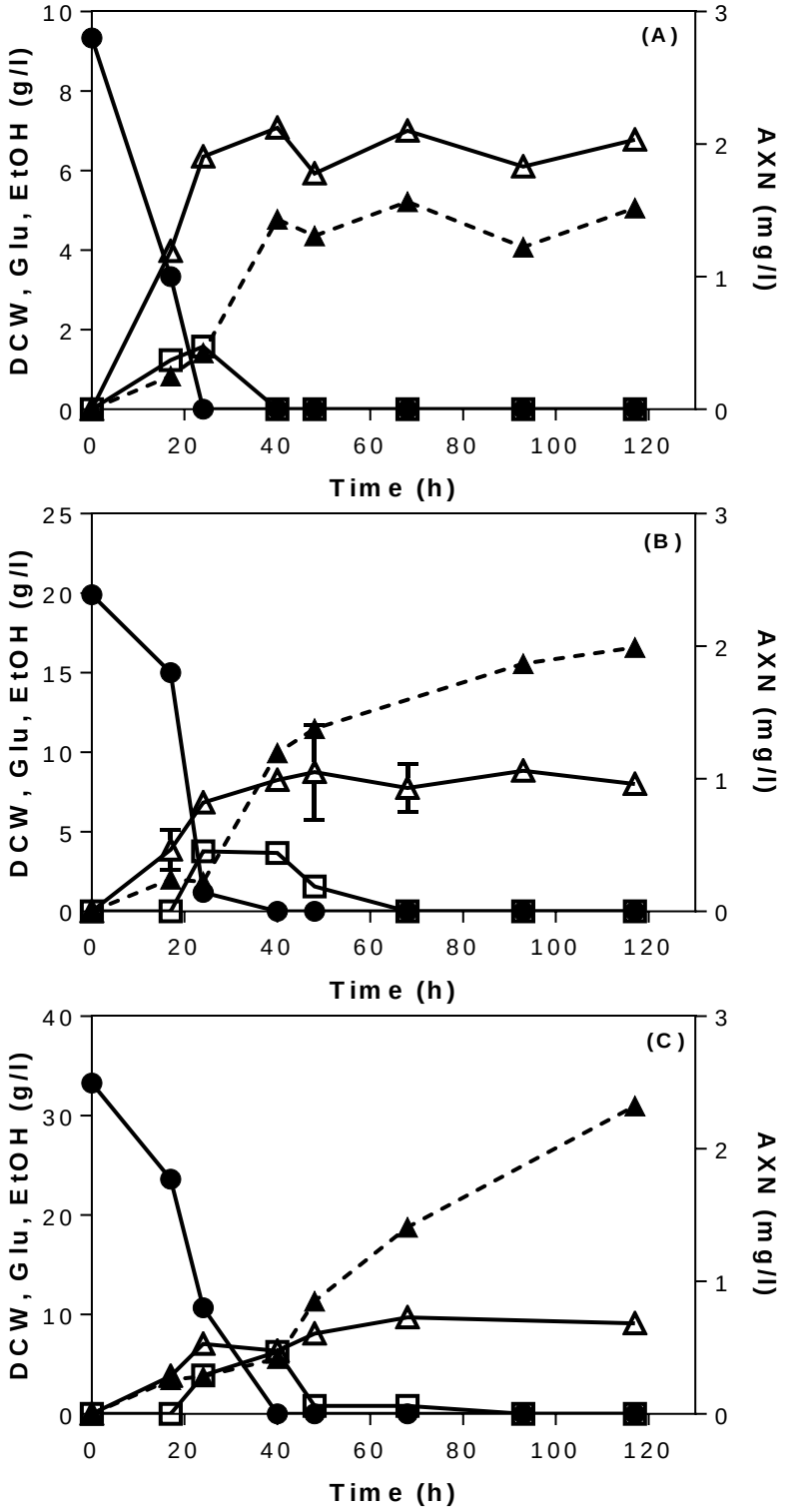

Figure 3-1: *X. dendrorhous* growth profile, sugar consumption, ethanol and astaxanthin production in during growth in shake flasks in semi-defined media containing different concentrations of glucose. (**A)**: 10 g/l, (**B)**: 20 g/l, **(C)**: 30 g/l . Fermentation condition as follows; temperature (20°C), agitation (200rpm), initial pH (pH 6). Symbols represents ●- glucose (g/l), □- ethanol (g/l), Δ – dry cell weight (g/l), ▲- astaxanthin (µg/ml).

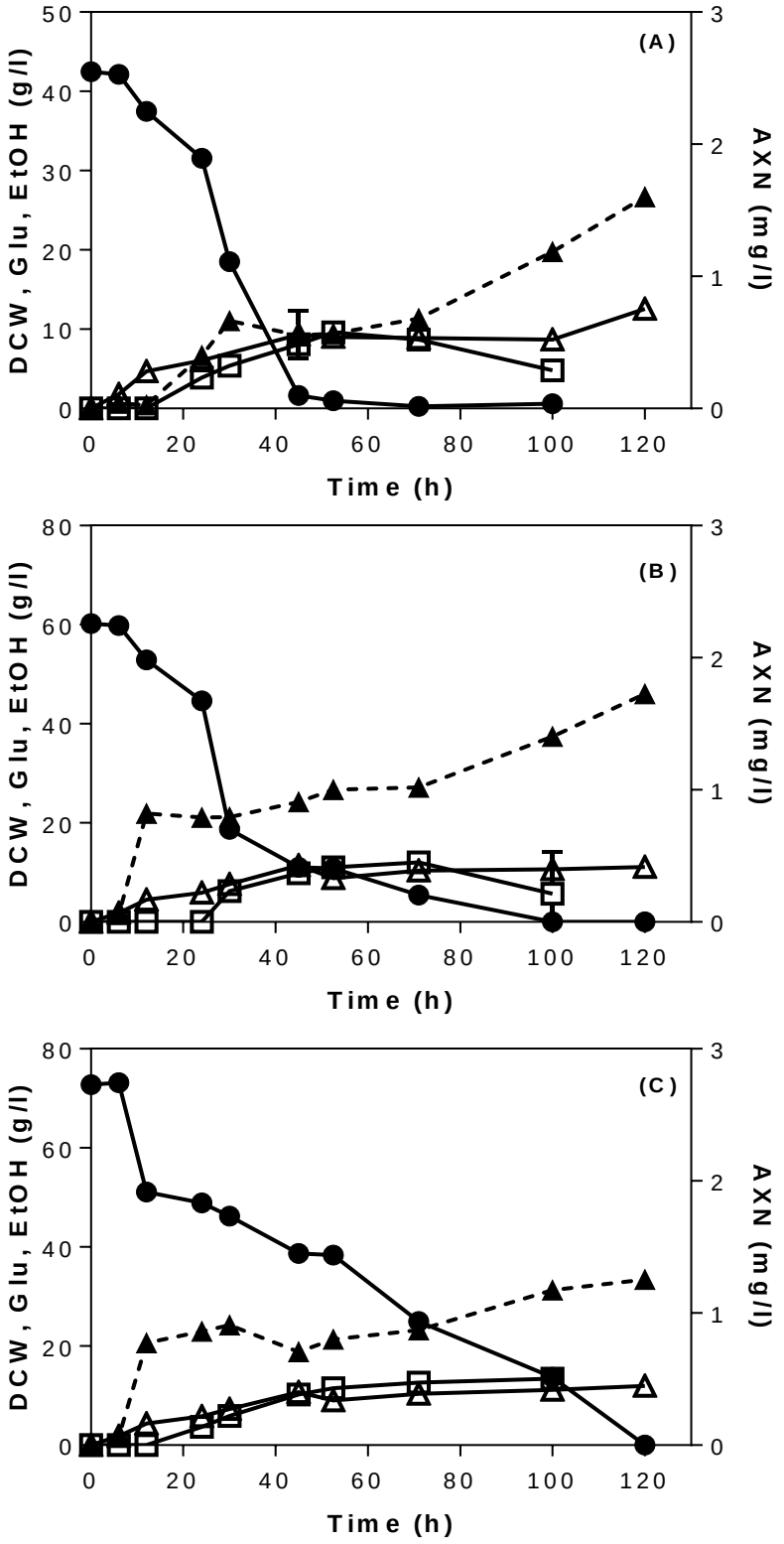

Figure 3-2: *X. dendrorhous* growth profile, sugar consumption, ethanol and astaxanthin production in during growth in shake flasks in semi-defined media containing different concentrations of glucose. (**A)**: 40 g/l, (**B)**: 60 g/l, **(C)**: 70 g/l . Fermentation condition as follows; temperature (20°C), agitation (200rpm), initial pH (pH 6). Symbols represents ●- glucose (g/l), □- ethanol (g/l), Δ – dry cell weight (g/l), ▲- astaxanthin (µg/ml).

Table 3-1: Experimental results of flask fermentation with *X. dendrorhous* cultivated in semi-defined media using different concentrations of glucose controlled at 20 °C under shaking (250 rpm).

Glucose (g/l)	**Time (h)**	**Cell biomass (g/l)**	***P* (mg/l)**	**$Y_{p/x}$ (µg/g)**	**$Y_{p/s}$ (µg/g)**	**$Y_{x/s}$ (g/g)**
10	117	6.78 (1.2)	1.52 (0.2)	247 (39)	0.15 (0.02)	0.72 (0.00)
20	117	8.00 (0.36)	1.99 (0.02)	249 (14)	0.09 (0.01)	0.40 (0.00)
30	117	9.13 (0.67)	2.32 (0.03)	255 (15)	0.08 (0.01)	0.28 (0.01)
40	120	12.55 (0.2)	2.30 (0.01)	156 (4)	0.06 (0.00)	0.30 (0.01)
60	120	11.1 (0.8)	1.73 (0.75)	153 (55)	0.02 (0.01)	0.19 (0.02)
70	120	11.95 (0.5)	1.25 (0.12)	105 (6)	0.03 (0.01)	0.16 (0.02)

* Values in bracket represent standard deviation.
P (mg/l) = product concentration
Yp/x (µg/g) = product conversion yield on biomass concentration
Yp/s (ug/g) = product conversion yield on substrate concentration
Yx/s (g/g) = biomass production on substrate consumption

Table 3-2 summarises the main fermentation parameters for the above experiments. Results showed that *X. dendrorhous* was able to grow in media containing glycerol and was able to inhibit the occurrence of the Crabtree effect. Results show that increasing glycerol concentration from 10 g/l to 75 g/l resulted in an increase in the amounts of biomass from 4 g/l to 17 g/l, respectively. When compared with glucose as a carbon source, higher biomass production was observed in glucose-based media (6 g/l) as compared to glycerol at similar concentrations (10 g/l).

At the same time, the astaxanthin production (*P*) followed a similar trend with that of biomass production. However, as for astaxanthin yield per biomass ($Y_{p/x}$), a negative correlation was observed as glycerol concentration increased. Results also show that astaxanthin yield per substrate ($Y_{p/s}$) was reduced as higher glycerol concentrations were applied. Kusdiyantini et al. (1998) reported that increasing the glycerol concentration from 9.4 to 38 g/l resulted in increased cellular astaxanthin yield ($Y_{p/x}$) from 1.4 mg/l to 1.8 mg/l. Furthermore, they reported that astaxanthin production (*P)* increased with increasing the initial glycerol concentrations, a fact which suggested that it was directly correlated with a constant increase in biomass production.

According to Klein et al. (2017), there are two possible pathways for glycerol catabolism in yeasts. One of them is phosporylative glycerol catabolic pathway, also known as the catabolic G3P pathway. Via this pathway, two main enzymes are involved; a glycerol kinase and FAD-dependent glycerol 3-phosphate dehydrogenase. This catabolic pathway is more likely to occur in yeast. Another possible pathway is the catabolic DHA pathway which

involves the oxidation of glycerol to dihydroxyacetone (DHA) via NAD^+-dependent glycerol dehydrogenase.

Silva et al. (2012) investigated the application of glycerol as an additional carbon source for astaxanthin production in *X. dendrorhous* NRRL Y-17268 in flask fermentation. They reported that a maximum of 8.9 g/l of biomass with 20 mg/l of astaxanthin when 40 g/l of pure glycerol was used as a carbon source. When approached more broadly, the utilisation of glycerol as a carbon source for yeast growth has been reported for metabolic products such as lipids and carotenoids by *Rhodotorula glutinis* (Liang et al. (2010), as well as in the case of strains of *Yarrowia lipolytica* (Papanikolaou & Aggelis, 2002) and *Cryptococcus curvatus* (Saenge, Cheirsilp, Suksaroge, & Bourtoom, 2011). The ability of *X. dendrorhous* to assimilate glycerol as the main carbon source shows a high potential for further studies.

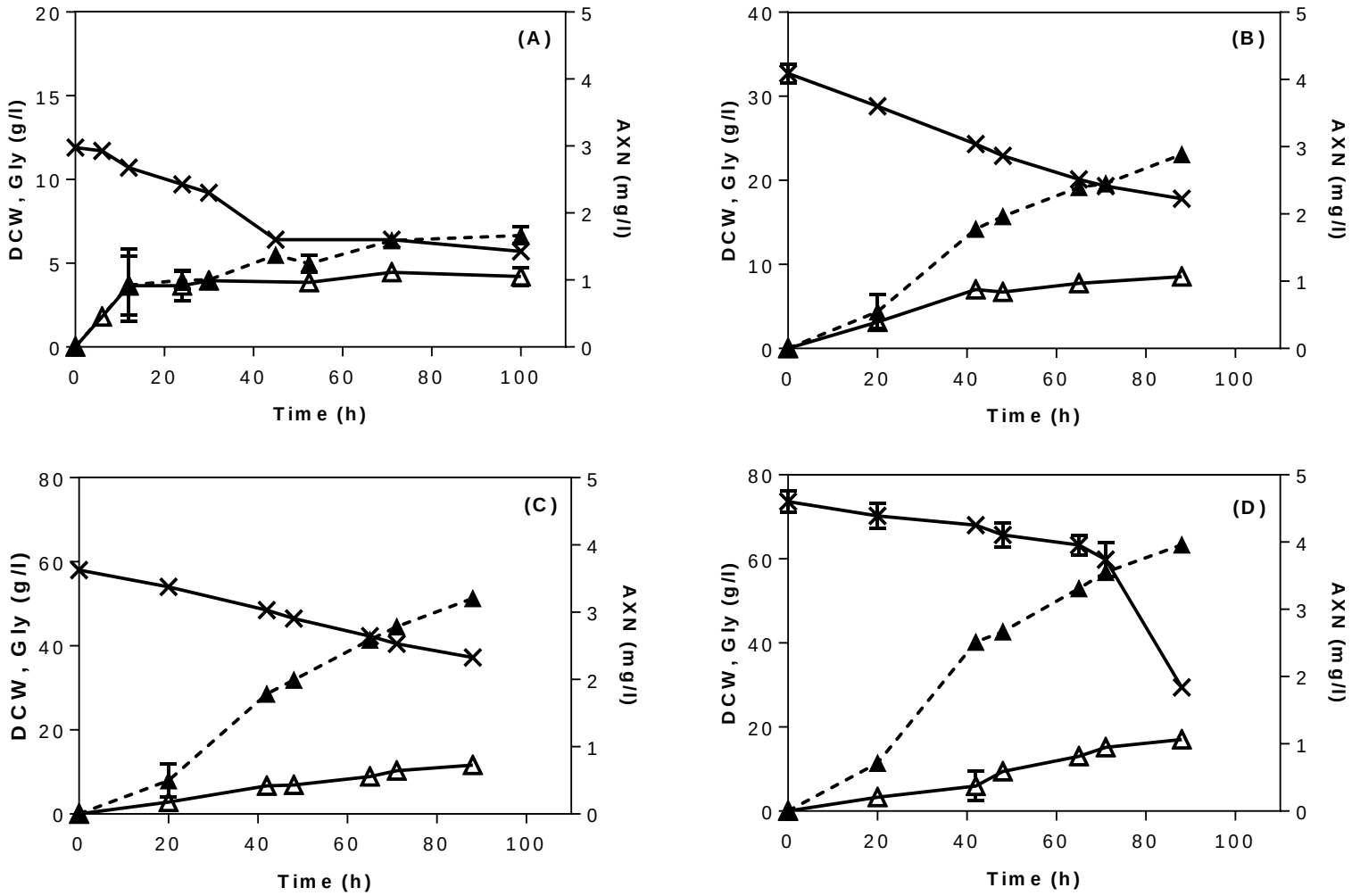

Figure 3-3: *X. dendrorhous* growth profile, glycerol consumption and astaxanthin production in flask fermentation in synthetic media consist of different concentrations of glycerol. A: 10 g/l, B: 30 g/l, C: 60 g/l, D: 70 g/l. Symbols represents: ×- glycerol (g/l); Δ- dry cell weight (g/l); ▲- astaxanthin (mg/l).

Table 3-2: Experimental results of flask fermentation with *X. dendrorhous* cultivated in synthetic media using different concentrations of glycerol controlled at 20 °C under shaking (250 rpm).

Glycerol (g/l)	Time (h)	X (g/l)	P (mg/l)	$Y_{p/x}$ (µg/g)	$Y_{p/s}$ (µg/g)	$Y_{x/s}$ (g/g)
10	100	4.2 (0.56)	1.67 (0.13)	402 (8.5)	0.29 (0.02)	0.69 (0.01)
30	88	8.55 (0.21)	2.88 (0.01)	337 (5.0)	0.16 (0.01)	0.57 (0.02)
60	88	11.6 (0.14)	3.21 (0.02)	276 (1.5)	0.09 (0.01)	0.56 (0.02)
75	88	17.1 (0.21)	3.96 (0.01)	232 (3.5)	0.07 (0.01)	0.97 (0.06)

* Values in bracket represent standard deviation.
P (mg/l) = product concentration
Yp/x (µg/g) = product conversion yield on biomass concentration
Yp/s (ug/g) = product conversion yield on substrate concentration
Yx/s (g/g) = biomass production on substrate consumption

3.3.2 Semi-Synthetic Media with Alternative Sugars

The aim of this particular experiment was to investigate the capability of *X. dendrorhous* DSMZ 5292 growing in different types of other carbon sources including galactose, cellobiose, xylose and arabinose (30 g/l) (Figure 3-4 and Figure 3-5). This would also demonstrate whether other sugars besides glucose would trigger a Crabtree effect. The hypothesis was that in the case of pentoses there should be no Crabtree effect, due to their assimilation via a different pathway (the pentose phosphate pathway).

X. dendrorhous was able to consume the different types of sugar tested in this study. Despite that, the growth rate and astaxanthin production were rather different. The yeast was able to consume cellobiose, xylose and fructose, with the concomitant accumulation of astaxanthin. The results showed that almost 11 g/l of cell biomass was produced when *X. dendrorhous* was cultivated in 30 g/l of fructose and 30 g/l of cellobiose as the carbon source. In media containing 30 g/l of xylose, 8.3 g/l of cell biomass was produced.

By comparing the performance of the yeast when various carbon sources were used, it can be deduced that astaxanthin accumulation by *X. dendrorhous* in fructose-, xylose- and cellobiose-based media was higher than in glucose-based medium. The highest astaxanthin yield (4.26 ug/ml) with 11 g/l of biomass was observed when 30 g/l of cellobiose was used. It was observed that cellobiose was hydrolysed into its monomers (glucose) during fermentation (Figure 3-4C) by the action of β-glucosidase. As a result, the consumption of cellobiose was carried out at a much slower rate than in glucose or fructose media, as 52 h were needed for the cellobiose to be exhausted. The slow consumption rate and the fact that the glucose levels in the fermentation medium were very low, most likely prevented the occurrence of the Crabtree effect, up that time point. However, after 52 h of fermentation, ethanol was detected in the

media (0.9 g/l) containing cellobiose as a carbon source. At this time point, the presence of glucose was detected in the media. In media containing 30 g/l of fructose, a maximum of 1.4 g/l of ethanol was produced after 27 h. Once fructose was exhausted in the medium (30 h), the cells started to consume the ethanol leading to astaxanthin production. Fructose, being a hexose, undergoes the same catabolic pathway as glucose.

As for pentose sugars (xylose), high biomass was produced with 13.6 g/l of dry biomass with 3.8 mg/l of astaxanthin produced after 92h of fermentation. In this fermentation medium, ethanol was not detected during fermentation, indicating that the Crabtree effect was suppressed. The metabolic pathways of hexoses and pentoses catabolism in yeasts are completely different. Pentoses are catabolised via the pentose phosphate pathway in yeasts; in the case of xylose, this is converted into D-xylulose-5-phosphate by a xylose transporter and subsequently enters the pentose phosphate pathway (Jeffries, 2006). Parajo et al. (1998) reported that *X. dendrorhous* NRRL-Y-17268 cultivated with xylose as a carbon source was able to support astaxanthin production, with maximal values of 5.2 mg/l. This finding contrasts what Wu & Yu (2013) reported. They studied the microbial astaxanthin production by *X. dendrorhous* strain AS 2.1557 cultivated in different types of carbon sources (glucose, sucrose and xylose) and found out that xylose is the least suitable carbon source for both cell biomass (4.4 g/l) and astaxanthin production (1.6 mg/l).

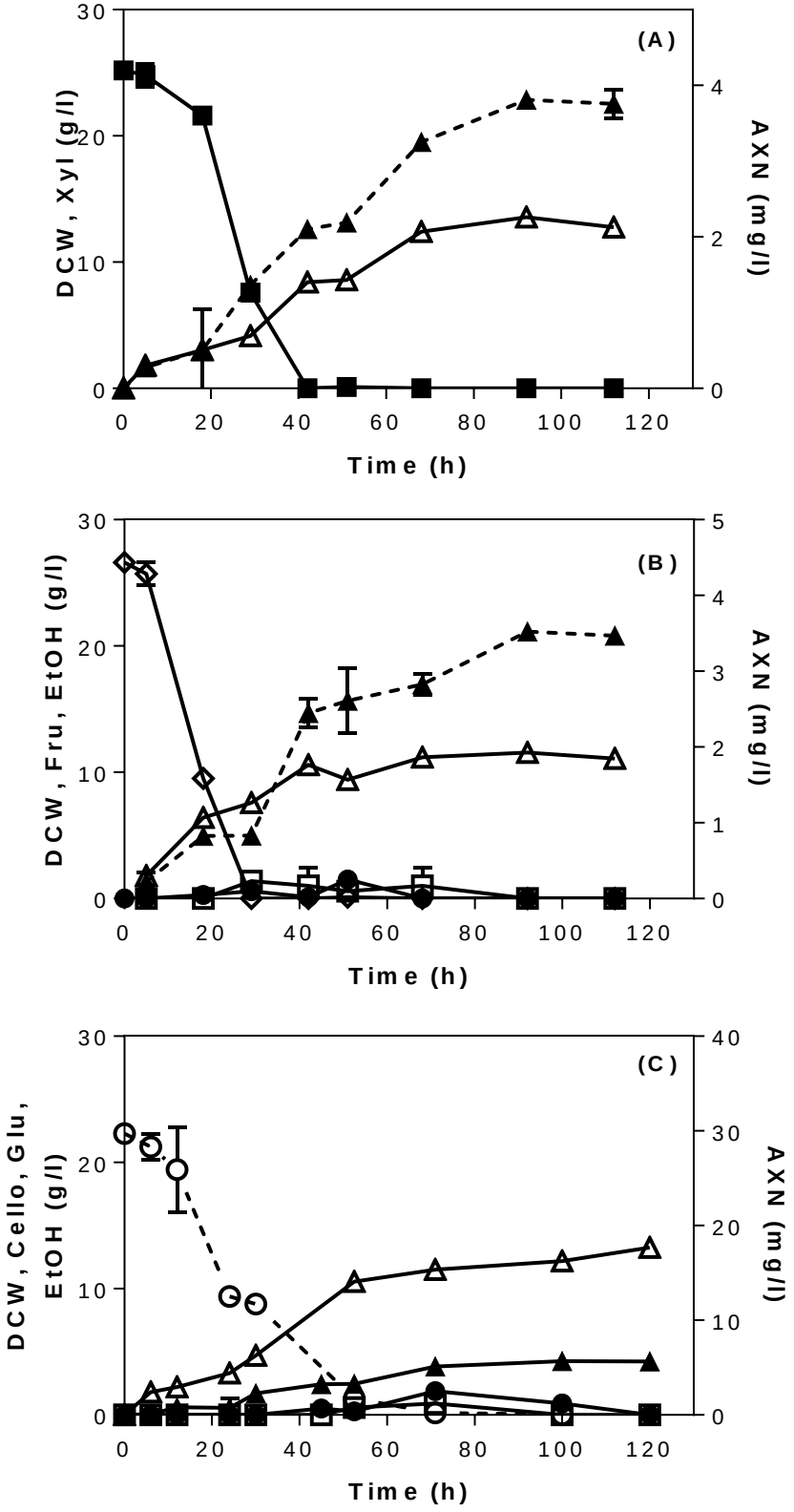

Figure 3-4: *X. dendrorhous* growth profile, glycerol consumption and astaxanthin production in flask fermentation in synthetic media consist of different types of sugars. A: xylose (xyl), B: fructose (fru), C: cellobiose (cello). Symbols represent: Δ- dry cell weight, ▲- astaxanthin, ■- xylose, ◊ - fructose, ◑ - cellobiose, □- ethanol

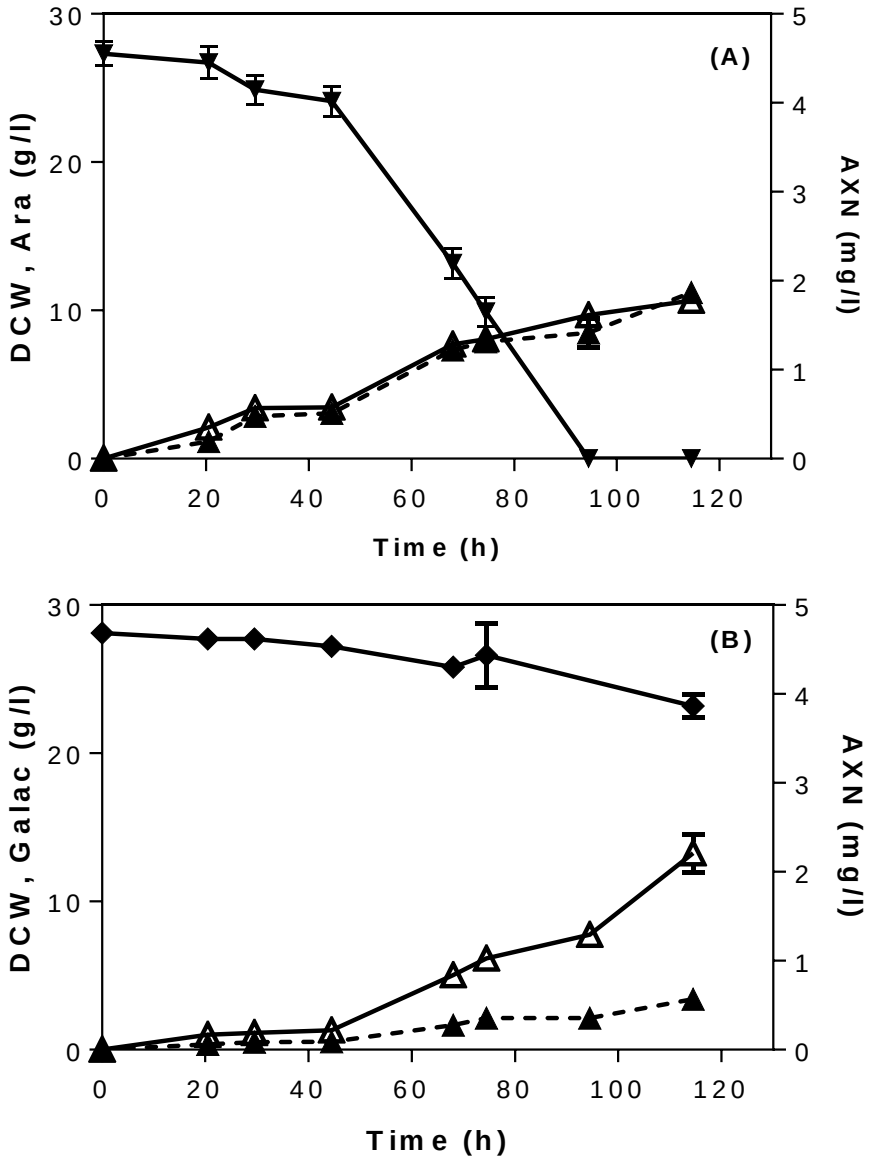

Figure 3-5: *X. dendrorhous* growth profile, glycerol consumption and astaxanthin production in flask fermentation in synthetic media consist of different types of sugars. A: arabinose (ara), B: galactose (galac). Symbols represent: Δ- dry cell weight, ▲- astaxanthin, ▼- arabinose, ◆- galactose.

With regards to arabinose and galactose, the adaptation phase was long. Only 3.2 g/l of arabinose were consumed after 45 h of fermentation. However, rapid consumption of arabinose occurred afterwards, yielding 10.7 g/l of biomass and complete consumption of arabinose at the end of fermentation. Galactose was the least favoured sugar by *X. dendrorhous* as only 1.5 g/l of galactose were consumed during 75 h of adaptation phase. However, at the end of fermentation (115 h), 13 g/l of biomass was produced with 23 g/l of galactose remaining in the fermentation media.

In terms of astaxanthin production (P_{max}), the highest astaxanthin was produced in media containing cellobiose (4.26 mg/l) and the least in media with galactose (0.36 mg/l). It is interesting to note that even when high biomass production was obtained (13.3 g/l) after 115 h in galactose-containing media, the astaxanthin concentration was very low (0.5 mg/l). The highest astaxanthin yield per biomass ($Y_{p/x}$) was obtained in media containing cellobiose (349 µg/g of biomass). Carotenoid pigmentation in *X. dendrorhous* follows the same trend regardless of the sugars used. Astaxanthin started to accumulate during the late exponential phase and continued in the stationary phase. This is in agreement with previous studies that reported astaxanthin production in *X. dendrorhous* is a partially growth dependent product.

Table 3-3: Experimental results of flask fermentation with *X. dendrorhous* cultivated in synthetic media using different types of carbon sources controlled at 20 °C under agitation (250 rpm).

Carbon source (30 g/l)	**Time (h)**	**X (g/l)**	***P* (mg/l)**	**$Y_{p/x}$ (µg/g)**	**$Y_{p/s}$ (µg/g)**
Cellobiose	120	13.3 (0.21)	4.24 (0.22)	319.70 (11.8)	0.14 (0.01)
Xylose	112	12.8 (0.2)	3.76 (0.19)	294.67 (10.1)	0.15 (0.01)
Fructose	112	11.1 (0.00)	3.47 (0.02)	312.38 (2.07)	0.13 (0.01)
Arabinose	115	10.7 (1.2)	1.87 (0.02)	174.75 (1.23	0.07 (0.01)
Galactose	115	13.3 (1.27)	0.57 (0.04)	46.51 (3.23)	0.12 (0.01)

* Values in bracket represent standard deviation.
P (mg/l) = product concentration
Yp/x (µg/g) = product conversion yield on biomass concentration
Yp/s (ug/g) = product conversion yield on substrate concentration

X. dendrorhous has been reported to be capable of assimilating different types of carbon sources including monosaccharides, disaccharides and alcohols (Schmidt et al., 2011). This study agrees that *X. dendrorhous* DSMZ 5626 was able to grow and produce astaxanthin on a wide range of simple sugars. Moreover, cellobiose, fructose and xylose were found to be suitable for astaxanthin production in *X. dendrorhous*. Considering the aim of this project, which is to use rapeseed meal hydrolysate as a fermentation feedstock, this study gave an overview of the performance of the yeast in complex media. Based on the obtained results, it can be estimated that *X. dendrorhous* should be able to consume cellobiose, glucose and xylose from the available sugars in rapeseed meal. The results also indicated that targeting the hydrolysis of rapeseed meal towards cellobiose rather than glucose could be an advantageous strategy to employ.

3.4 Addition of Inducers

3.4.1 Ethanol

In order to investigate the effect of ethanol addition on astaxanthin production, two different concentrations of ethanol (0.5 g/l and 1.5 g/l) were added to the fermentation media at two different time points: at the beginning of the fermentation, and once the sugars were completely exhausted (48h). Figure 3-6 shows the effect of ethanol addition into the fermentation media on the biomass and astaxanthin production in *X. dendrorhous*.

Increasing the concentration of ethanol from 0.5 g/l to 1.5 g/l negatively affected the biomass production regardless of the feeding point. It was observed that a significant reduction of biomass production occurred when 1.5 g/l of ethanol were added to the fermentation media as compared to control. About 4.2 g/l of biomass was produced when 1.5 g/l of ethanol was added at the beginning of the fermentation. However, when lower ethanol concentrations (0.5

g/l) were added into the media, it was observed that biomass production increased slightly as compared to control. This shows that high ethanol concentrations during the early stage of the fermentation suppressed biomass production but at lower concentrations, it assisted the biomass production.

As for astaxanthin yield per biomass ($Y_{p/x}$), significant high amounts of astaxanthin were produced (379 µg/g of biomass) when ethanol was added after 48 h of fermentation, compared to control (300 µg/g). This demonstrated that ethanol addition after the available sugars was depleted in the media assisted in astaxanthin production. Gu et al (1997) reported that the addition of ethanol at different stages of growth caused an increase in the total amount of carotenoids in *Phaffia rhodozyma* UCD-FST-67-385. The mechanism involved where ethanol is converted into acetate by aldehyde oxidase may have produced superoxide radicals. As a defensive mechanism in yeast against superoxide radical formation, the synthesis of carotenoids was induced. Alternatively, the produced acetate was channelled towards the generation of acetyl coenzyme A, which later served as a precursor in the mevalonate pathway. According to Marcoleta et al. (2011), the addition of ethanol (0.2% w/v) resulted in a significant increase in the expression of genes involved in astaxanthin synthesis through the mevalonate pathway in *X. dendrorhous*, fortifying carotenoid production in the cells. This positive effect of ethanol in terms of carotenoids production has also been reported for other yeast species apart from *X. dendrorhous*; Bhosale (2004) reported that 20 g/l of ethanol supplementation stimulated β-carotene and torulene formation in a carotenoid-producing yeast, *Rhototorula glutinis*.

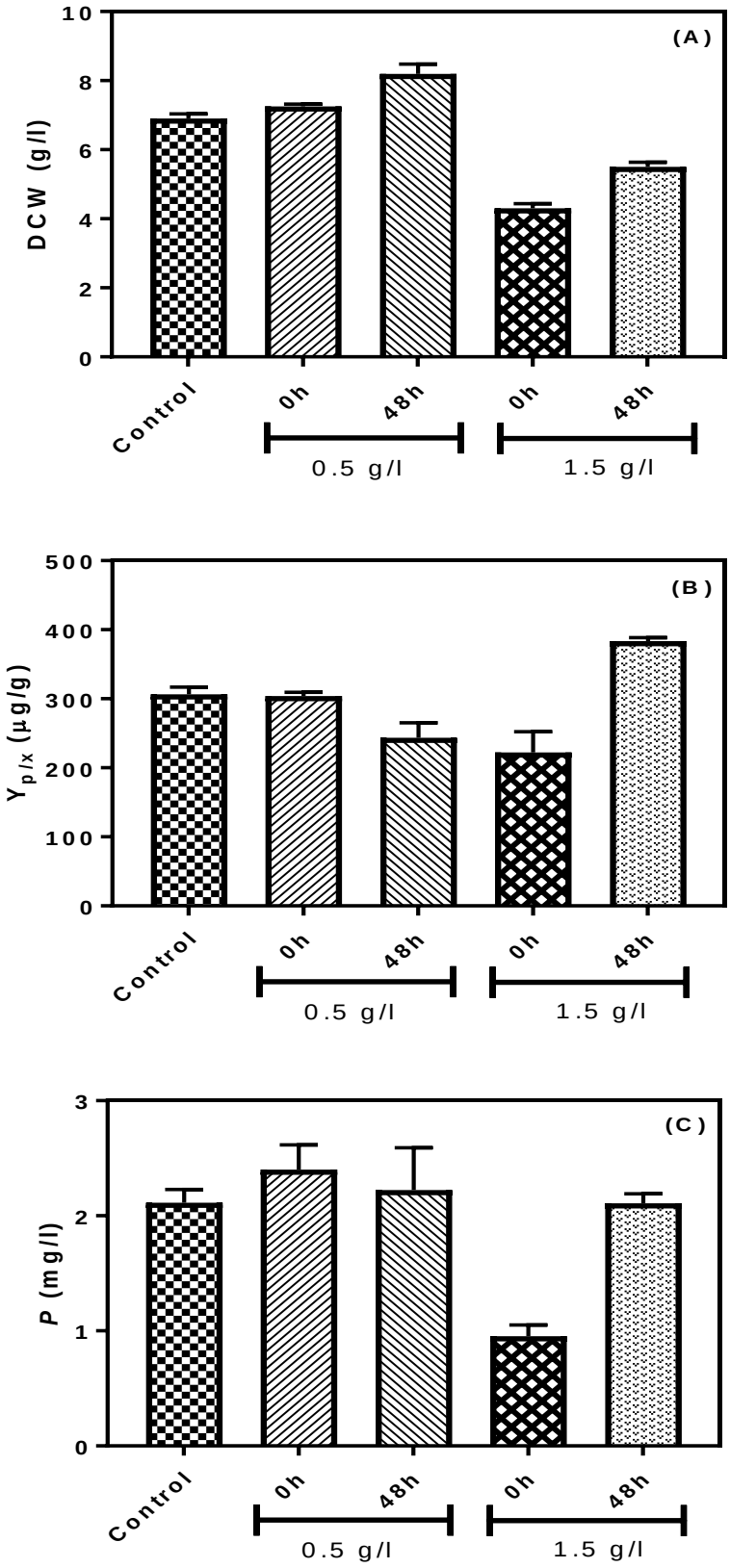

Figure 3-6: Effects of ethanol addition (0.5 and 1.5 g/L) at different feeding time (0 and 48h) during *X. dendrorhous* fermentation in semi-defined media. (A): Dry cell weight, DCW (B): Astaxanthin yield on biomass (µg/g) and (C): Astaxanthin concentration (µg/ml). Fermentation conditions: Temperature (20 °C), pH (not controlled) and agitation (250 rpm). Abbreviations: $Y_{p/x}$ (µg/g)- Yield of astaxanthin on biomass, *P* (mg/l)- astaxanthin concentration.

3.4.2 Citric Acid

Figure 3-7 shows the effects of citrate addition into semi-defined media for astaxanthin production in *X. dendrorhous*. Generally, the addition of citrate enhanced the cell growth. The biomass production increased by 21% against the control when 10 mM of citric acid were added to the fermentation media at the beginning of the fermentation. As for the astaxanthin yield, it was observed that the addition of 20 mM of citric acid during fermentation increased the astaxanthin production up to 330 µg/g biomass. On the other hand, the astaxanthin yield (Yp/x) increased at least 27 % in media added with 10 mM citric acid as compared to control.

Citrate supplementation has been reported to assist in carotenoid production in *X. dendrorhous* (Flores-Cotera & Sanchez, 2001). Citric acid acts as a precursor for the biosynthesis of carotenoid molecules in the cells, as an intermediate of the TCA cycle. The stimulation of citric acid on the TCA cycle might occur due to the fact that the key enzymes involved in the carotenoids biosynthesis pathway are affected by the TCA cycles intermediates and subsequently enhance carotenoid production. The other possible explanation is that citric acid increases the acetyl coA concentration and promotes pigmentation via the mevalonate pathway. Previous studies have reported that TCA intermediates supplementation helps to induce carotenoid production in several microorganisms including yeast (*X. dendrorhous)* and bacteria (*Dietza natronolimnea)* (Liu, 2006; Nasrabadi & Razavi, 2010).

In addition, previous studies have shown that supplementation of a-ketoglutarate, oxaloacetate and succinate have the greatest stimulatory effects on canthaxanthin production in *Dietza natronolimnaea* HS-1 (Nasrabadi & Razavi, 2010). Zeaxanthin production in *Flavobacterium* sp. was stimulated by the supplementation of citrate, α-ketoglutarate and oxaloacatetae (Alcantara & Sanchez, 1999). This current study indicates that supplementation of citric acid to fermentation media helps astaxanthin accumulation in *X. dendrorhous*.

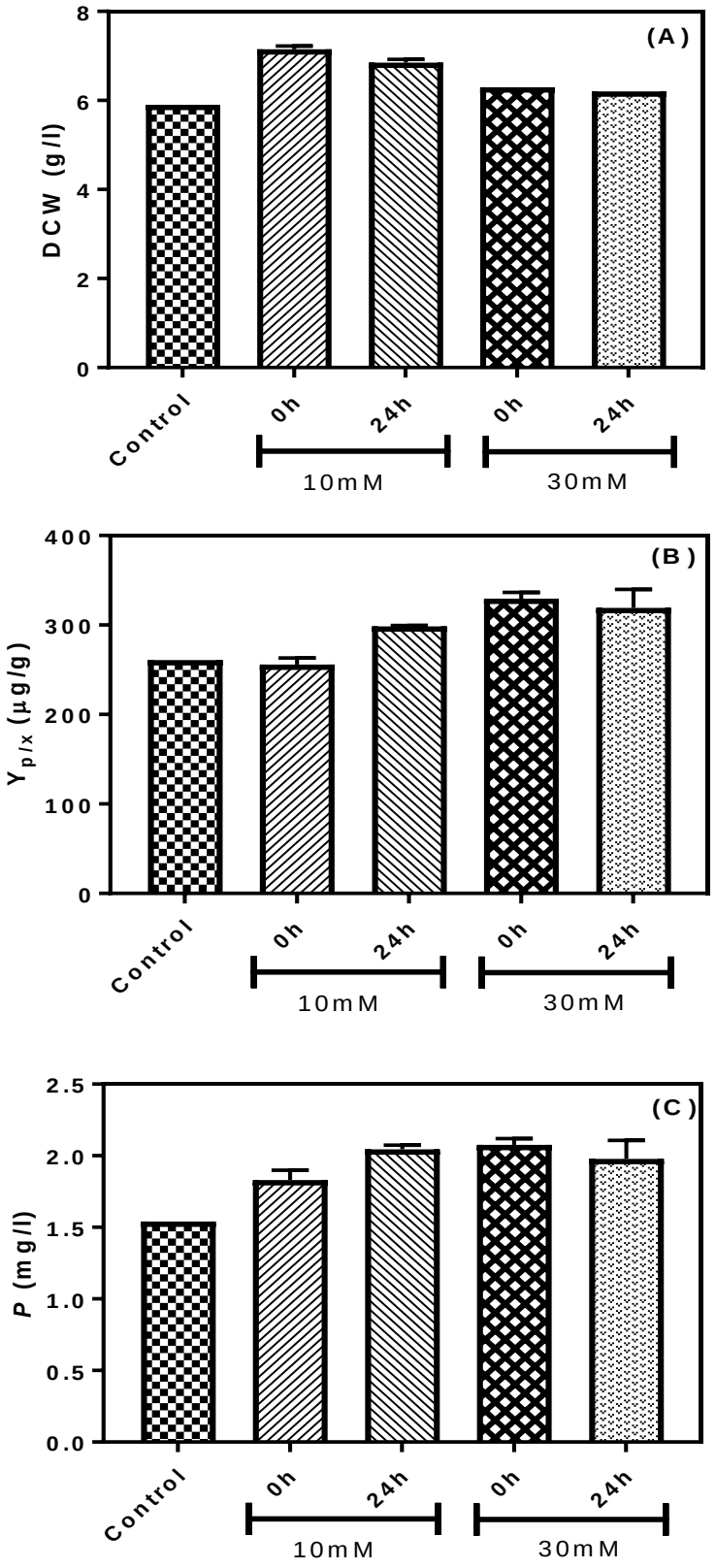

Figure 3-7: Effects of citric acid addition (10 and 30 mM) at different feeding time (0 and 24h) during *X. dendrorhous* fermentation in semi-defined media controlled at 20 °C under agitation (250 rpm). (A): Dry cell weight, (B): Astaxanthin yield on biomass (µg/g) and (C): Astaxanthin concentration (µg/ml). Abbreviations: $Y_{p/x}$ (µg/g)- Yield of astaxanthin on biomass, *P* (mg/l)- astaxanthin concentration

3.4.3 Hydrogen Peroxide (H_2O_2)

Astaxanthin is known for having a high antioxidant capacity which may have protective effects on the microorganisms against oxidative damage (Fassett & Coombes, 2011; Guerin, Huntley, & Olaizola, 2003; Wang, Yang, Yan, & Yao, 2012). Therefore, introducing oxygen to the fermentation media is one way to improve carotenoid accumulation in the yeast by stimulating the oxidative stress response of H_2O_2 on the cells.

X. dendrorhous in flask cultures were exposed to 10 and 20 mM H_2O_2 at two different stages (0 and 24h) of fermentation (Figure 3-8). Generally, results showed that H_2O_2 addition negatively affected the biomass production with more notable cell suppression occurring when H_2O_2 was introduced at the beginning of fermentation (0 h). However, astaxanthin yield per biomass was enhanced with H_2O_2 supplementation, (355 µg/g) compared to control (276 µg/g). The addition of H_2O_2 was found to increase catalase activity in *X. dendrorhous* which is expected to subsequently be used for the conversion of H_2O_2 into liquid oxygen in the cell. However, the limitation is that the catalase activity in *X. dendrorhous* is lower than in other yeast species such as *Saccharomyces cerevisiae* (Schoreder and Johnson, 1993). The addition of reactive oxygen, H_2O_2, resulted in the creation of a stressful and toxic environment. The yeast will then display a defence mechanism to protect the cells from cellular damage which occurs due to the presence of H_2O_2. Feeding reactive oxygen into the fermentation media is a simple way to increase astaxanthin accumulation in the cells (Kobayashi et al., 1997; Liu, Wu, & Ho, 2006)

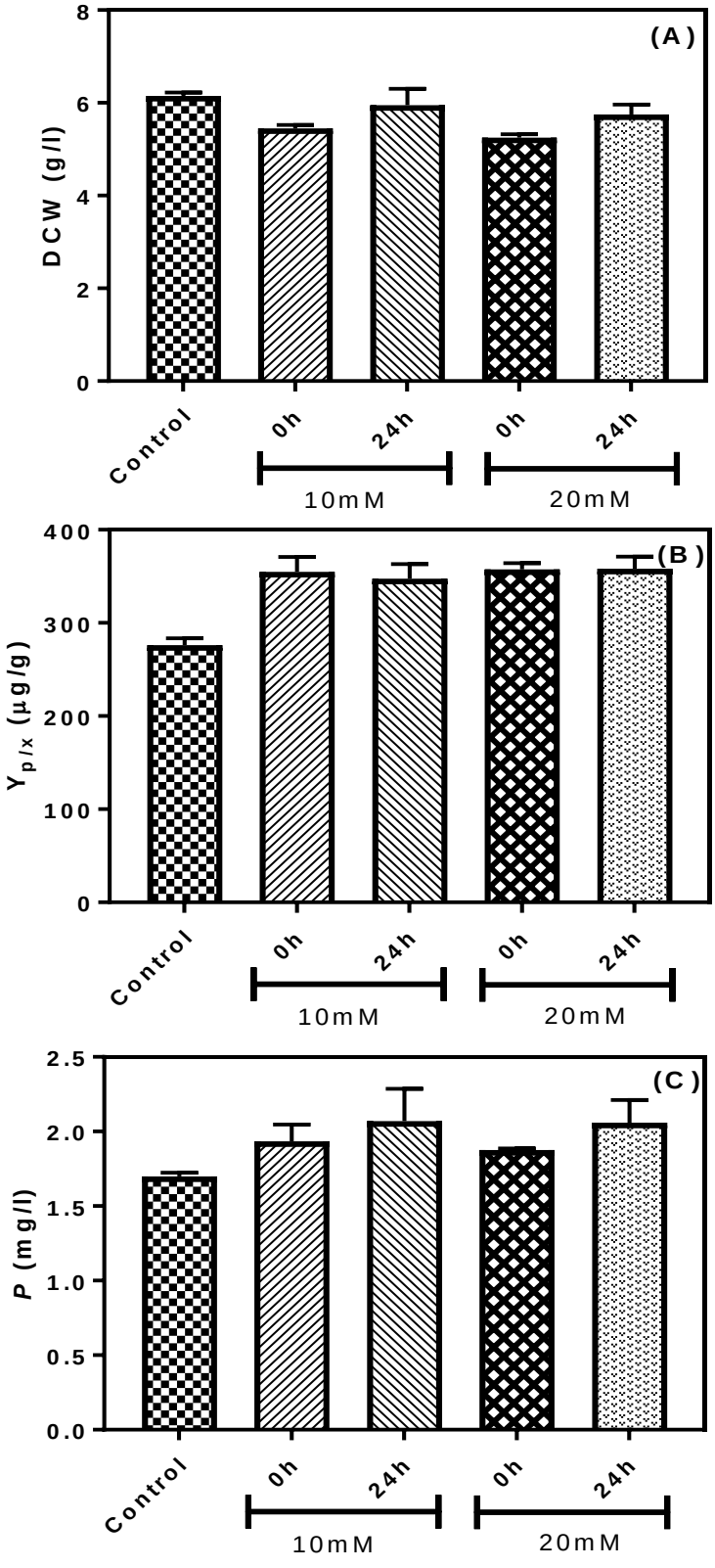

Figure 3-8: Effects of hydrogen peroxide addition (10 and 20 mM) at different feeding time (0 and 24h) during *X. dendrorhous* fermentation in semi-defined media. (A): Dry cell weight, (B): Astaxanthin yield on biomass (µg/g) and (C): Astaxanthin concentration (µg/ml). Fermentation conditions: Temperature (20 °C), pH (not controlled) and agitation (250 rpm). Abbreviations: $Y_{p/x}$ (µg/g)- Yield of astaxanthin on biomass, *P* (mg/l)- astaxanthin concentration

3.5 Conclusions

This study has shown that the selection of carbon sources and their respective concentrations can lead to substantial changes in biomass and astaxanthin production in *X. dendrorhous*. High yeast growth and astaxanthin production were observed when cellobiose, xylose and fructose (30 g/l) were used with astaxanthin production > 3 mg/l. Utilising glucose as a carbon source (>40 g/l) clearly shows inhibitory effects on astaxanthin production as well as suppression of yeast growth due to the occurrence of the Crabtree effect. However, high biomass concentration did not always correlate to high astaxanthin production as observed in the media containing galactose and arabinose as carbon sources. To improve astaxanthin production in yeast, selected chemicals (citric acid and hydrogen peroxide) showed positive effects on astaxanthin yield on biomass (Yp/x) when added at suitable doses and time points. The application of these chemicals to induce astaxanthin production should provide a simple and inexpensive strategy to increase astaxanthin yield in *X. dendrorhous*. Finally, this study gave an initial overview on the yeast behaviour in a media containing various sugars and the subsequent rapeseed meal hydrolysis in the next chapters will be tailored based on the preferred carbon source for the yeast, *X. dendrorhous* DSMZ 5626.

4 Valorisation of Rapeseed Meal as Substrate for the Microbial Production of Astaxanthin

Abstract

Rapeseed meal, which is a by-product of the oil processing industry, was evaluated as a substrate for the microbial production of astaxanthin using the yeast strain *Xanthophyllomyces dendrorhous* DSMZ 5626. The meal demonstrated high protein (25%, w/w), lignin (18%, w/w) and total carbohydrate (34%, w/w) contents, with the latter consisting primarily of glucose (20.0%, w/w) and to lesser extent arabinose (6.0%, w/w), galactose (2.9, w/w) and also uronic acids (3.2%, w/w). During the experiment, four commercial enzymes, namely (i) Viscozyme L, (ii) Accellerase 1500, (iii) pectinase and (iv) cellulase (from *Aspergillus niger*) were tested at different concentrations (1 – 15 %, v/v) for the individual assessment of their ability to break down the cellulosic and hemicellulosic compounds of rapeseed meal into monomeric fermentable sugars. Specifically, Viscozyme L and Cellulase demonstrated the highest glucose recovery yields (47 – 52% yield for 15 % (v/v) of enzyme used) and rapeseed meal derived total sugar concentration (74-77 g/l). A thermal pre-treatment step (126 ^{o}C, 30 min) prior to enzyme hydrolysis by Accellerase 1500 was also evaluated and was found to improve the hydrolysis rate of rapeseed sugars by 25%, which can be associated with the highly dispersed rough surface obtained after the thermal treatment, as demonstrated by electron scanning microscopy. All hydrolysates were tested as substrates for the growth of *X. dendrorhous* in shake flasks and it was demonstrated that the pectinase and Accellerase hydrolysates (with and without thermal treatment) supported best cell growth and astaxanthin production, thereby leading to astaxanthin concentrations of 6.7 mg/l in the former and approximately 3 mg/l in the latter case, with astaxanthin yields ($Y_{p/x}$) ranging from 258 to 332 µg per g of biomass. The

higher astaxanthin production in the case of the pectinase hydrolysate was attributed to the presence of high amounts of glycerol (originating from the enzyme formulation) which served as energy and carbon source for the cells. Overall, this research demonstrated that rapeseed meal hydrolysate, produced through the use of specific enzymes, is a nutrient rich medium that can support optimum *X. dendrorhous* growth without the need for additional supplements and could potentially be used as a fermentation feedstock for the efficient microbial production of astaxanthin.

Keywords: *Rapeseed meal, Astaxanthin, Hydrolysate, Xanthophyllomyces dendrorhous, Pre-treatment*

4.1 Introduction

Oilseed crops are extensively cultivated for their oil, similar to the planting of rapeseed, sunflower and soybean. The European Union (EU) is the largest producer of rapeseed with a reported total production of 32 million tonnes in 2015/16 and these figures are expected to increase annually (Krautgartner et al., 2017). In the United Kingdom (UK), rapeseed (*Brassica napus*) is a major oilseed crop, which is cultivated alongside sunflower with a reported total of 579 kHa of land planted in 2016 (Department for Environment, Food and Rural Affairs, 2016). Among rapeseed cultivars, some varieties are not suitable for human consumption as they contain high amounts of erucic acid (> 60%, w/w) and also glucosinolates that are responsible for the bitter taste of the oil. However, in the UK, rapeseed is cultivated mainly for cooking purposes with the use of improved cultivars that contain lower than 2% (w/w) erucic acid. Generally, rapeseed contains about 40-45% (w/w) of oil, which is low in saturated fatty acids (7-10%, w/w) but high in monounsaturated fatty acids (oleic acid). Rapeseed oil has also as a well-balanced ratio of n-6 to n-3 fatty acids (2.3-2.1%) as well as high phytosterol (4.5-11.3 g/kg) and tocopherols (430-2680 mg/kg) contents (Rekas et al., 2015). In the EU countries, non-edible varieties of rapeseed are cultivated as starting material for non-edible products such as grease, lubricants and biodiesel (Szydłowska-Czerniak, 2013). Large-scale extraction of rapeseed oil involves several steps including seed cleaning, dehulling, flaking and mechanical extraction via pressing end extrusion, which is often followed by solvent extraction (Dworakowska, Szczepan, & Bogdal, 2011). However, the method of using a single or double step cold-pressing process without the solventing step has gained increasing interest, due to the superior nutritional and sensory characteristics of the final product (oil). The cold-pressed method involves mild extraction temperatures that yield high-quality oils which do not require a refining step. However, the oil extraction yields are usually low, in the range of 40% (w/w). The oil extraction process leaves behind a solid residue, known as rapeseed meal, which is

generally underutilised and considered as a low value by-product (Wang et al., 2010). The world production of rapeseed meal in 2016/17 was reported at an estimated 40 million tonnes, with 13.9 million tonnes generated in the EU (Krautgartner et al., 2017). Currently, rapeseed meal is used as an organic fertiliser and as a supplement in livestock feeds due to its high protein content (26-31%) (Jeong et al., 2013; Pustjens et al., 2013). However, the rapeseed meal protein is not easily digested by monogastric animals compared to other protein sources such as soy meal, thus rendering it less valuable as a feed component (Kiran et al., 2012). Moreover, rapeseed meal may contain anti-nutritional compounds such as glucosinolates, erucic acid and phenols, thus making it less favourable by animals (El-Beltagi & Mohamed, 2010; Lomascolo et al., 2012).

There is significant potential for the valorisation of rapeseed meal, particularly as a substrate for microbial bioconversions, as besides protein, it also contains large amounts of carbohydrates (34-36%, w/w) (Jeong et al., 2014; Pustjens et al., 2013) as well as minerals such as calcium, phosphorous and iron (Pustjen et al., 2013.; Chatzifragkou et al., 2014). The utilisation of rapeseed meal in microbial bioconversions is not a single step process, which can be attributed to the fact that most microorganisms lack the necessary enzymes for catalysing cellulose hydrolysis. As such, the transformation of rapeseed meal into a fermentation feedstock requires the application of physicochemical and/or enzymatic approaches, aiming to break down the complex structure of the biomass and concurrently generate sugar and nitrogen components that are directly assimilable by microorganisms (Kiran et al., 2012; Chatzifragkou et al., 2014). The soybean hydrolysate can be potentially used for several microbial conversions, including for the production of platform and speciality chemicals, biopolymers and natural colourants.

Astaxanthin belongs to the xanthophyll group of carotenoids and is a powerful antioxidant, which acts by donating the electrons from the conjugated double bonds and reacts with free radicals to produce high stability products and terminate the free radical chain reaction in a wide range of living organism (Ambati et al., 2014). It also possesses anti-inflammatory activities and has potential as a therapeutic agent against cardiovascular diseases in humans (Fassett & Coombes, 2011). Additionally, in aquaculture, astaxanthin is used as feed additive because it contributes to the attractive colouration in the bodies of salmons, shrimps and crustaceans as well as maintains their growth and survival (Ip et al., 2004; Baker & Günther, 2004). Currently, the commercial demand for astaxanthin is met by its synthetic production (petroleum derived) via the Wittig reaction and Grignard condensation strategies (Panel, 2014). However, natural astaxanthin is more favourable as it has been reported to possess high stability due to its esterified nature, which prevents oxidation and thus contributes to prolonged shelf life (Chandra Bhatt et al., 2012). The natural source of astaxanthin has been approved to be used as food colouring (E161j) in the EU. Currently, the naturally produced astaxanthin by microalgae (*Haematococcus pluvialis*) and yeasts (*Xanthophyllomyces dendrorhous*) face a competitive market price compared to synthetically produced astaxanthin due to the complex production process.

Trials to extract rapeseed protein have been conducted using various methods such as commercial cellulolytic enzymes (Rodrigues et al., 2014) as well as the use of proteolytic fungi, *Aspergillus oryzae* (Chatzifragkou et al., 2014). However, studies involving carbohydrates hydrolysis from rapeseed meal are scarce in comparison to protein hydrolysis (Rodrigues et al., 2014; Sari et al., 2013). The aim of this study was to enzymatically hydrolyse the lignocellulosic structure of rapeseed meal to produce a nutrient-rich hydrolysate, which was subsequently evaluated for its potential to be used as a fermentation substrate for the astaxanthin production by the yeast *X. dendrorhous* DSMZ 5626.

4.2 Materials and Methods

4.2.1 Microorganism and Growth Medium

The yeast strain *Xanthophyllomyces dendrorhous* DSMZ 5626 was used in this study and was purchased from the Leibniz Institute DSMZ, Germany. The strain was proliferated and maintained in Yeast and Mold (YM) media containing (in g/L): Yeast extract, (3.0); malt extract, (3.0); peptone from soybean, (5.0); glucose, (10.0); and agar (15).

4.2.2 Materials

The rapeseed meal that was used in this study was kindly provided by Stainswick Farm (Oxfordshire, UK) and was generated via the cold pressing oil extraction process. Rapeseed meal samples were grinded using a dry-grinder and sieved to obtain uniform sized particles (< 850 μm). The oil was removed using a supercritical CO_2 extraction rig (SciMed, UK) at 60 °C and 300 bar pressure for 1 hr, with ethanol (10%, v/v) as co-solvent. The residual defatted meal was kept at 4 °C prior to use.

4.2.3 Enzymatic Hydrolysis

The enzymatic hydrolysis of rapeseed meal was performed using four different commercial enzymes, namely Accellerase 1500 (DuPont), Viscozyme (Novozymes), Cellulase from *Aspergillus niger* (Sigma-Aldrich) and pectinase (Novozymes) (Table 4-1). Enzymatic hydrolysis was carried out in 2 ml Eppendorf tubes containing 0.150 g rapeseed meal with different enzyme concentrations (1-15%, v/v). Optimum temperatures for each enzyme and adequate mixing (1600 rpm) were maintained in a thermomixer F 1.5 (Eppendorf, Germany). The reaction time varied from 2 hrs to 24 hrs and the reactions were terminated by incubation

at 95 °C for 10 mins. The mixtures were then centrifuged at 10 845 x *g* (Heraeus Multifuge X3R, Thermo Fisher, USA) for 10 mins and the supernatants were collected and analysed for sugar composition.

Table 4-1: Details of the industrial enzymes used in the study

Enzyme	Source	Main Activity	Side activities	Activity	Stabiliser/ Protectant
Viscozyme L	*Aspergillus aculeatus*	Endoglucanse	Xylanase Cellulase Hemicellulase	100 FBG/g	Sucrose, NaCl
Cellulase	*Aspergillus niger*	Endo-glucanase	-	~ 0.8 unit/mg	Not given
Pectinase	*Aspergillus aculeatus*	Polygalacturonase	Cellulase, β-Galactosidase	≥ 3800 unit/ml	Glycerol
Accellerase 1500	*Trichoderma reesei*	Exoglucanase, Endoglucanase and β-Glucosidase	Hemicellulases	2200-2800 CMC unit/g	Not given

4.2.4 Rapeseed Meal Pre-Treatment

The thermal pre-treatment of rapeseed meal was carried out prior to enzymatic hydrolysis by the commercial enzyme Accellerase 1500. Various substrate loadings (10-50%, w/v) were prepared in 30 ml of distilled water and subjected to autoclaving at different temperatures (126 and 135 °C, pressure ~2 bar) for 30 min. Once the heat treatment was completed, 10% (v/v) of enzyme (Accellerase 1500) was added to the mixture and hydrolysis was carried out at 50°C for 24 hrs under continuous stirring (250 rpm) in an orbital shaker (GFL, 3015, SciQuip, UK).

4.2.5 Microbial Fermentations

In order to investigate the suitability of the rapeseed meal hydrolysates as substrates for *X. dendrorhous* growth, flask experiments were conducted. Rapeseed meal hydrolysates were prepared using different enzymes under identified optimised conditions. The produced hydrolysates were subsequently filter-sterilised using 0.25 µm Millipore stericup filters, prior to the fermentation. Suspensions of *X. dendrorhous* pre-cultures were then aseptically added to the 50 ml of hydrolysates in 250 ml conical flasks in order to obtain an initial OD of ~0.1. All flask fermentations were carried out for 5 days at 20 °C under constant agitation (250 rpm), in duplicate. Data shown are the mean values of these measurements.

4.2.6 Analytical Methods

The carbohydrate content of the rapeseed meal was analysed according to the experimental work of Sluiter et al., (2004). Briefly, 300 mg of rapeseed meal were pre-hydrolysed with 3 ml of 72 % (v/v) H_2SO_4 at 30 °C for 1 hr. Subsequently, 84 ml of distilled water were added to the mixture in order to dilute the sulphuric acid content to 4 % (v/v) and further in an autoclave, hydrolysis was carried out at 121 °C for 30 mins. On the completion of hydrolysis, the mixtures were neutralised with calcium carbonate to pH 5-6. The samples were then analysed using a High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) system (Dionex, Thermo, UK). In this step, the samples were passed through a CarboPac PA1 column (4 x 250mm) at a flow rate of 1 ml/min and the mobile phase consisted of 0.016 mM of sodium hydroxide (NaOH) (eluent A) and 0.5 mM of sodium acetate (NaOAc) (eluent B) in a gradient method. Operating conditions were as follows: Sample volume: 20µl; mobile phase: 0.5 M NaOH, 0.1 M NaOAc; column temperature: 65°C; flow rate: 0.6 ml/min; gradient conditions: $T_{0\text{-}40}$, 100% A, $T_{41\text{-}50}$, 60% A, 40% B, T_{51}, 100% A.

The protein and fat content were measured by the Kjeldahl and Soxhlet method, respectively (AOAC, 1990). The lignin content was determined according to Sluiter et al. (2011). Briefly, after hydrolysis with 72 % (w/w) H_2SO_4 for 1 hr at 30°C, samples were hydrolysed with 1.0 M H_2SO_4 at 121 °C for 30 mins in an autoclave. Samples were then filtered and the filtrate was measured for acid soluble lignin spectrophotometrically at 240 nm. Acid Soluble Lignin (ASL) was calculated according to the formula below (Eq. 4-1). The washed residue was dried at 100 °C for 18 hrs. Subsequently, the dried samples were placed in a furnace (500 °C; 5 h) and the ash was weighed and classified as ASL. Total lignin was calculated as the sum of acid soluble and acid insoluble lignin.

$$ASL = \frac{Absorbance \times Dilution\ factor \times filtrate\ volume\ (ml)}{Extinction\ coefficient \times Weight\ of\ sample\ (g)} \qquad \text{Eq. 4-1}$$

In the case of yeast fermentations, samples of 2 ml were periodically withdrawn at regular time intervals. 1 ml of sample was added into a pre-weigh tube and centrifuged at 10845 x g for 10 min. The supernatant was collected for sugar and ethanol analysis via HPLC. The pellet was washed twice with distilled water and kept frozen at -20 °C prior to freeze drying (Virtis Sentry 2.0, UK). Biomass was measured by weight difference. The sugar composition of the hydrolysates was determined by HPLC analysis (Agilent 1260 Infinity) with an Aminex HPX-87H column coupled to a differential refractometer and a diode array detector. Operating conditions were as follows: Sample volume: 20µl; mobile phase: 5 mM H_2SO_4; flow rate: 0.6 ml/min; column temperature: 65 °C. Quantitation of monosaccharides (glucose, galactose, xylose, arabinose), uronic acids and ethanol were achieved on the basis of standard curves, which were conducted using standard solutions.

Total carotenoid extraction was carried out according to the experimental work of Sedmak et al. (1990). Briefly, 1 ml of Dimethyl Sulfoxide (DMSO) was preheated at 55 °C and added to the freeze dried biomass, followed by vortexing for 30-40 secs. Subsequently, 0.2 ml of 20 % Sodium Chloride (NaCl) and 1.0 ml of acetone were added to the mixture to extract the intracellular carotenoids. The aqueous and organic phases were separated by centrifugation at 5423 x g for 5 mins. The extraction process was repeated until a colourless biomass was obtained. The organic phases were then pooled together, and the absorbance measured at 480 nm in a spectrophotometer. Values were then divided by the extinction coefficient of 2150. The equation for total carotenoids concentration is given below:

$$Carotenoid\ content\ (\mu g/g) = \frac{A\ \times V\ (ml) \times\ 10^4}{A_{1cm}^{1\%}\ \times P\ (g)} \qquad \text{Eq. 4-2}$$

Where, *A* is the absorbance at 480 nm, *V* is the volume, $A_{1cm}^{1\%}$ is the coefficient (2150) and *P* is the weight of biomass.

4.2.7 Scanning Electron Microscopy

The structure of rapeseed meal samples after pre-treatment and enzymatic hydrolysis was analysed by environmental scanning electron microscope (FEI Quanta FEG 600). All samples were subjected to freeze drying in order to remove the moisture prior to the imaging. The freeze-dried cells were deposited on carbon tape, fixed on stubs and then coated with gold. To ensure reproducibility, a large number of images (~10) were captured.

4.3 Results and Discussion

4.3.1 Compositional Analysis of Rapeseed Meal

In cold pressing, rapeseed undergoes a mild oil extraction process (< 40 °C) without the use of solvents. Approximately 50-60 % of the initial oil is normally extracted from the seed and in line with this, the rapeseed meal used in this study contained substantial amounts of oil (16.7 ± 0.1 %). Table 4-2 shows the composition of the rapeseed meal on a dry basis. The rapeseed meal was rich in protein and carbohydrates, accounting for 26 % and 35 % (w/w) of each, respectively. The protein content was lower than that reported by Pustjens et al. (2013) (i.e., 30 %, (w/w)), possibly due to differences among rapeseed cultivars as well as due to differences in the oil extraction process (Leming and Lember, 2002).

The carbohydrate content was similar to that reported by Pustjens et al. (2013), accounting for 36 % (w/w). The monosaccharide composition indicated that the main polysaccharides present in the rapeseed meal were cellulose, hemicellulose and pectin. Glucose was the most abundant monosaccharide, i.e., equal to 20.2 % (w/w). The arabinose content was ~ 6 % (w/w), the highest among the other sugars that are normally present in hemicellulose, namely galactose and xylose. This finding is similar to the works of Pustjen et al., (2013) who found that arabinose is the major hemicellulosic-derived sugar present in rapeseed meal. It suggests the presence of arabinans, which might be attached on the side chains of pectin. Moreover, the presence of xylose and arabinose could also originate from the arabinoxylan present in rapeseed meal. Arabinoxylan is a hemicellulose consisting of a linear backbone of β-1,4 xylose residues with arabinose substitution (Butardo & Sreenivasulu, 2016).

The presence of galactose (2.9 %, w/w) could indicate the presence of galactomannan and arabinogalactan in the rapeseed meal. Moreover, the presence of galacturonic acid (2.5 %,

w/w) and glucuronic acid (0.6 %, w/w) further supported the presence of pectin in the rapeseed meal. Pectins have been reported as major polysaccharides in rapeseed meal apart from hemicellulose and cellulose (Bell, 1984; Pustjens et al., 2013). Small amounts of fructose (0.3 % w/w) were detected in the acid treated fraction of the rapeseed meal, in agreement with Bell (1984) and Lomascolo et al. (2012). In the water soluble fractions of rapeseed meal, which was extracted using hot water, sucrose was also detected (data not shown). Sucrose and fructose have been previously reported in the water soluble fractions of rapeseed meal and they normally act as an energy reserve in plants (Lomascolo et al., 2012; Pustjens et al., 2013).

The analysis indicated the presence of ~ 18 % (w/w) of total lignin, consisting of 1.9 ± 0.1 % (w/w) of acid soluble lignin and 16.1 ± 4. 6% (w/w) of Klason lignin. The amount of total lignin in rapeseed meal was similar to the values (~16 %) reported by studies investigating the production of biodiesel from rapeseed meal (Briones et al., 2012; Egües et al., 2010).

Table 4-2: Chemical composition of rapeseed meal

Composition	Percentage (%, w/w)
Oil	16.7 ± 0.1
Protein	25.3 ± 0.2
Lignin	18.0 ± 4.6
Ash	5.3 ± 0.1
Moisture	6.8 ± 0.1
Sugars (% carbohydrate)	34.2 ± 6.2
Glucose	20.2 ± 2.3
Galactose	2.9 ± 0.5
Arabinose	5.9 ± 0.7
Xylose	1.6 ± 0.2
Fructose	0.3 ± 0.2
Fucose	0.2 ± 0.1
Glucuronic acid	0.4 ± 0.3
Galacturonic acid	2.8 ± 0.1

4.3.2 Enzymatic Hydrolysis of Rapeseed Meal

4.3.2.1 Effect of Enzyme Concentration

The rapeseed meal contained high amounts of carbohydrates and protein, however, most microorganisms cannot directly assimilate these compounds due to inability to produce protease and cellulase enzymes (Chatzifragkou et al., 2014, Wang et al, 2010). Therefore, commercial enzymes were employed in this study, targeting the conversion of cellulose and hemicellulose into their monomeric sugars (glucose, galactose, xylose and arabinose). The generated hydrolysates were subsequently tested for their capability to support astaxanthin production by the yeast *X. dendrorhous*.

Figure 4-1 shows the effect of different enzyme concentrations on the sugars released after 24 hrs of hydrolysis using four different commercial enzymes (cellulase, pectinase, Viscozyme L and Accellerase 1500). The highest glucose yield (g of glucose released after hydrolysis per g of glucose in rapeseed meal) was observed when Viscozyme L (53 %, w/w), followed by cellulase (47 %, w/w), pectinase (41.8 %, w/w) and Accellerase 1500 (30 %, w/w) were used. Viscozyme L is a multi-enzyme complex containing mixtures of cellulases, hemicellulases and xylanase and has also a proteolytic activity (Guan & Yao, 2008; Hanmoungjai, Pyle, & Niranjan, 2002; Rodrigues et al., 2014). Hence, the synergistic effect of these enzymes rendered the rapeseed meal structure more accessible to enzymatic attack, thereby resulting in the production of a hydrolysate rich in cellulose- and hemicellulose-derived monomeric sugars. Galactose and arabinose were obtained at their highest yields when Viscozyme L was used, equal to 74 % (w/w) and 79 % (w/w), respectively (Figure 4-1A).

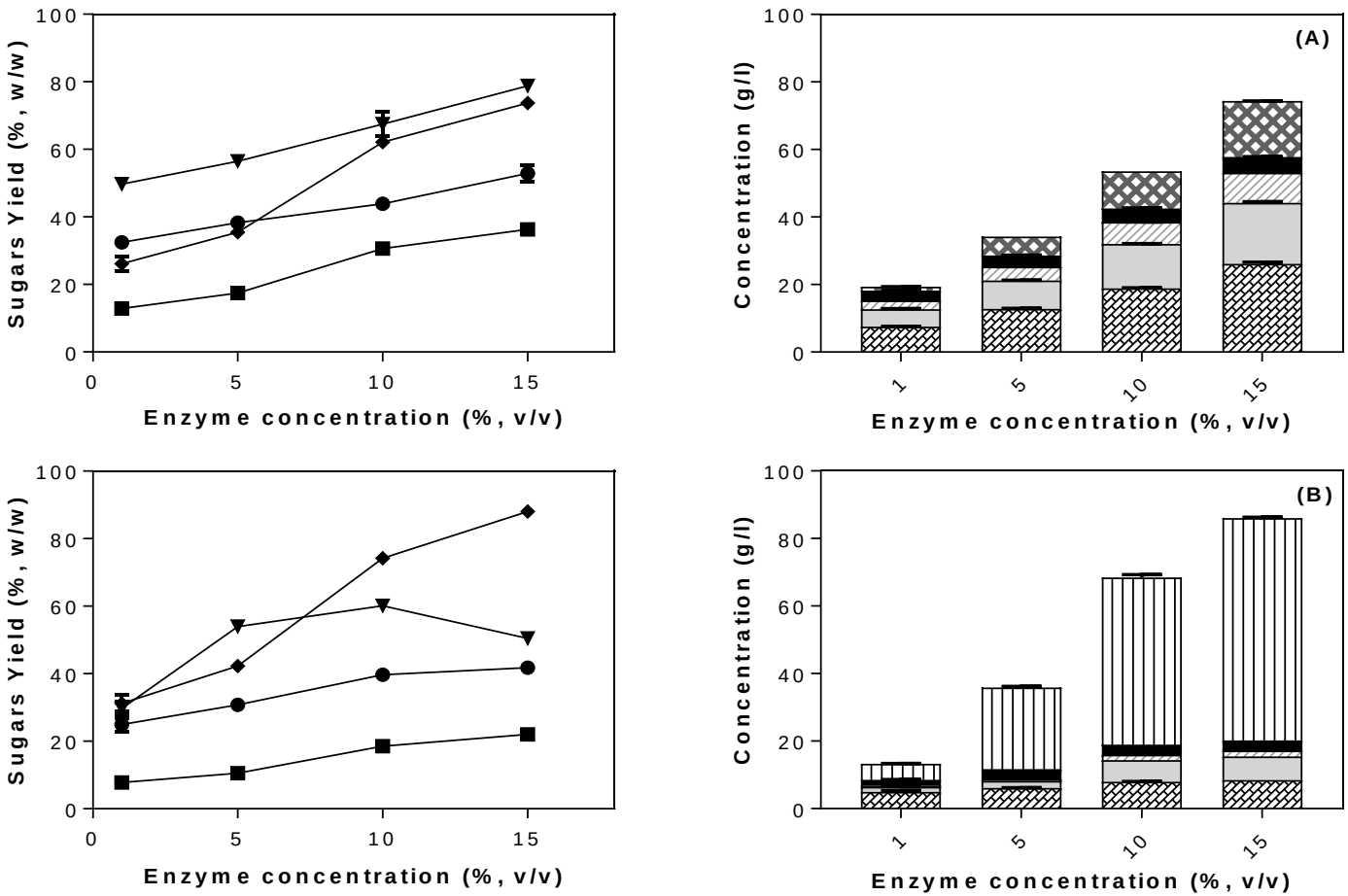

Figure 4-1a: Effect of different enzyme concentrations on the total sugars released after 24 hrs of hydrolysis of 10% (w/v) rapeseed meal using four different commercial enzymes [Viscozyme (A), pectinase (B)]. Line graph represents the sugars yield (%), whereas the bar graph represents the concentration of sugars in rapeseed meal hydrolysates. Symbols represent: ●- glucose, ■- xylose, ▼- arabinose, ♦- galactose, -glucose (g/l) - galactose (g/l), - xylose (g/l), - arabinose (g/l), - glycerol (g/l) and - fructose (g/l). * Sugars yield is calculated based on the sugar present in the hydrolysates after deducting the initial sugars that were present in the enzyme solution per total sugar before the treatment.

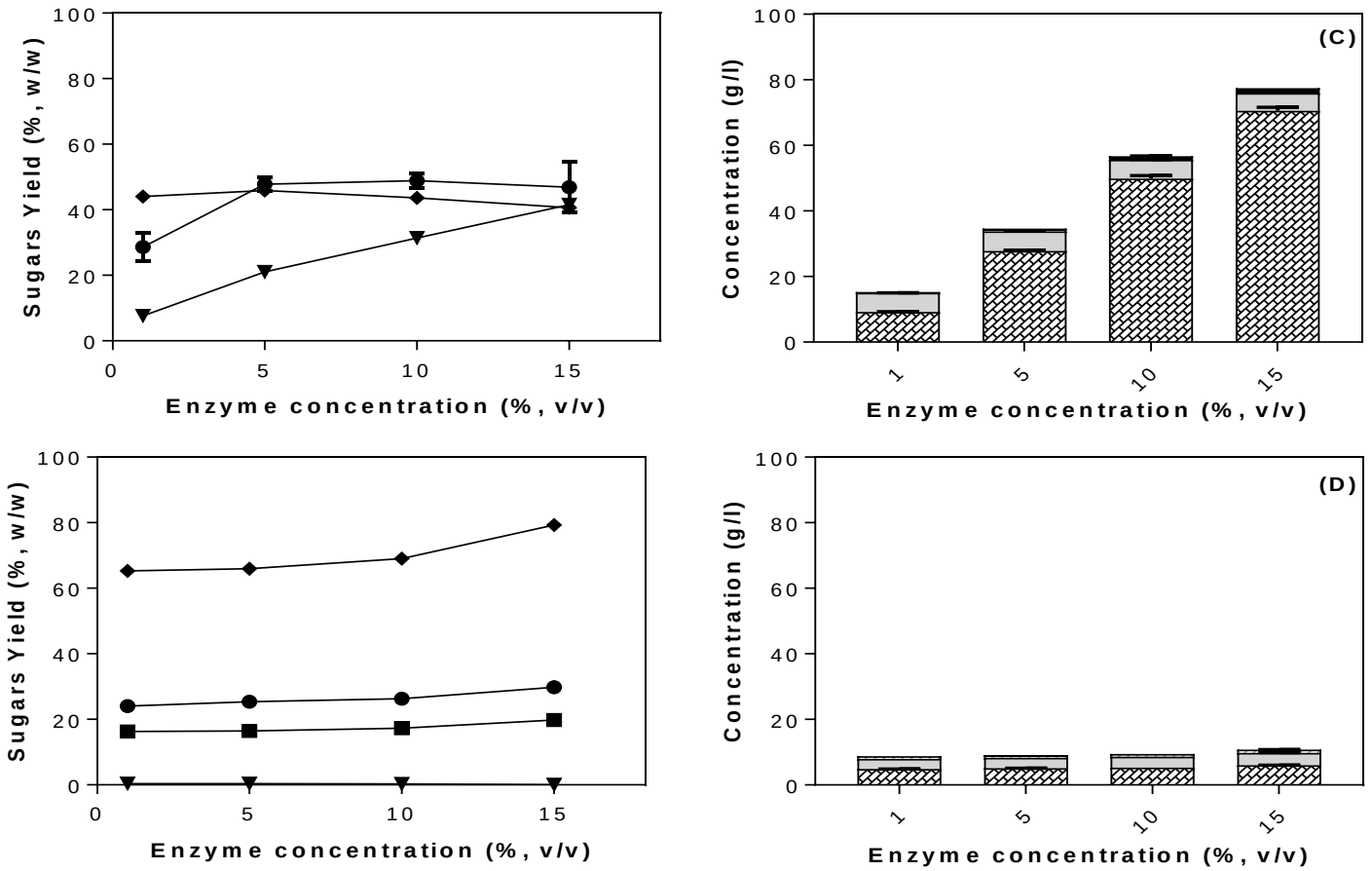

Figure 4-1b: Effect of different enzyme concentrations on the total sugars released after 24 hrs of hydrolysis of 10% (w/v) rapeseed meal using four different commercial enzymes [cellulase (C) and Accellerase 1500(D)]. Line graph represents the sugars yield (%), whereas the bar graph represents the concentration of sugars in rapeseed meal hydrolysates. Symbols represent: ●- glucose, ■- xylose, ▼- arabinose, ♦- galactose, -glucose (g/l) - galactose (g/l), - xylose (g/l), - arabinose (g/l) and - fructose (g/l). * Sugars yield is calculated based on the sugar present in the hydrolysates after deducting the initial sugars that were present in the enzyme solution per total sugar before the treatment.

According to the manufacturer's datasheet (Sigma Aldrich), Cellulase contained mainly endo- and exo- cellulases, thus their documented synergistic activities led to the effective hydrolysis of cellulose and β-glucans (Taherzadeh & Karimi, 2007; Yoo, 2012). Endo-cellulases attack the β-1,4-glycosidic bonds randomly, targeting the amorphous region along the cellulose structure and produce either cellobiose or glucose. As for exo-cellulases, they act on the linkages from the non-reducing end of cellulose releasing cellobiose as the main reaction product (Yoo, 2012). Similar patterns to those obtained for glucose were observed for the hemicellulose-derived monosaccharides during the experiment. Generally, increased enzyme concentrations resulted in increased amounts of galactose, xylose and arabinose in the hydrolysate. Xylose was detected in all hydrolysates except for those with Cellulase activity (Figure 4-1C).

In terms of the sugar concentrations in the hydrolysates, glucose and galactose were mainly present. In the case of Viscozyme L hydrolysates (Figure 4-1A), the total sugar concentration when high enzyme loadings (15 %, v/v) were used was ~74 g/l, with glucose being ~ 26 g/l and fructose ~ 17 g/l. It must be noted that approximately ~16.5 g/l of glucose and ~17 g/l of fructose were derived from the enzymatic hydrolysis of sucrose that was present in the Viscozyme L formulation and is generally used as a preservative for enzyme formulations. In the case of pectinase (Figure 4-1B), the total concentration of monomeric sugars in the hydrolysates accounted for approximately 20 g/l, with glucose and galactose being the principle sugars. However, 66 g/l of glycerol was also detected in the hydrolysates that originated from the enzyme formulation itself, which could potentially act as an additional carbon source for microbial fermentation. As for Cellulase (Figure 4-1C), the total sugar concentration was ~76 g/l, the majority of which was glucose (71 g/l), although ~64 g/l of glucose were attributed to the enzyme formulation. In Accellerase 1500 hydrolysates, 11 g/l of total sugars were present, with glucose (~ 6 g/l) and galactose (~4 g/l) being the most prominent

sugars. Due to the absence of additional preservatives in the formulation, the Accellerase 1500 enzyme was chosen for further investigation, as the composition of its hydrolysate is a direct reflection of the activities of the commercial enzyme.

The synergistic action of enzyme mixtures assists in the further break down of carbohydrate polymers, as indicated by the action of pectinase on rapeseed meal. This was depicted by the higher hydrolysis yield of rapeseed meal as 41 % of glucose was extracted using pectinase enzyme, which contains mixtures of activities including cellulases as well as hemicellulases. Comparing to Accellerase 1500 treatment, which did not contain the side activity of pectinase, this extraction yielded lower glucose yields (30 %, w/w). This suggests that selecting the right enzymes for rapeseed meal hydrolysis is important to obtain high hydrolysis yield.

Monitoring the hydrolysis kinetics of rapeseed meal by Accellerase 1500 (Figure 4-2) demonstrated that after 24 hrs of hydrolysis period, the sugars released during hydrolysis reached a plateau with no further significant increase. Therefore, the enzymatic hydrolysis of rapeseed meal in this study was performed up to 24 hrs. As expected, glucose was the principal sugar, released during enzymatic hydrolysis with Accellerase 1500, accounting for 6 g/l after 24 hrs of hydrolysis. This value correlates with the composition of carbohydrates in rapeseed meal, which primarily consisted of glucose, at ~20 % (w/w).

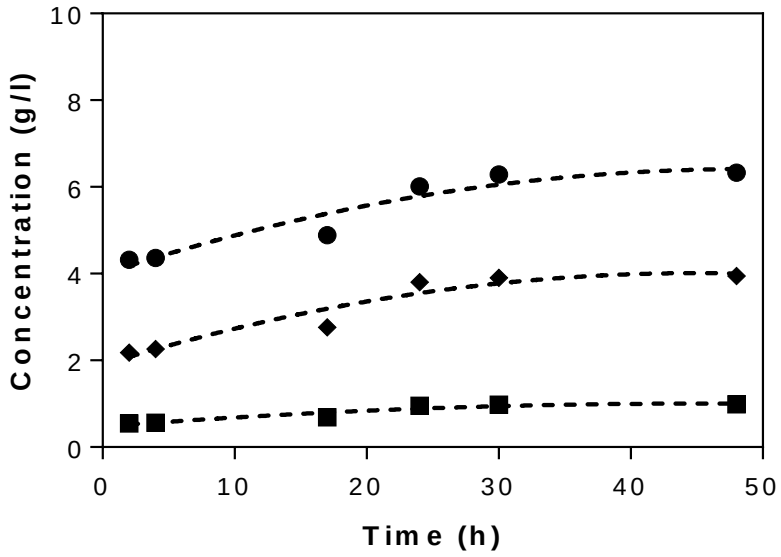

Figure 4-2: Kinetics of enzyme hydrolysis of rapeseed meal by Accellerase 1500. Experimental conditions: Temperature (50°C), enzyme concentration (15%, v/v) and substrate concentration (10%, w/v). Symbols represent: Glucose (●, g/l), xylose (■, g/l), galactose (♦, g/l)

The enzymatic hydrolysis of rapeseed meal has been widely reported for the extraction of proteins rather than of carbohydrates. Chen et al. (2011), tested three different enzymes (Celluclast, Viscozyme and Pectinase G), both singly and in combination, in order to hydrolyse dilute acid pretreated rapeseed meal that was subsequently used for succinic acid production by *Actinobacillus succinogenes*. They reported that about 28.6 g/l of total sugars were released from rapeseed meal after Pectinase G (2 %, w/w) hydrolysis consisting of sucrose, glucose, fructose and arabinose. In a different study, Viscozyme L was found to assist in the protein hydrolysis of rapeseed meal with high protein yield (68 %) and total carbohydrates yield (80 % of total reducing sugars) (Rodrigues et al., 2014).

4.3.2.2 Effect of Thermal Pretreatment on Rapeseed Meal

Lignocellulosic materials have complex structures, due to the presence of polysaccharides (cellulose, hemicellulose, pectin), lignin and structural proteins. Previous studies have shown that lignin and pectin hinder the hydrolysis of cellulose and hemicellulose in lignocellulosic biomass (Chen et al., 2011; Sun and Cheng, 2002; Taherzadeh & Karimi, 2008). The presence of lignin may cause non-productive adsorption of cellulase (Düsterhölt, Engels & Voragen 1993; Taherzadeh & Karimi, 2007) and hinders the accessibility of cellulase to cellulose content (Cui et al., 2014). Generally, thermal treatment has been acknowledged as a choice of pre-treatment method for lignocellulosic materials as it does not require catalysts and does not cause corrosion issues (Jönsson & Martín, 2016). The presence of heat and water disrupts the hydrogen bonds that hold together the crystalline structure of cellulose and lignin matrices, resulting in swelling of the biomass and disruption of the cellulose matrix. The increase in the solubility of the lignin regions allows a greater accessibility of enzymes into the polymer structure (Brodeur et al., 2011; Wan & Li, 2011). Additionally, a neutralisation step is not required as no harsh chemicals are used and the generated liquid fraction could also be used for further applications such as microbial bioconversions (Brodeur et al., 2011).

In this study, a thermal pre-treatment step was carried out to evaluate its effectiveness in enhancing the enzymatic hydrolysis of rapeseed meal. Table 4-3 presents the composition of rapeseed meal hydrolysates following the heat pre-treatment and enzymatic digestion by Accellerase 1500 (10 % v/v). Heat pre-treatment at 126 °C for 30 mins was found to significantly increase ($p < 0.05$) the glucose hydrolysis yield by 17 % compared to control (no pre-treatment). However, higher temperature conditions (135 °C for 30 mins) resulted in lower sugar yields compared to those at 126 °C. This was most likely due to the more prominent degradation of glucose to furfural and 5-hydroxymethyl-2-furaldehyde (HMF) under the higher

temperature in the presence of mild acid conditions (Li et al., 2015). The acidic environment was generated due to the partial acetylation of hemicellulose, leading to the hydrolysis of acetyl ester bonds into acetic acid during the heat pre-treatment process (Sun et al., 2015). Similar to glucose, the recovery of the hemicellulosic-derived sugars also decreased in the case of higher pre-treatment temperature.

Table 4-3: Effect of thermal pre-treatment (at 126 ºC and 135 °C) of a rapeseed meal suspension (10 %, w/v) followed by 15% (v/v) of Accellerase 1500 treatment for 24 hrs on hydrolysis yield and sugar concentrations.

Temperature (T°C)	Time (min)	Hydrolysis Yield (%)			Concentration (g/l)		
		Glu	Xyl	Gal	Glu	Xyl	Gal
Control (no pre-treatment)		29.82 (0.31)^{a}	19.82 (0.36)^{a}	79.28 (1.43)^{a}	6.01 (0.06)^{a}	0.95 (0.02)^{a}	3.81 (0.07)^{a}
126	**15**	37.37 (0.22)^{cd}	15.59 (0.32)^{a}	62.37 (1.27)^{a}	7.54 (0.04)^{cd}	0.74 (0.02)^{a}	2.97 (0.06)^{a}
126	**30**	40.44 (2.12)^{bc}	16.97 (0.82)^{b}	67.88 (3.27)^{b}	8.15 (0.43)^{bc}	0.81 (0.04)^{b}	3.24 (0.16)^{b}
126	**60**	34.25 (0.15)^{c}	16.67 (0.09)^{b}	66.69 (0.36)^{b}	6.91 (0.03)^{c}	0.79 (0.01)^{b}	3.18 (0.02)^{b}
135	**15**	38.06 (0.66)^{b}	16.74 (0.33)^{b}	66.95 (1.32)^{b}	7.67 (0.13)^{b}	0.80 (0.02)^{b}	3.19 (0.06)^{b}
135	**30**	38.46 (0.31)^{b}	15.72 (0.16)^{a}	62.89 (0.63)^{a}	7.75 (0.03)^{b}	0.75 (0.01)^{b}	3.00 (0.01)^{b}

Different letters (a-c) represent significant differences among the data in the same column ($p<0.05$)

Figure 4-3 shows the scanning electron microscope images of rapeseed meal before and after pre-treatment/ hydrolysis. Each process stage resulted in structural changes in the rapeseed meal. The untreated sample (Figure 4-3A) showed a firm, smooth, flat and highly ordered surface. Treatment with Accellerase 1500 (Figure 4-3B) mildly changed the surface of the rapeseed meal rendering it slightly rougher compared to the non-treated sample. Thermal pre-treatment (126 °C, 30 min) resulted in a highly dispersed structure with a rough surface (Figure 4-3C), indicating that the open and rough surface of the rapeseed meal could be more susceptible to enzymatic hydrolysis. The combination of heat and enzymatic treatment lead into the highly porous surface of the rapeseed meal (Figure 4-3D), indicating a greater degree of cellulosic disruption under these treatment conditions.

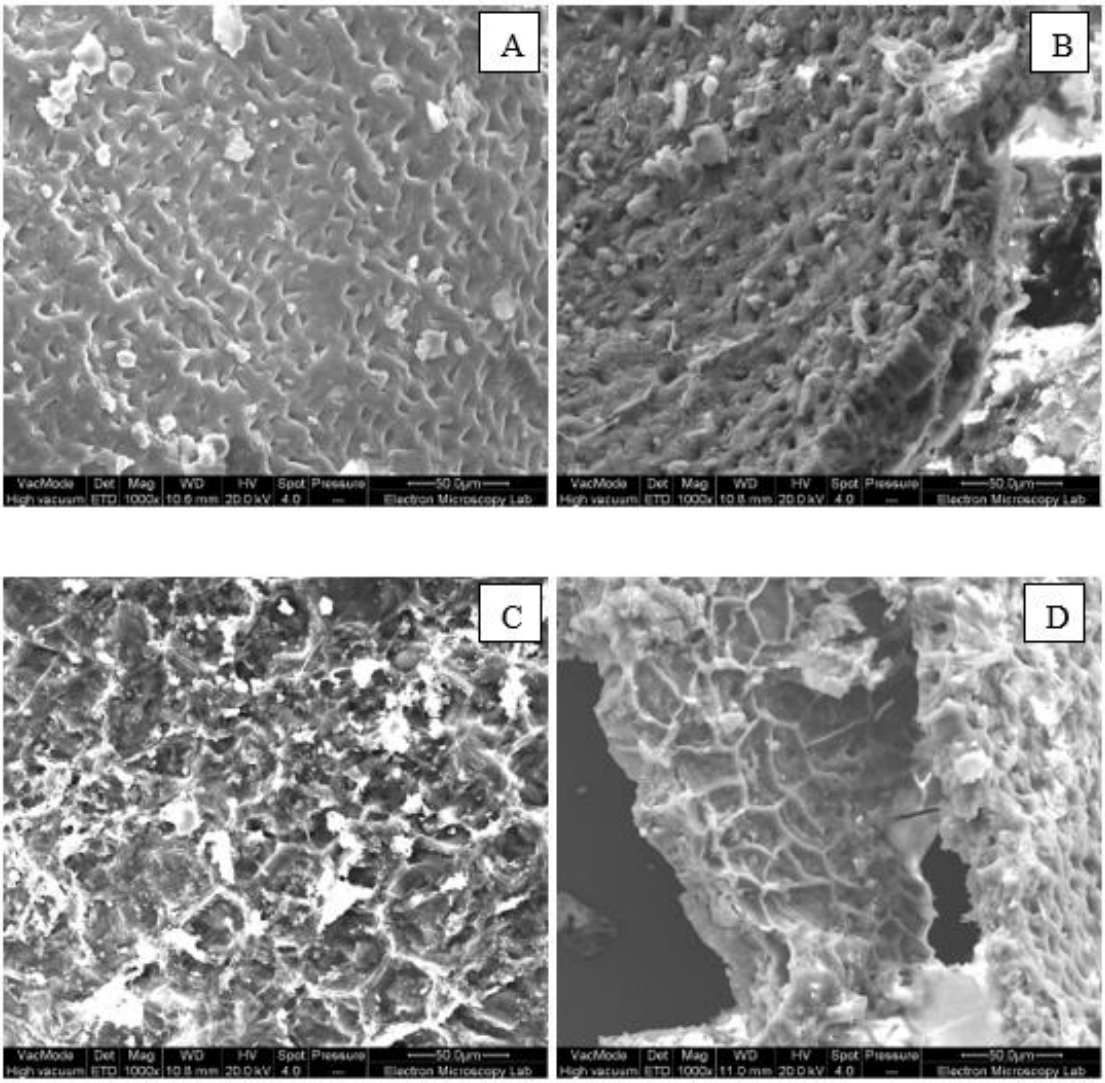

Figure 4-3: Scanning electron microscopy images of rapeseed meal: A) untreated; B) after treatment with 10% Accellerase 1500 for 24 hrs; C) after thermal pre-treatment (126 °C; 30 mins); and D) after thermal pre-treatment (126°C; 30 mins) followed by enzymatic hydrolysis with 10 % (v/v) Accellerase 1500.

The effect of the solid substrate on the enzymatic hydrolysis of rapeseed meal is depicted in Figure 4-4. Overall, an increase in the substrate concentration resulted in lower sugar yield for both the control and the pretreated samples. More specifically, as the rapeseed meal concentration increased from 10 to 40 % (w/v) the glucose hydrolysis yield decreased gradually, reaching a value of 24 % (w/w) for the 40 % w/v sample. In the case of the 50 % (w/v) rapeseed meal concentration, it was noted that the liquid of the reaction mixture was absorbed and retained by the solids, resulting in low water activity. Consequently, inadequate enzymatic hydrolysis with 18 % (w/w) of glucose yield was obtained for pretreated samples. These findings are in accordance with the study by López-Linares et al. (2014) who reported that increasing the solid/liquid ratio (from 20 to 30 %) resulted in decreased of enzymatic efficiency (by ~10%) in rapeseed straw, which could be attributed to enzyme inhibition by glucose released (product inhibition). Furthermore, increased substrate concentration might lead to insufficient mixing and thus heat and mass transfer limitations, particularly with the use of greater than 20 % (w/v) of substrate (Kristensen et al., 2009). It is important to note that even though increasing the substrate concentration resulted in lower sugars recoveries, high substrate loadings (e.g. > 30 % w/v) could be considered as an option to increase the sugar concentrations in the produced hydrolysates (Wanderley, Martín, Rocha, & Gouveia, 2013).

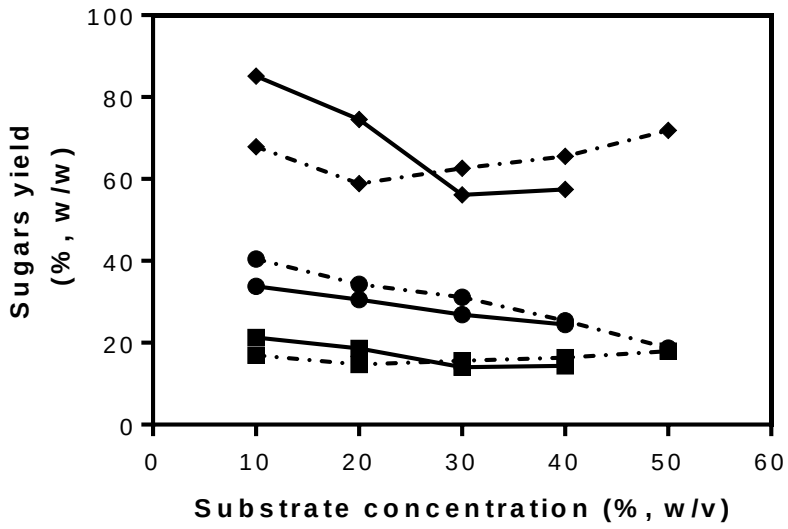

Figure 4-4: Effect of substrate concentration on the enzymatic hydrolysis of pretreated rapeseed meal using 10 % (w/v) of Accellerase 1500. Conditions of pre-treatment: Temperature (126 °C), pressure (~2 bar) for 30 mins. Conditions of Accellerase hydrolysis: Temperature 50°C for 24 hrs. Symbols: Glucose (●, g/l), xylose (■, g/l), galactose (♦, g/l). The same symbols on the dotted line represent the same sugars following pre-treatment at 121°C for 30 mins.

4.3.3 Microbial Production of Astaxanthin in Rapeseed Meal Hydrolysates

A key objective of this study was to investigate the suitability of rapeseed meal hydrolysates as potential fermentation media for the microbial production of astaxanthin. Therefore, rapeseed meal hydrolysates produced by the action of the four commercial enzymes were initially tested in flask cultures for their capability to support *X. dendrorhous* growth and astaxanthin production.

As shown in Table 4-4, significantly different cell biomass and astaxanthin production values were obtained depending on the hydrolysate. In terms of cell and astaxanthin concentrations, the best substrate was the pectinase hydrolysate (~26 g/l of cell biomass, 6.7 g/l of astaxanthin) and the worst was the Viscozyme hydrolysate (~3 g/l of cell biomass, 0.4 g/l of

astaxanthin). In terms of astaxanthin yield on biomass ($Y_{p/x}$), the highest value (332 µg/g) was obtained when Accellerase 1500 hydrolysate was used. In the pectinase hydrolysate, the $Y_{p/x}$ value was 258 µg/g, whereas, lower values were obtained for the Viscozyme hydrolysate (156 µg/g) and the cellulase hydrolysate (71 µg/g). The low astaxanthin concentrations obtained in the Viscozyme and cellulase hydrolysates were most likely associated with the relatively high hexose concentrations (> 50 g/l). Such high sugar concentrations can initiate the Crabtree effect in *X. dendrorhous* which changes the yeast metabolism from respiration to alcoholic fermentation, leading to ethanol production, which inhibits cell growth and astaxanthin accumulation in the cells (Liu, 2006; Marova, Certik, & Breierova, 2011; Yamane et al., 1997). Indeed, in the case of the Cellulase hydrolysate the highest amount of ethanol was produced (6.9 g/l) and the second highest in the case of Viscozyme (3.8 g/l). In the former, the high glucose concentration of the hydrolysate (45 g/l) benefited cell biomass production (14 g/l) but compromised the production of astaxanthin during secondary metabolism (1.1 mg/l); a similar trend was observed in the latter.

However, in the case of the pectinase hydrolysate, no ethanol was detected, indirectly indicated that the Crabtree effect was suppressed as glycerol was the predominant carbon source in the medium rather than hexose sugars. In the case of Accellerase 1500 hydrolysis, two types of rapeseed meal samples were used (untreated samples and thermal pretreated samples). Between these two samples, different fermentation results were obtained with pretreated samples resulting in higher biomass (12 g/l) and astaxanthin production (3.2 mg/l). It is interesting to note that the maximum ethanol detected in this fermentation was in very small amounts (< 1 g/l).

Table 4-4: Growth of *X. dendrorhous* and astaxanthin production in different rapeseed meal hydrolysates at 20 °C

Rapeseed meal hydrolysates	Time (h)	Biomass (g/l)	*P* (mg/l)	Yp/x (µg/g)	$EtOH_{max}$ (g/l)
Viscozyme L	115	3.07 ± 0.07	0.4 ± 0.03	156 ± 11.75	3.8 ± 0.13
Cellulase	115	14.63 ± 0.64	1.10 ± 0.18	71 ± 11.4	6.92 ± 0.00
Pectinase	120	25.83 ± 1.52	6.71 ± 0.44	258 ± 1.83	-
Accellerase 1500	118	8.2 ± 0.01	2.69 ± 0.05	332 ± 12	2.02 ± 0.15
Thermal treated (126 °C, 30 min) plus Accellerase 1500	140	11.95 ± 0.35	3.2 ± 0.4	271 ± 26.4	0.5 ± 0.01

Yp/x: Yield of astaxanthin on biomass produced.
$EtOH_{max}$: Maximum ethanol concentration produced during the fermentation
P: Astaxanthin production (mg/l)

Figure 4-5 depicts the kinetic profile of *X. dendrorhous* in the Accellerase 1500 hydrolysate of the thermally pretreated rapeseed meal. The total initial sugar concentration of the hydrolysate accounted for 16 g/l (in the form of glucose, galactose and xylose). The adaptation period for the yeast in this hydrolysate was rather long (~ 68 hrs) before the cells started to consume the available sugars. This probably could be attributed to the presence of growth inhibitors that most likely had accumulated in the hydrolysate during the thermal pre-treatment step, such as HMF and furfural, as shown previously for lignocellulosic materials (Sun and Cheng, 2002). During the exponential phase (68 hrs - 92 hrs), the cells consumed all sugars simultaneously and in parallel to cell growth also produced astaxanthin, which continued to be produced during the stationary phase (93 hrs - 140 hrs). During the exponential phase, a small amount of ethanol was produced (~2 g/l), which was later consumed during the stationary

phase after all the available sugars were exhausted. Whilst high ethanol concentrations are undesirable during the initial fermentation stages as it inhibits the yeast growth, low ethanol concentrations have been reported to stimulate astaxanthin pigmentation in yeast (Gu et al., 1997; Marcoleta et al., 2011).

Marcoleta et al. (2011) suggested the involvement of two possible mechanisms in the induction of pigmentation by ethanol. The first proposed mechanism involves the enzyme aldehyde oxidase that generates superoxide radicals, which are responsible for the induction of carotenoid synthesis. The second mechanism is the conversion of ethanol by alcohol dehydrogenase to acetate; in the presence of energy (ATP), acetate can then be converted to acetyl CoA, which is the major precursor for both the tricarboxylic acid (TCA) cycle and the mevalonate pathway, the latter being responsible for carotenoid production (Marcoleta et al., 2011; Thomson et al., 2005). In addition, ethanol might induce the production of pigments by inducing the expression of phytoene β-carotene synthase (crtYB) and astaxanthin synthase (crtS) genes, which are responsible for the production of carotenoids in the mevalonate pathway (Marcoleta et al., 2011). The observed decrease in the ethanol concentration indicated the presence of alcohol dehydrogenase in this particular *X. dendrorhous* strain and the generated acetyl-CoA was used for astaxanthin synthesis (hence the increase in astaxanthin concentration during the stationary phase) rather than for the production of biomass through the TCA cycle.

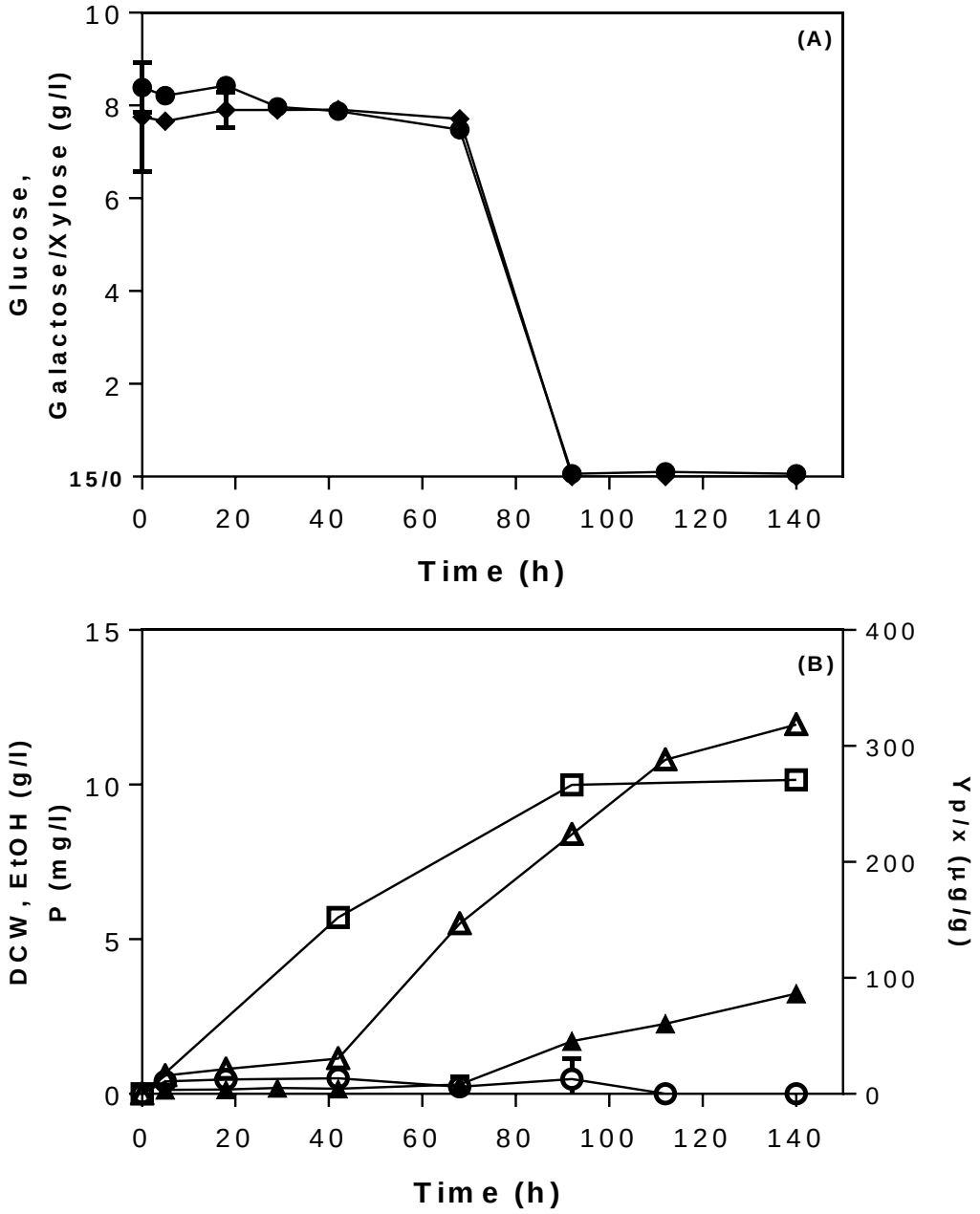

Figure 4-5 : Kinetic profile of (A) carbon sources consumption and (B) product formation during *X. dendrorhous* growth in pectinase rapeseed meal hydrolysate. Symbols represent: (●, g/l), galactose/xylose concentration (◆, g/l), dry cell weight (DCW) (Δ, g/l), ethanol concentration (EtOH) (○, g/l), astaxanthin concentration, *P* (▲, mg/l) and astaxanthin yield on biomass, $Y_{p/x}$ (□, µg/g of); Enzymatic hydrolysis conditions: Thermal pre-treatment of rapeseed meal (10 % w/v) at 126 ºC for 30 mins, followed by Accellerase 1500 treatment (10% v, v) for 24 hrs at 50°C. Fermentation conditions: Temperature, 20°C; agitation, 250rpm.

Figure 4-6 depicts the kinetic profile of *X. dendrorhous* cultivated in the pectinase hydrolysate. The hydrolysate consisted of ~15.7 g/l of sugars (glucose, galactose, xylose and arabinose) as well as 40 g/l of glycerol, which was originally present in the enzyme formulation. All sugars were utilised by the cells, with glucose being the most preferred one (hence utilised first), followed by the hemicellulose derived sugars. Once all sugars were exhausted, glycerol was utilised as an energy and carbon source. In contrast to the Accellerase 1500 hydrolysate, the cells did not experience a lag phase in the pectinase hydrolysate (as the rapeseed meal was not thermally treated in this a case). The cells continued to grow for the whole of the fermentation period (1 hr - 120 hrs) and showed a growth pattern similar to diauxic growth, reflecting the sequential consumption of sugars and glycerol. The cell biomass achieved in the case of the pectinase hydrolysate was the highest amongst all hydrolysates (~ 26 g/l). This was due to the relatively low sugar concentration and the fact that glycerol, which was present in high amounts does not initiate a Crabtree effect. Astaxanthin was produced throughout the fermentation, including during the glycerol consumption phase. The astaxanthin concentration at the end of the fermentation was 6.7 mg/l, significantly higher than in the case of the two Accellerase 1500 hydrolysates (2.7 and 3.2 mg/l) although the astaxanthin yield was slightly lower (Figure 4-6). The reason for this could be the fact that in the case of the Accellerase 1500 hydrolysates, small amounts of ethanol were produced which (as discussed above) can potentially stimulate astaxanthin production, whereas in the pectinase hydrolysate fermentation ethanol was not detected. Another reasoning for the lower astaxanthin yield could be the absence of certain minerals, which are important for carotenoid production in the pectinase hydrolysate (Sanpietro, 1998). More specifically, several metals including zinc, iron, copper and magnesium have been reported to act as carotenoid inducers in certain yeast species such as *Rhodotorula glutinis* and *Rhodotorula graminis* (Mata-Gómez et al., 2014). Moreover, Wang et al. (2000) suggested that high levels of antinutritional components such as glucosinolates and phenols, which are present in rapeseed meal might also inhibit carotenoid synthesis.

To our knowledge, no analogous works are currently available in the literature investigating the use of rapeseed meal for the growth of *X. dendrorhous*. However, previous studies have reported that *X. dendrorhous* was able to grow well on enzymatic hydrolysates of wood supplemented with glucose and produce under optimised conditions approximately 1.8 mg of total carotenoids/l (Parajó et al., 1997). In this study, the rapeseed meal hydrolysates were able to support higher astaxanthin production concentration and yields, indicating that this medium had the sufficient nutrients present for optimal cell growth. In a different study, 5% molasses supplemented with urea (30 g/l) and sodium phosphate (4.5 g/l) were used to cultivate *X. dendrorhous* GM807 in shake flasks. Results showed that the use of molasses demonstrated positive effects on yeast growth (3.5 g/l), but not on the astaxanthin formation (105 µg/g) (Jirasripongpun et al., 2007). In a study by Ramírez et al., (2006) it was reported that a mutant strain of *Xanthophyllomyces dendrorhous* was cultivated in a Yucca plant-based medium supplemented with date juice in a fed-batch fermentation system and resulted to very high cell biomass (39 g/l), astaxanthin production (24 mg/l) and astaxanthin yield (600 µg/g). This indicates the potential to genetically engineer *X. dendrorhous* strains in order to achieve high conversion yields, which will render the potential commercialisation of the microbial production of astaxanthin from low value agri-food materials such as rapeseed meal more economically viable.

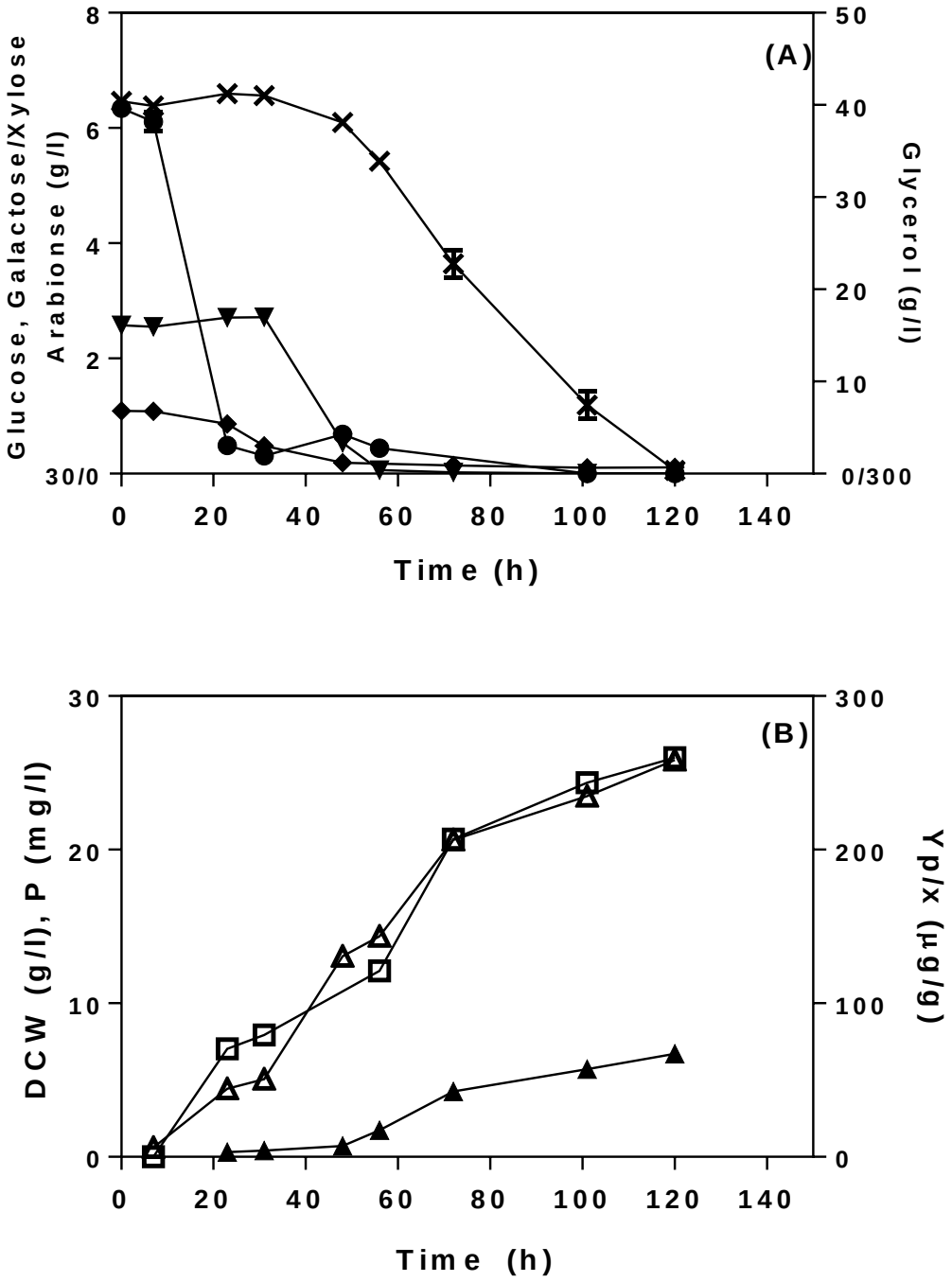

Figure 4-6 : Kinetic profile of (A) carbon sources consumption and (B) product formation during *X. dendrorhous* growth in pectinase rapeseed meal hydrolysate. Symbols represent: Glucose (●, g/l), arabinose (▼, g/l), yield of astaxanthin on biomass Yp/x (□, µg/g), galactose/xylose (♦, g/l), astaxanthin production *P* (▲, mg/l), dry cell weight (Δ, g/l), glycerol (×, g/l). *Galactose and xylose are indicated as one in the graph due to similar retention time when analysed using HPLC (Aminex HPX-87H column).

4.4 Conclusions

The rapeseed meal by-product generated from the rapeseed oil processing industry has the potential to be used for the microbial production of astaxanthin. Thermal treatment of the meal followed by treatment with specific enzymes can result in a nutrient rich medium that is able to support high growth of *X. dendrorhous* and astaxanthin production. Controlling the sugar levels in the rapeseed meal hydrolysate through an effective selection of the primary processing steps is critical to ensure the prevention of the Crabtree effect. This approach offers a sustainable alternative to chemically synthesise astaxanthin and can stimulate the circular bioeconomy through the utilisation of low value abundant resources.

5 Bioconversion of Rapeseed Meal into Astaxanthin by *Xanthophyllomyces dendrorhous* DSMZ 5626 in a 2-Litre Stirred Tank Bioreactor

Abstract

Rapeseed meal, a by-product of oil seed processing industry, was evaluated as an alternative, low-cost substrate for microbial astaxanthin production in yeast *Xanthophyllomyces dendrorhous* DSMZ 5626. This study is divided into two parts; the first part aimed to investigate the effect of processing fermentation parameters (pH and agitation) using semi-defined media. These defined the basis for the second part that involved the use of enzymatically produced rapeseed meal hydrolysates as fermentation media. In semi defined media, the effect of controlling pH and agitation were evident as significant reduction in both biomass and astaxanthin production was observed when the culture pH was not controlled and low agitation speed was applied. By combining pH control at 6 and agitation speed at 250 rpm, the yeast produced 8 g/l of biomass and 2 mg/l of astaxanthin. When agitation speed was increased to 600 rpm, significant increase in biomass (16.4 g/l) and astaxanthin production (3.6 mg/l) was obtained due to efficient mixing that ensured better oxygen supply as well as maintained satisfactory levels of heat and mass transfer inside the reactor during fermentation. In the second part of study, rapeseed meal hydrolysates were tested as fermentation media for microbial astaxanthin production using separate hydrolysis and fermentation (SHF) approach in batch and fed-batch fermentation modes. Batch fermentation with pectinase derived rapeseed meal hydrolysates supported both biomass (42 g/l) and astaxanthin production (11 mg/l) as the presence of glycerol from the enzyme formulation acted as additional carbon source for yeast growth. In Accellerase 1500 derived hydrolysates, 14 g/l of biomass were produced with 3.6

mg/l of astaxanthin. However, the astaxanthin yield on biomass ($Y_{p/x}$) did not differ significantly (242-251 µg/g) between these two hydrolysates. In fed-batch fermentations using Accellerase 1500 derived hydrolysates supplemented with 13 g/l of glycerol, astaxanthin production was lower (3.2 mg/l), indicating that glycerol addition to the fermentation resulted in adverse effects on astaxanthin metabolism. Overall, this study demonstrated that rapeseed meal has a potential as fermentation medium in supporting yeast growth and astaxanthin production in *X. dendrhorhous* DSMZ 5626.

Keywords: rapeseed meal hydrolysate, SSF, batch, fed-batch, bioreactor, astaxanthin, *X. dendrorhous*, yeast

5.1 Introduction

Carotenoids are pigments that are responsible for orange, yellow, red and purple colours in a wide variety of plants, animals and microorganisms. β-Carotene, lycopene, astaxanthin and lutein are among the most prominent carotenoids reported. The growing demands for carotenoids in various industries such as aquaculture, feed, pharmaceutical, cosmetics, food and pet food industries has gained interest among researchers to increase the natural production of these compounds.

Among carotenoids, astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione; $C_{40}H_{52}O_4$) belongs to xanthophylls group and has high demand in the market for its colouring and antioxidant properties as well as its health benefits (Rodríguez-Sáiz et al., 2010). It is an orange-pinkish pigment that can be found in a wide range of animals including fish (salmon, trout), shrimp and bird's feather (flamingo) (Guerin et al., 2003). The demand of astaxanthin is high as animals do not have the ability to synthesize astaxanthin and as such, astaxanthin is added into their feed. The market values for astaxanthin were $369 million in 2014 and are forecasted to reach $423 million in 2019 (BBC Research, 2015). Currently, the demand of astaxanthin is fulfilled by chemical synthesis through petroleum based reactions. Since the demand for astaxanthin is high, the research interest on alternative pathways of natural astaxanthin production has increased. One such way represents the biotechnological route, through the utilisation of algae, bacteria and yeast.

Apart from microalgae, (*Haematococcus pluvialis*), the yeast *Xanthophyllomyces dendrorhous* (previously known as *Phaffia rhodozyma*) is one of the most promising microorganisms for the commercial production of natural astaxanthin. This is due to the fact that the growth rate in yeasts is shorter than in microalgae and that yeasts have the ability to grow on various carbon sources including glucose, cellobiose, xylose and glycerol. Attempts

on using low-cost substrates such as agricultural by-products for microbial astaxanthin production have been previously made (Jirasripongpun et al., 2007; Parajó, Santos, & Vázquez, 1998; Vázquez et al., 1998). To achieve this, the agriculture residues need first to be hydrolysed by chemical and/or enzymatic treatment into fermentable sugars. Several studies have utilised various agricultural by-products as fermentation substrates including pine wood (Parajó et al., 1997), *Eucalyptus* wood hydrolysates (Vázquez et al., 1998), molasses (Jirasripongpun et al., 2007), by-products of wet corn milling (Hayman, Mannarelli, & Leathers, 1995) and mussel waste water processing (Amado & Vázquez, 2015) as substrates for carotenoid production.

In the standard approach of lignocellulosic bioconversion strategy, separate saccharification and fermentation (SHF) process is performed. In this approach, lignocellulosic hydrolysis is carried out separately and the obtained hydrolysates are then subjected to microbial fermentation (Mosier et al., 2004; Öhgren et al., 2007; Taherzadeh & Karimi, 2007). Simultaneous saccharification and fermentation (SSF) is another method that can be applied for bioconversion of lignocellulosic materials into value added products. In this process, sugars are produced during hydrolysis step and directly consumed by the microorganism present in the reactor. This technique provides the possibility to overcome the main disadvantages of enzyme hydrolysis, including the reduction of the potential of product inhibition (glucose and cellobiose) on cellulase activity by maintaining a low concentration of the end-products of enzymatic reaction (Taherzadeh and Karimi 2007). Furthermore, this method could reduce the risk of contaminations compared to separate hydrolysis and fermentation (SHF). This approach has been reported to enhance bioethanol production in yeast, *Saccharomyces cerevisiae* by utilising lignocellulosic biomass from paper manufacturing industry (Kádár, Szengyel, and Réczey 2004), corn stover (Öhgren et al. 2007) and sugarcane bagasse (Zhao, Song, and Liu 2011).

Various cultivation modes including batch, fed-batch and continuous have been investigated for carotenoid production in yeasts, either in lab or pilot scale (Meyer & Du Preez, 1994; Montanti et al., 2011; Valduga et al., 2014). In the case of some yeast species cultivated on batch mode, high initial carbon concentrations (usually glucose) result in suppression of yeast growth as well as product formation due to Crabtree effect (Liu & Wu, 2008; Yamane, Higashida, Nakashimada, Kakizono, & Nishio, 1997). It has been demonstrated that *X. dendrorhous* undergoes Crabtree effect, a phenomenon where cells metabolically switch to fermentative metabolism leading to ethanol production even under ample oxygen supply when initial glucose present is above a given threshold (strain dependent) (Reynders et al., 1997; Yamane et al., 1997). Fed-batch cultivation mode is considered as an appropriate strategy to overcome such issues, as it allows the addition of one or more nutrients to the reactor during fermentation in order to maintain the concentration of the substrate below its inhibitory levels (Yen, Liu, & Chang, 2015).

This study aimed to investigate the production of astaxanthin in *Xanthophyllomyces dendrorhous*, using enzymatically hydrolysed rapeseed meal as fermentation feedstock. Initially, the experiments were conducted using semi-defined media to optimise the fermentation parameters. The optimised conditions were then applied in lab-scale bioreactor cultures using rapeseed meal hydrolysates under batch and fed-batch mode. Besides that, the feasibility of using pre-hydrolysis and simultaneous saccharification and fermentation (pSSF) was also investigated. Thus, the main contribution of this chapter is to provide detailed information on the suitability of low-cost substrates as in the case of rapeseed meal as sole fermentation feedstock for astaxanthin production in *X. dendrorhous*.

5.2 Materials and methods

5.2.1 Microorganism

The yeast strain *Xanthophyllomyces dendrorhous* DSMZ 5626 was used in this study and was purchased from Leibniz Institute DSMZ. The strain was proliferated and maintained in Yeast and Mold (YM) media containing (in g/l): yeast extract (3.0); malt extract (3.0); peptone from soybean (5.0); glucose (10.0) and agar (15). Stock yeast cultures were stored at -80 °C until further use.

For the preparation of the inoculum, a loopful of stock yeast culture was inoculated on sterilised commercial YM agar (Sigma Aldrich, UK) and incubated at 20 °C for 5 days. After that, a single colony of yeast *X. dendrorhous* was transferred in 30 ml of YM broth media (similar composition as above) for cell proliferation and was incubated for 3 days prior to inoculation in semi defined media. Finally, a suspension of *X. dendrorhous* was transferred into 50 ml of semi defined media to a final optical density (OD) measurement of 0.1. The optical density measurement was conducted using a Biomate 3 UV/VIS Spectrophotomer (Thermo Spectronic, NY). The composition of semi defined media was as follows (in g/l): carbon source (30), yeast extract (2.0), malt extract (2.0), KH_2PO_4 (7.0), $(NH_4)_2SO_4$ (1.0), $MgSO_4.7H_2O$ (1.5), $FeCl_3.6H_20$ (0.15), $ZnSO_4.7H_2O$ (0.02), $MnSO_4.H2O$ (0.06), $CaCl_2.2H_2O$ (0.15).

5.2.2 Raw materials

The rapeseed meal that was used in this study was kindly provided by Stainswick Farm (Oxfordshire, United Kingdom) and was generated via cold pressing oil extraction technique. The fresh rapeseed meal (Chapter 4) had the following composition (w/w): oil, 16.7%; protein, 25.3%; lignin, 16.1%; ash, 5.3% and carbohydrate, 34.2%. Rapeseed meal samples were

grinded using dry-grinder and sieved to obtain uniform size of particles (< 850 μm). After that, oil was removed using a supercritical carbon dioxide (CO_2) extractor rig (SciMed, UK) at 70 °C and pressure at 350 bar for 1 h, with ethanol (10%, v/v) as the co-solvent. The residual defatted meal was kept at 4 °C prior to use.

5.2.3 Production of Rapeseed Meal Hydrolysates

In this work, two commercial cellulase mixtures were used for enzymatic hydrolysis of rapeseed meal; Accellerase 1500 (kindly provided by DuPont, Leiden, Netherlands) and pectinase (Novozymes). Both enzymes contained a mixture of cellulolytic activities that were given in detail in Chapter 4.

150 g of defatted rapeseed meal (10 % w/v) was heat pretreated in a 2-litre glass bottle containing 1.35 litre of distilled water in an autoclave at 126 °C for 30 min. After cooling down the rapeseed meal, 150 ml of enzyme (10% v/v) were added to the vessel. Enzymatic hydrolysis was carried out in an orbital shaker (GFL 3015, SciQuip, UK) at 250 rpm at 50 °C for 24 h. Heat inactivation was applied at 95 °C for 10 min in a water bath (GD 120, Grant, Cambridge). The produced hydrolysates were then filter-sterilised using 0.25 μm Millipore stericup unit (EMD Millipore Stericup^{TM}) and were kept at 4 °C prior to use.

5.2.4 Bioreactor setup

In this study, a 2-litre stirred tank bioreactor (BIOSTAT B, Sartorious AG, German) was used with a working volume of 1.5 litres. This unit consisted of a double jacketed 2-litre vessel with an integrated digital control unit (DCU) that controlled the speed, air flow, temperature and pH of the fermentation. The vessel was equipped with baffles, 3 units of 6-bladed impellers as well as multiple ports for sensors for fermentation control. The temperature was controlled

by circulating warm/cold water through the double jacket vessel whereby air was supplied via compressed air. The pH of the fermentation was controlled by Easy Ferm pH probe (Hamilton, Switzerland) and the pH corrective solutions used were 5 M sodium hydroxide and 5 M hydrochloric acid. Antifoam 204 (10% v/v) (Sigma Aldrich) was used to prevent foaming during fermentation. Dissolved oxygen (DO) was controlled by a DO-probe (OxyFerm, Hamilton, Switzerland). Prior to each fermentation experiment, the bioreactor was sterilised at 121 °C for 20 min, unless stated otherwise. In all fermentation studies, samples were taken at regular time intervals for 5 days and analysed as described in section 5.2.7.

5.2.5 Fermentation with semi defined media

The *X. dendrorhous* cultivation in semi defined media was performed in a 2-litre bioreactor with a working volume of 1.5 litres as described in section 5.2.4. The composition of semi defined media was as described in section 5.2.1. Initially, 1.5 litres of synthetic media were prepared, added in the reactor and autoclaved at 121 °C for 20 min prior to yeast inoculation. Batch fermentation was carried out at different process parameters to investigate the best conditions for microbial astaxanthin production in *X. dendrorhous* including: (i) pH (pH 6 and uncontrolled pH) and (ii) agitation speed (250, 400 and 600 rpm). Other processing parameters were maintained as follows: temperature, 20 °C; aeration, 1 l/min.

Fed-batch cultivation approach was also conducted using semi defined media as described in section 5.2.1. Two different refined carbon sources, glucose and glycerol, were used as feeding solutions. 10 g/l of feed solution were added to the bioreactor once the values of dissolved oxygen increased during fermentation (DO-stat approach). Fermentation conditions were controlled as follows: temperature, 20 °C; pH 6; agitation speed, 600 rpm; aeration, 1 l/min.

5.2.6 Fermentation with Rapeseed Meal Hydrolysates

5.2.6.1 Separate hydrolysis and fermentation (SHF)

Filter sterilised rapeseed meal hydrolysates (1.5 litres) were prepared as described in section 5.2.3 and aseptically transferred to a 2-litre bioreactor, previously autoclaved at 121°C for 20 min. In fed-batch fermentations, experiments were initiated in similar way as in batch ones. 10 g/l of glycerol solution was manually and aseptically added to the bioreactor after dissolved oxygen values showed an increasing trend during fermentation (DO-stat approach). Fermentation conditions were as follows; temperature, 20 °C; pH 6; agitation speed, 600 rpm; aeration, 1 l/min.

5.2.6.2 Pre-hydrolysis and fermentation (pSSF)

In pre-hydrolysis and fermentation (pSSF) approach, the enzymatic hydrolysis of rapeseed meal and the subsequent fermentation were performed in one reactor. Two different enzymes were separately tested in this study (pectinase and Accellerase 1500). Firstly, 150 g/l of rapeseed meal (10% w/v) were added to the reactor with 1.35 litre of distilled water. The bioreactor unit was then subjected to autoclaving at 126 °C for 30 min. After cooling, 150 ml of enzyme were added to the fermentation vessel and the hydrolysis step was initiated at 50 °C for 24 h. Agitation was controlled at 250 rpm during this period. Once the hydrolysis step was completed, the vessel was cooled down to 20 °C followed by inoculation of 5% (v/v) of *X. dendrorhous* suspension. Fermentation conditions were as follows; temperature, 20 °C; pH 6; agitation speed, 600 rpm; aeration, 1 l/min.

5.2.7 Analytical methods

In the case of batch bioreactor cultures using semi-defined media and SHF fermentations, samples of ~2 ml were periodically withdrawn from the bioreactor. 1 ml of sample was added into a pre-dried tube (24 h in drying oven at 100°C) and centrifuged at 10845 *x* g for 10 min. The supernatant was collected for sugar and ethanol analysis. The pellet was washed twice using distilled water and was frozen at -20°C prior to freeze drying for 2 days (Virtis, UK). The dry weight of yeast biomass was calculated as the difference between the weight of the tubes before and after freeze drying process.

As for SSF technique, cell growth was observed by total plate count method. Hence, 100 µl of serially diluted samples were plated on Yeast and Mold agar plates (YM agar) and incubated at 20°C for 5 days. The calculation for colony forming unit (CFU) were calculated as in Eq 5-1:

$$CU\ ml^{-1} = \frac{No\ of\ colonies\ \times Dilution\ Factor}{Volume\ of\ sample\ (ml)} \qquad \text{Eq 5-1}$$

Sugars (glucose, xylose, galactose and arabinose), glycerol and ethanol were analysed by high performance liquid chromatography (HPLC) using an Agilent Infinity 1260 system (Agilent Technologies, USA) with Aminex HPX-87H column (Bio-rad, CA) column coupled to a differential refractometer and a DAD detector. Operating conditions were as follows: sample volume: 20µl; mobile phase: 0.5 mM H_2SO_4; flow rate: 0.6 ml/min; column temperature: 65 °C. The quantification of each chromatogram peak was achieved on the basis of external standard curves, using standard solutions of known concentrations.

Astaxanthin extraction was carried out according to Sedmak et al (1990). Briefly, 1 ml of dimethyl sulfoxide (DMSO) was preheated at 55 °C and added to the freeze-dried biomass, followed by vortexing for 30-40 sec. Subsequently, 0.2 ml of 20 % sodium chloride (NaCl) and 1.0 ml of acetone were added to the mixture to extract the intracellular carotenoids. The aqueous and organic phases were separated by centrifugation at 5423 x g for 5 min. The extraction process was repeated until colourless biomass was obtained. The organic phases were pooled together and their absorbance was measured at 480 nm in a spectrophotometer. DMSO solution was used as blank. Values were then divided by the extinction coefficient of 2150. The equation for total carotenoids concentration is given in equation below (Eq 5-2);

$$Crotenoids\ content\ (\mu g/g) = \frac{A\ \times V\ (mL) \times\ 10^4}{A_{1cm}^{1\%}\ \times W\ (g)} \qquad \text{Eq 5-2}$$

where, A is the absorbance at 480 nm, V is the volume, $A_{1cm}^{1\%}$ is the coefficient (2150) and W is the weight of biomass.

5.3 Results and discussion

5.3.1 Fermentations using semi-defined media

Cultures of *X. dendrorhous* were performed in a 2- litre bioreactor using semi defined media with glucose (30 g/l) as carbon source. The semi defined media were selected to investigate the yeast growth in a controlled environment with a view to optimise the process parameters (pH and agitation) of the bioreactor operation. The optimised parameters were then

applied in bioreactor fermentations using rapeseed meal hydrolysates as sole fermentation feedstock.

5.3.1.1 Batch fermentations - effect of pH

Figure 5-1 depicts the fermentation profile of *X. dendrorhous* DSMZ 5626 using semi defined media in 2-L stirred tank bioreactor. Around 8 g/l of dried biomass and 2 mg/l of astaxanthin were produced after 105 h under controlled pH. In the case of uncontrolled pH, the initial pH of the culture was 5.97, and was progressively decreased to 4.2 after 48 h of fermentation, at time point in which cell growth was suppressed, as demonstrated by poor biomass (1.5 g/l) and astaxanthin production (0.2 mg/l). At the end of fermentation, the pH of the culture had dropped to 3.8, indicating a highly acidic culture environment. Low pH environment is known to cause acid stress to yeasts that results in reduced membrane permeability, anion extrusion and alters expression of genes that are key for yeast growth (Lopes Brandão et al., 2014; Yalcin & Ozbas, 2008).

As observed in Figure 5-1A, glucose consumption was strongly influenced by the pH. In uncontrolled pH cultures, around 10 g/l of glucose were consumed by the yeast before cell growth was interrupted at 36 h due to acidic conditions (pH 4.2). This is in contrast with yeast growth in controlled pH cultures, where 30 g/l of glucose were fully consumed within 34 h of fermentation. Additionally, yeast metabolism by-products such as ethanol (2 g/l) and glycerol (1.2 g/l) were produced during fermentation. Hu et al., (2006) reported that the optimal pH for *X. dendrorhous* growth is at 6. When the pH largely deviates from its optimal range, it prevents the cells from maintaining their optimal intracellular pH, which in turn results in failure of key intracellular enzymatic functions (Narendranath & Power, 2005).

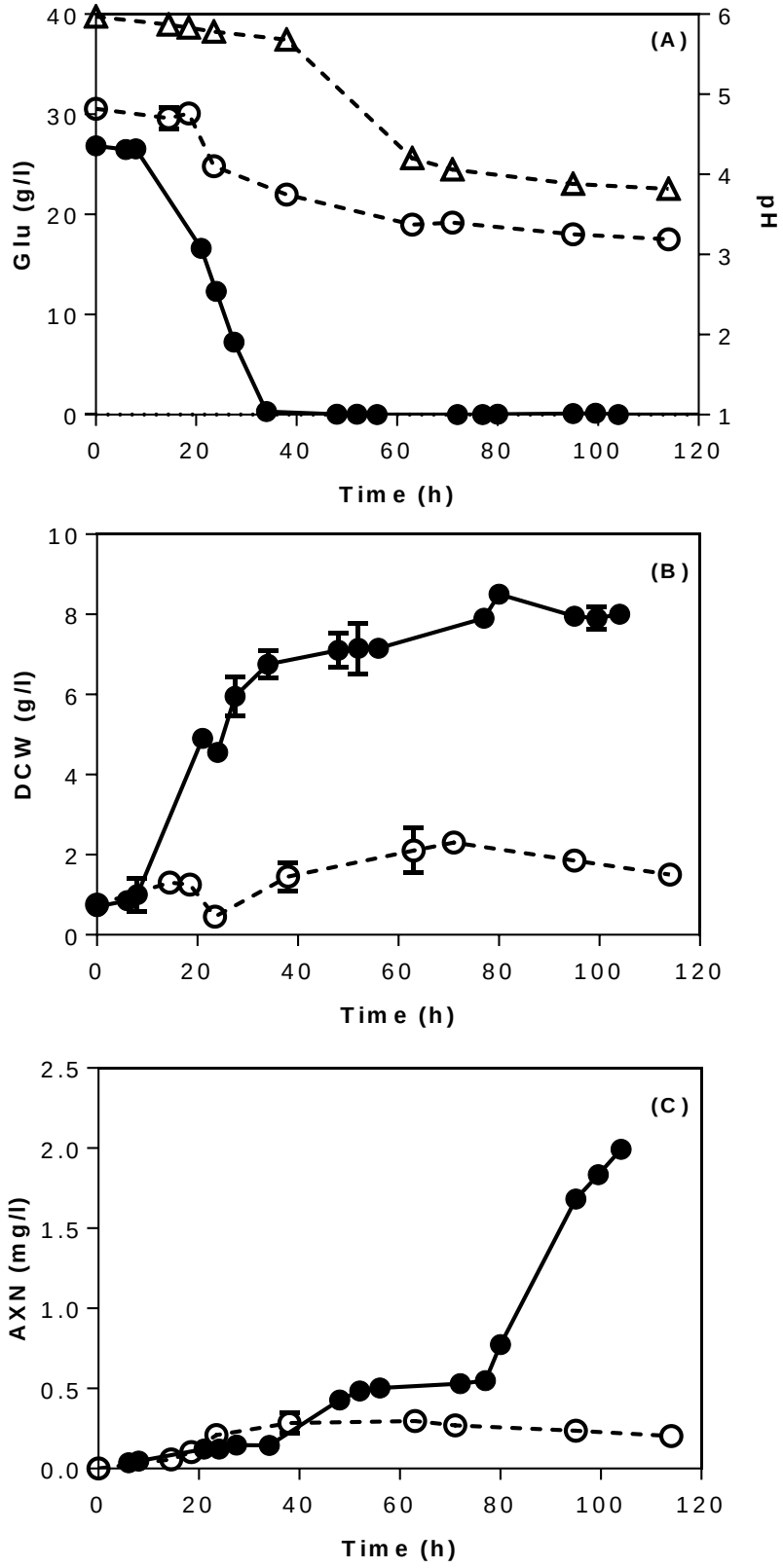

Figure 5-1: Growth profile of *X. dendrorhous* cultivated in 2-litre stirred tank reactor using semi defined media at different pH conditions. Symbols represent: (●)- controlled at pH 6, (○) - uncontrolled pH and (Δ)- pH profile for uncontrolled experiment. Fermentation conditions; aeration, 1 l/min; temperature, 20 °C; agitation, 250 rpm. Abbreviations: AXN - astaxanthin; Glu – glucose.

5.3.1.2 Batch fermentations - effect of agitation speed

The effects of three different agitation speeds (250, 400 and 600 rpm) on astaxanthin production were investigated under controlled pH (pH 6) and constant air flow rate (1 l/min). Figure 5-2 depicts the fermentation profile of *X. dendrorhous* in terms of yeast growth, astaxanthin production, dissolved oxygen, glucose consumption and ethanol production, in cultures with semi defined media and at different agitation speeds. It was clear that astaxanthin production was positively correlated with increasing agitation speed. Specifically, the highest agitation speed (600 rpm), supported both the highest astaxanthin yield (3.61 mg/l) and biomass production (16.35 g/l of dry weight) at 118 h of fermentation. In contrast at the lowest agitation speed (250 rpm), 2.4 mg/l of astaxanthin were produced with 8 g/l of biomass. As for yeast growth, *X. dendrorhous* entered into the stationary phase after 36 h of fermentation when 250 and 400 rpm were applied, due to oxygen limitations in the bioreactor (< 20 %). On the other hand, at high agitation (600 rpm), the cell growth had a longer exponential phase, up to 70 h of fermentation (Figure 5-2C).

Observing the dissolved oxygen values when agitation at 250 rpm was applied, DO readings fell at 0% after 20 h of fermentation and were maintained at low levels (<20%) until 72 h. After that, DO levels increased to >80%, coinciding with the stationary growth phase of *X. dendrorhous*. However, when the agitation rate was increased to 400 and 600 rpm, the DO levels remained at > 40% for most of the fermentation duration. High agitation rates ensured sufficient dissolved oxygen supply that is required for cells growth. In terms of metabolites production, maximum ethanol concentration ($EtOH_{max}$) was observed at 2.1 g/l after 31 h of fermentation when agitation at 250 rpm was applied. However, at agitation speeds of 400 and 600 rpm, minimal amount of ethanol was detected in the culture (< 1 g/l). This reduction in ethanol production indicated that Crabtree effect was reduced at higher agitation rates. It should

be noted that in all experiments, any produced ethanol was subsequently consumed by the yeast once glucose was depleted from the media. High agitation rates ensure better oxygen uptake rate by microorganisms as well as heat and mass transfers, enabling a satisfactory supply of nutrients and facilitating the removal of carbon dioxide from the culture medium (Valduga et al., 2011). It has been reported that medium to high agitation rates (300 - 900 rpm) increased yeast growth and astaxanthin production in *X. dendrorhous* (Luna-Flores, Ramírez-Cordova, Pelayo-Ortiz, Femat, & Herrera-López, 2010; Vázquez et al., 1998; Zheng et al., 2006). Sufficient supply of oxygen can enhance astaxanthin production by preventing NADH accumulation and supplying oxygen molecules to re-oxidize NADH into NAD+ as a starting material for astaxanthin biosynthesis (Yamane et al., 1997). Johnson & Shroeder (1996) have described the following stoichiometric equation for astaxanthin synthesis from glucose by *X. dendrorhous* (Eq 5-3).

$$12\ \text{Glucose} + 4O_2 + 14\text{NADPH} + 48\text{NAD}^+ = 1\ \text{Astaxanthin} + 32CO_2 + 14\text{NADP}^+ + 48\text{NADH} + 12H_2O + 34H^+ \quad \text{Eq 5-3}$$

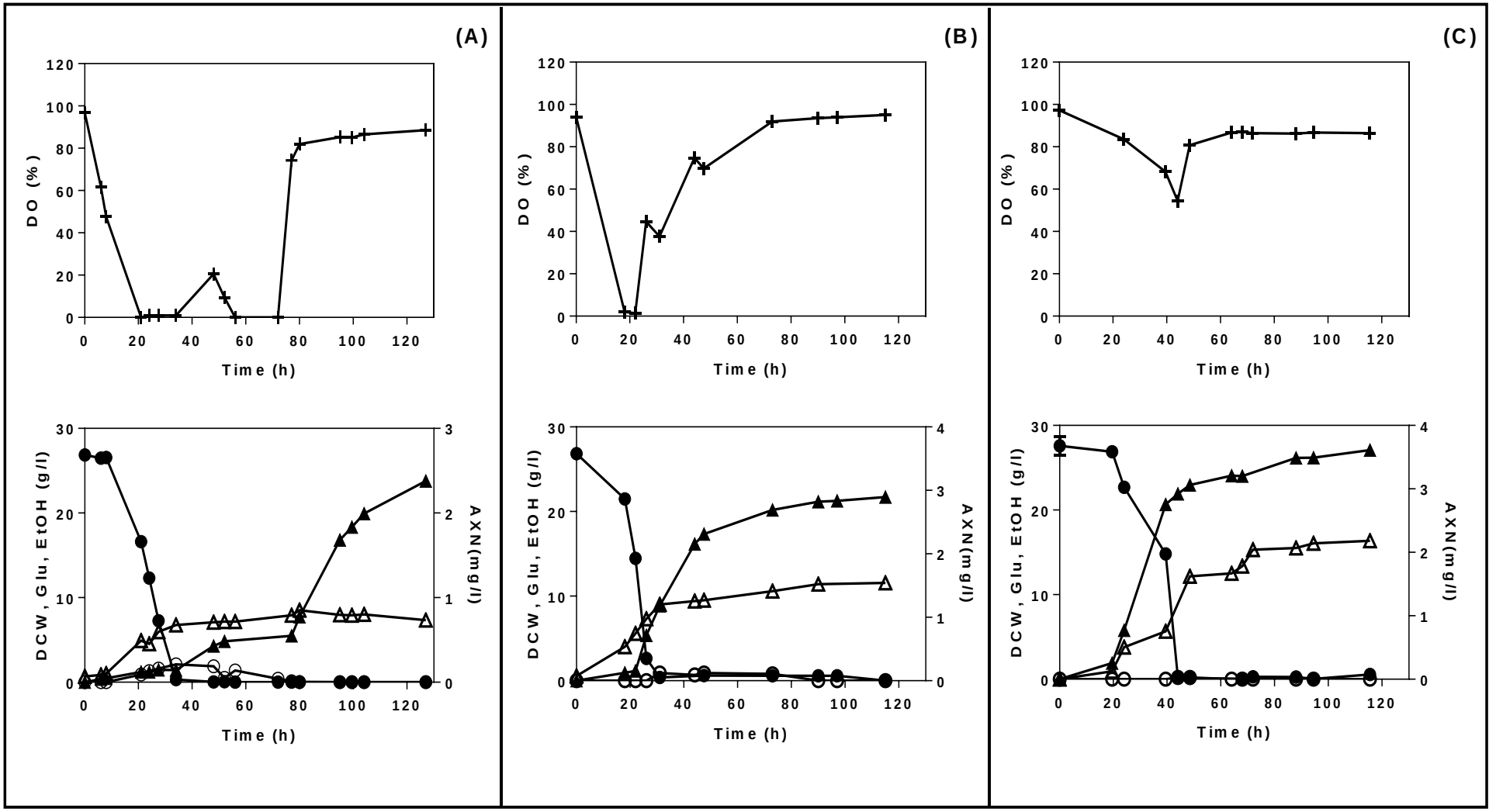

Figure 5-2: Effect of agitation speed on cell growth, astaxanthin production, ethanol production and glucose consumption in a 2-L bioreactor using semi defined media with 30 g/l of glucose as carbon source. (A) 250 rpm; (B) 400 rpm; (C) 600 rpm. Fermentation conditions: aeration, 1 l/min; temperature, 20 °C; pH 6. Symbols represent: ▲-astaxanthin, AXN (µg/ml); ●- glucose, Glu (g/l); Δ- dry cell weight, DCW (g/l); ○- ethanol, EtOH (g/l); + - dissolved oxygen, DO (%)

Based on Eq 5-3, the presence of oxygen is required to metabolise glucose into astaxanthin. Yamane et al. (1997) investigated the effect of oxygen supply on growth and astaxanthin production of *X. dendrorhous* by controlling the DO levels in the fermenter. They found out that increasing the DO (from 20% to 80%) benefited both biomass and astaxanthin production and could inhibit the occurrence of Pasteur and Crabtree effect which are related to high glucose concentrations. The Pasteur effect refers to the inhibition of glycolysis by respiration whereas Crabtree effect refers to the inhibition of respiration by glycolysis (Rodrigues, Ludovico, & Leão, 2006; Yamane et al., 1997).

5.3.1.3 Fed-batch fermentations

A key-objective of this study was to investigate the feasibility of fed-batch strategy on the improvement of biomass and astaxanthin yield in *X. dendrorhous*. To this end, dissolved oxygen control (DO-stat) was used as a substrate feeding indicator, due to the reverse dependence of carbon source with dissolved oxygen in the culture. Two types of feeding solutions were used, that of glucose (10 g/l) and glycerol (10 g/l). In batch fermentations, a sharp increase in dissolved oxygen levels was observed demonstrating a slow oxygen uptake by the microorganism, as a response to the starvation of nutrients in the fermenter. That was the point of feeding intervention applied in fed-batch cultivations.

Figure 5-3 depicts the fed-batch fermentation kinetics for *X. dendrorhous* cultivated in semi-defined media with glucose (A) and glycerol (B) as feeding solutions. As shown in Figure 5-2(A), glucose (10 g/l) was added at two time points (48 and 68 h) where DO values spiked from < 68% to 89% coinciding with the depletion of glucose in the fermenter (residual glucose concentration, 0-1 g/l). Following feeding of glucose, DO values reduced again indicating cell

activity. After 100 h of fermentation, 18 g/l of biomass and 4.8 mg/l of astaxanthin were observed after two feeding cycles.

Fed batch fermentations with glycerol (10 g/l) as feeding solution were also performed (Figure 5-3B). After 100 h of fermentation, biomass (19 g/l) and astaxanthin production (4.8 mg/l) were similar to those in fed batch cultures with glucose as feeding solution. Astaxanthin accumulation was initiated in the mid-exponential growth phase (~30 h) and went on until the end of the fermentation (~100 h). Overall, astaxanthin production increased by 25 % compared to the respective fermentation with glycerol in batch mode (3.6 mg/l).

Fed-batch strategy has been implemented before to improve both yeast production and astaxanthin pigmentation in *X. dendrorhous*. Liu & Wu (2008) studied various feeding schemes including constant, exponential and optimal feeding (based on a mathematical model) with glucose as carbon source. They found out that the optimal feeding scheme resulted in high biomass (29 g/l) and astaxanthin production (27 mg/l) compared to batch mode (16.8 g/l of biomass and 15 mg/l of astaxanthin). Besides that, fed batch approach has been widely implemented as a strategy to improve biomass and astaxanthin production using low cost substrates as carbon sources such as mollases, wood hydrolysates and dates juice in *X. dendrorhous* (Luna-Flores et al., 2010; Moriel et al., 2005; Vázquez et al., 1998).

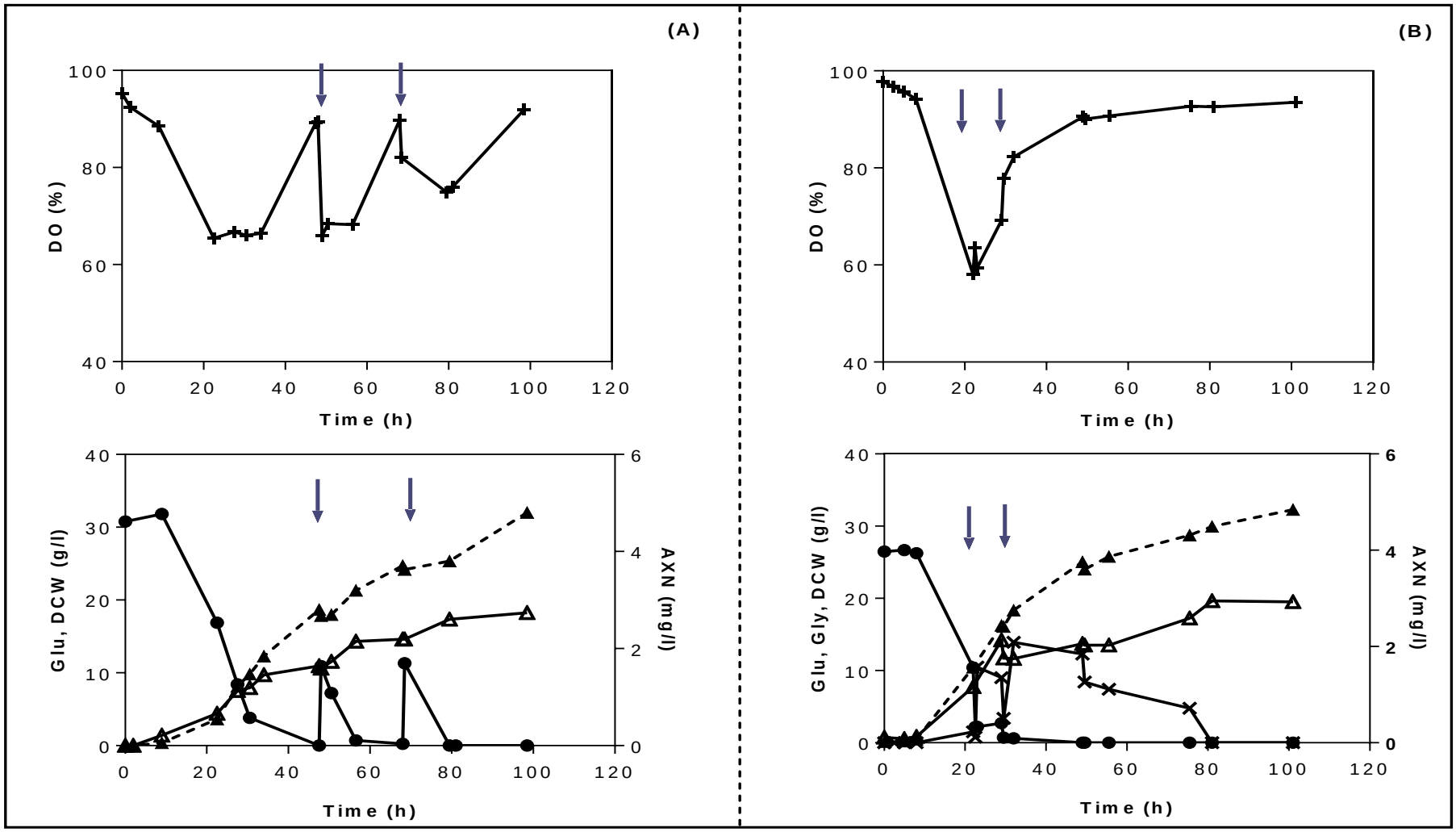

Figure 5-3: Fed-batch cultivation of *X. dendrohous* in a 2- litre stirred tank bioreactor. (A) Fed-batch with glucose, (B) Fed-batch with glycerol. Fermentation conditions: aeration, 1 l/min; temperature, 20 °C; pH 6. Symbols represent: ▲-astaxanthin, AXN (µg/ml); ●- glucose, Glu (g/l); Δ- dry cell weight, DCW (g/l); ○- ethanol, EtOH (g/l); + - dissolved oxygen, DO (%). Arrows indicate feeding points.

5.3.2 Fermentations using rapeseed meal hydrolysate

5.3.2.1 Batch Fermentations

This section aimed to investigate *X. dendrorhous* growth and astaxanthin production using enzymatically hydrolysed rapeseed meal hydrolysates as the sole substrate with no nitrogen-based additives. Based on the results obtained in semi-defined media, the process parameters that were applied were: pH 6, agitation speed at 600 rpm, under constant aeration of 1 l/min and 20 °C. Besides that, separate saccharification and fermentation (SHF) strategy was implemented, whereby rapeseed meal hydrolysis was performed in a separate vessel, and prior to *X. dendrorhous* fermentation. Two types of rapeseed meal hydrolysates derived from different enzymatic treatments (Accellerase 1500 and pectinase) were used. The details of the enzymatic hydrolysis treatments are given in detail in Chapter 4.

Figure 5-4 shows the growth profile of batch fermentation of *X. dendrorhous* in two different rapeseed meal hydrolysates and Table 5.1 presents the main fermentation data of these trials. The composition of rapeseed meal hydrolysates derived from Accellerase 1500 consisted of 13 g/l of total sugars (glucose, xylose and galactose). The utilisation of this hydrolysate as fermentation medium resulted in 14 g/l of biomass and 3.6 mg/l of astaxanthin (*P*). By observing the substrate consumption profile, it can be noted that glucose was used as a primary carbon source, and upon its depletion, hemicellulosic derived sugars were then consumed. As for astaxanthin yield on biomass ($Y_{p/x}$), 250 µg/g of biomass were produced. These values were higher compared to those obtained in flask fermentations utilising rapeseed meal hydrolysates as substrate (12 g/l of biomass and 3.2 g/l of astaxanthin) as discussed in Chapter 4. It is evident that the optimised process conditions led to higher biomass and astaxanthin production, due to the provision of better dispersion of nutrients and adequate oxygen supply in the bioreactor, in which had a positive impact on cell growth and astaxanthin production.

As for batch bioreactor fermentations using pectinase derived hydrolysates, it consisted of 20 g/l of total monomeric sugars with the additional presence of glycerol (52 g/l) that was sourced from the enzyme formulation as previously mentioned (Chapter 4). *X. dendrorhous* cultivation in this hydrolysate was most preferable for biomass (42 g/l). The concentration of astaxanthin, *P* (10.2 mg/l) was positively correlated with high biomass production. These data clearly showed that glycerol acted as additional carbon source for biomass production; upon exhaustion of the main sugars in the media, glycerol started to be consumed as demonstrated by the diauxic growth curve on this occasion (indicated by an arrow in Figure 5-4B). The metabolic pathway for glycerol catabolism is discussed in detail in Chapter 3.

Biomass production was positively correlated with increased sugars concentration in all tested hydrolysates. Observing the astaxanthin yield on biomass ($Y_{p/x}$), the values for both hydrolysates were similar (~ 250 µg/g), indicating that the type of carbon source did not affect the accumulation of astaxanthin. In comparison with semi-defined media with 30 g/l of glucose as initial carbon source, it was expected that biomass production was higher (16 g/l) compared to Accellerase 1500 hydrolysates (14 g/l) that contained less carbon source available (13 g/l), however, astaxanthin production (*P*) were similar (3.6 mg/l) in both fermentation. This suggests the ability of the particular yeast to produce intracellular astaxanthin was higher in Accellerase 1500 hydrolysates ($Y_{p/x}$ = 251 µg g) as compared to semi-defined media ($Y_{p/x}$ = 221 µg/g). The complex composition of the rapeseed meal hydrolysates that composed of mixtures of sugars, protein and other nutrients helps to promote in astaxanthin production in *X. dendrorhous.*

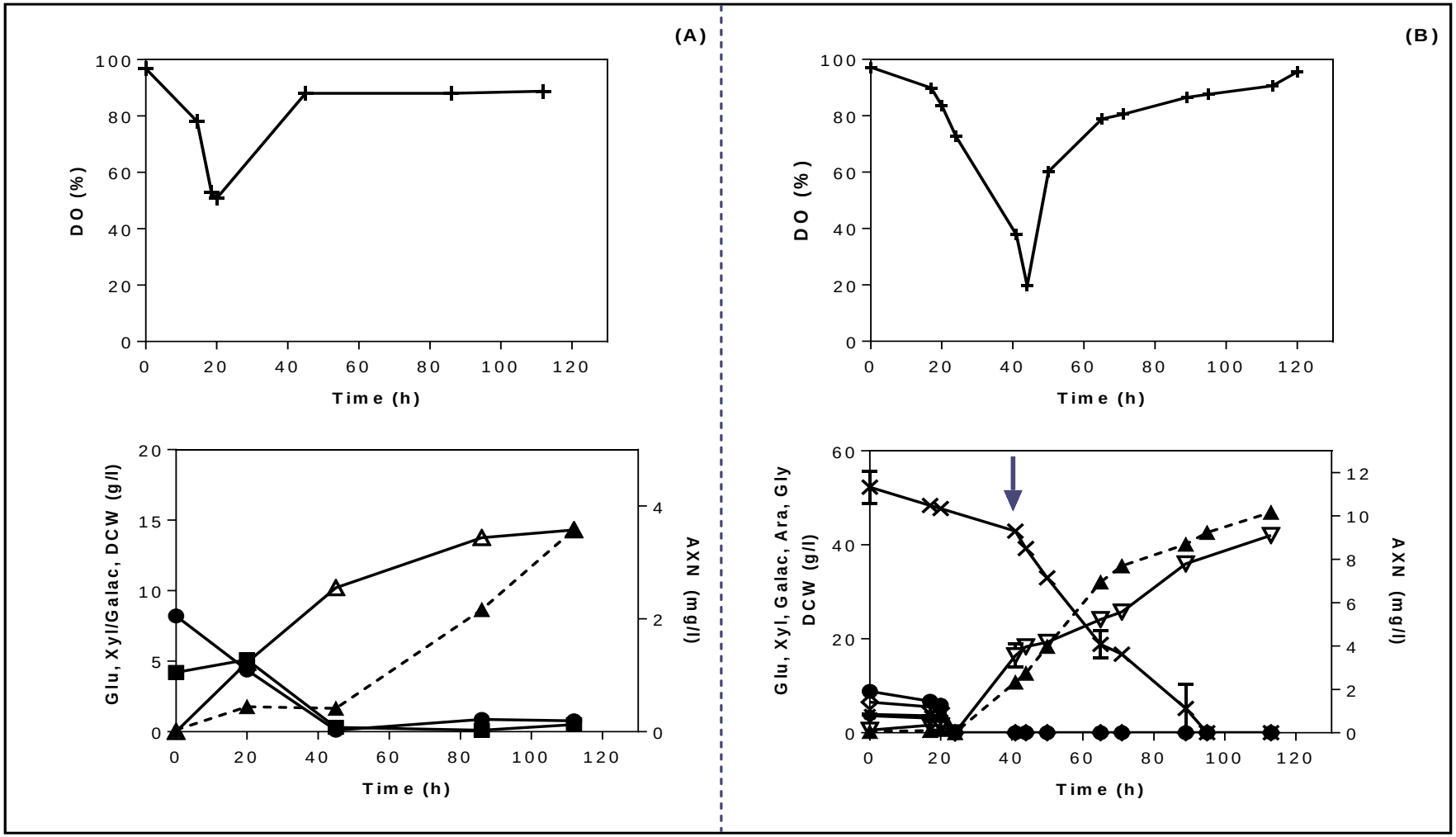

Figure 5-4: Growth profile of *X. dendrorhous* cultivated in 2-L bioreactor with rapeseed meal hydrolysates as sole fermentation media. A) Accellerase 1500 derived hydrolysates (15% v/v of enzyme and 10% w/v of substrate); B) pectinase derived rapeseed meal hydrolysates (10% v/v of enzyme and 10% w/v of substrate). Fermentation conditions: aeration, 1 l/min; temperature, 20 °C; pH 6. Symbols represent: ▲- astaxanthin, AXN (µg/ml); ●- glucose, Glu (g/l); ◆- xylose, xyl (g/l), ◇- galactose, galac (g/l), ⁎- arabinose, Ara (g/l); Δ- dry cell weight, DCW (g/l); ○- ethanol, EtOH (g/l), × - glycerol, Gly (g/l); + - dissolved oxygen, DO (%).

Table 5-1: Fermentation data of *X. dendrorhous* in batch bioreactor cultures of rapeseed meal hydrolysates and semi-defined media

Hydrolysates	**Time (h)**	**Total carbon concentration (g/l)**	***X* (g/l)**	***P* (mg/l)**	**$Y_{p/x}$ (µg/g)**
RSM+10% Accellerase	112	13.3 ± 0.2	14.3 ± 0.9	3.6 ± 0.1	251 ± 5
RSM + 10% Pectinase	113	72.1 ± 4.3	42.0 ± 0.4	10.2 ± 0.2	242 ± 6
Semi – defined media (30 g/l glucose)	123	27.6 ± 1.1	16.3 ± 0.4	3.6 ± 0.1	221 ± 8

*Fermentation conditions: agitation, 1 l/min; temperature, 20 °C; pH 6; agitation, 600 rpm

5.3.2.2 Fed-batch fermentations

Given that rapeseed meal hydrolysate was proved a promising nutrient source for both biomass and astaxanthin production in *X. dendrorhous*, we further investigated the cultivation of the yeast in Accellerase 1500 hydrolysates which were composed of sugars deriving solely from the hydrolysis of rapeseed meal (no additional carbon sources from the enzyme formulation). To this end, a similar fed-batch mode approach was taken (DO-stat mode) as in semi defined media experiment (section 5.3.1.3), with the addition of glycerol as feed (Figure 5-5).

Results showed that feeding of glycerol after the main carbon sources (glucose, xylose, galactose) were exhausted from the media, had some slight effects on the growth of *X. dendrorhous* compared to batch fermentations with Accellerase 1500 rapeseed meal hydrolysates alone. Some amounts of glycerol were consumed until 50 h of fermentation. After

this point, the concentration of glycerol was maintained at 10 g/l until end of fermentation. The addition of glycerol at this point failed to induce shifting towards glycerol metabolism possibly due to the fact that the yeast cells were already under stress environment. It might also occur due to possible deficiency of some key micronutrients such as protein or amino acids in the fermentation media (Chatzifragkou et al., 2014). Previous studies reported that fed-batch cultivation mode is the best approach for mass production of carotenoids, however this was not the case in our study. Total biomass (14 g/l) and astaxanthin production, *P* (3.2 mg/l) were slightly lower as compared in batch fermentation (biomass, 16 g/l and astaxanthin, 3.6 mg/l).

Fed batch strategies utilising low-cost substrates have been applied for microbial astaxanthin production in yeasts before. *Eucalyptus* wood containing xylose rich hydrolysates has been used for microbial astaxanthin production in *X. dendrorhous* NRRL Y-17268 continuous feeding of fresh hydrolysates. Around 10.3 g/l of biomass were obtained with 8.2 mg/l of astaxanthin produced in this case (Vázquez et al., 1998). Besides that, Moriel et al., (2005) investigated fed batch strategy with continuous feeding utilising sugar cane juice and urea for astaxanthin production in *X. dendrorhous* ATCC 24202. They reported that about 19 g/l of biomass with 5.7 mg/l of astaxanthin were produced when continuous feeding strategy was applied.

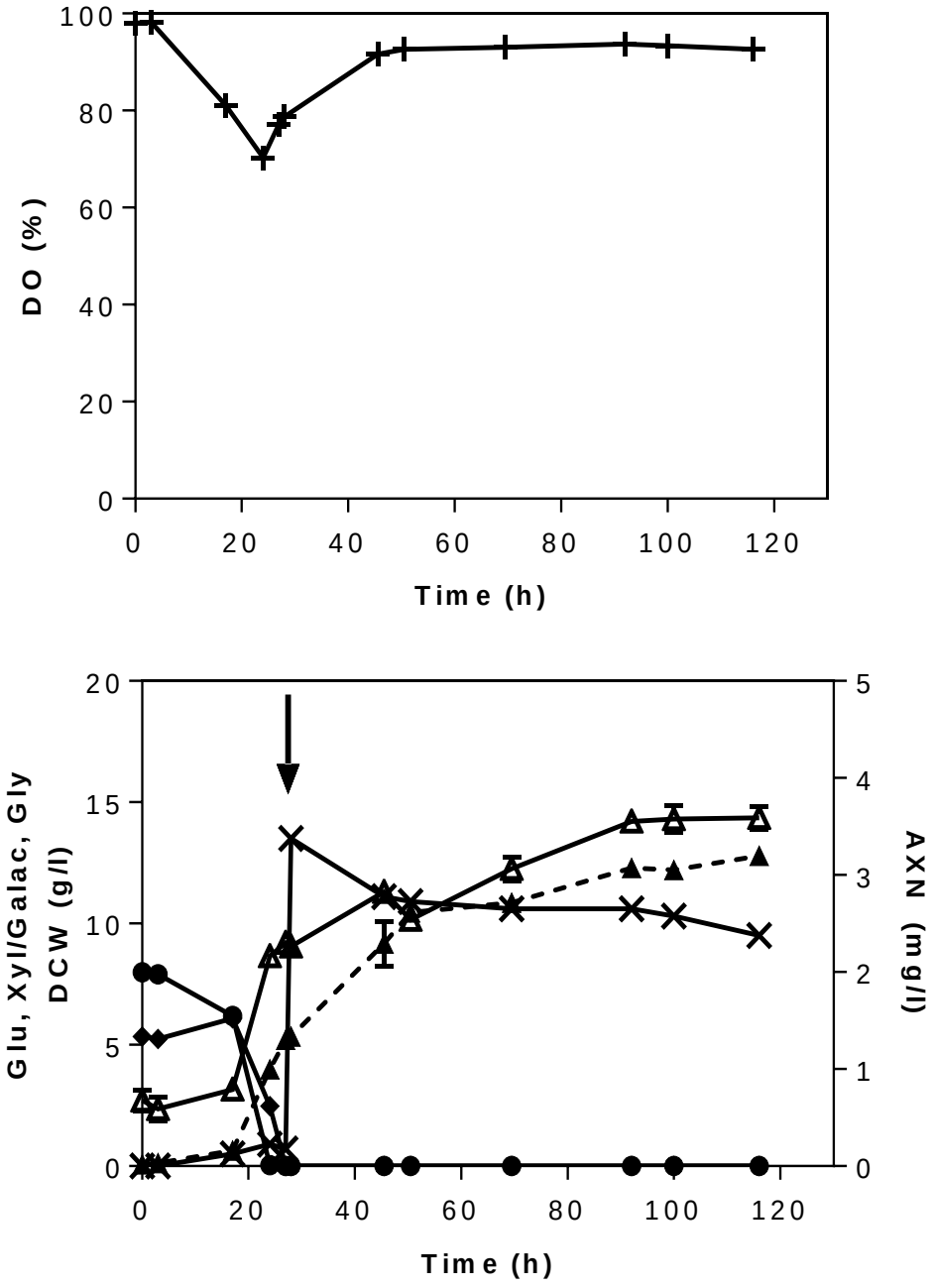

Figure 5-5: Fed-batch cultivation of *X. dendrorhous* utilising rapeseed meal hydrolysates as substrates supplemented with glycerol as feed solution. Fermentation conditions: aeration, 1 l/min; temperature, 20 °C; pH 6. Symbols represent: (+)-DO, (▲)- astaxanthin (mg/l), (◆)-galactose/xylose, (Δ)- dry cell weight (g/l), (●)- glucose, (×)-glycerol + - dissolved oxygen, DO (%). Arrows indicate feeding point.

5.3.3 Prehydrolysis and fermentation (pSSF) in 2-l bioreactor

This study was carried out in order to investigate the possibility of conducting pSSF strategy with a view to improve yeast growth as well as astaxanthin production in *X. dendrorhous*. pSSF strategy is an improvement of simultaneous saccharification and fermentation (SSF) strategy that has been widely used in bioethanol production by *Saccharomyces cerevisiae* (Taherzadeh & Karimi, 2007). In SSF strategy, the enzymatic hydrolysis of cellulosic material and microbial fermentation are performed simultaneously. However, problems often arise mainly due to the difference in the optimal temperatures of the enzymatic hydrolysis and yeast fermentation. In our case, the significant difference between optimal temperature for enzymatic hydrolysis of rapeseed meal and *X. dendrorhous* growth rendered the SSF approach not feasible. To this end, the vessel temperature was set at 50 °C for 24 h (the optimal temperature for enzyme hydrolysis), followed by reducing the temperature to 20 °C (optimal temperature for *X. dendrorhous*), prior to inoculation.

Results showed that Accellerase 1500 and pectinase derived rapeseed meal hydrolysates were able to support both growth and astaxanthin production. Figure 5-6 depicts the growth profile of *X. dendrorhous* cultivated in a 2-litre bioreactor using pSSF approach in Accellerase 1500 hydrolysates (Figure 5-6A) and pectinase hydrolysates (Figure 5-6B). In the case of pSSF using Accellerase 1500 hydrolysates, total astaxanthin production was 30 % lower than in SHF cultivation process utilising the same hydrolysates. This might occur due to the presence of rapeseed biomass in the bioreactor, in which led into collisions between rapeseed meal biomass and yeast cells during agitation and eventually cell disruption.

In the case of pectinase derived rapeseed meal hydrolysates, it was observed that the sugars released during hydrolysis step were used as primary carbon sources. Generally, about 70 g/l of total carbon sources (~ 22 g/l sugars and ~48 g/l glycerol) were available for yeast

consumption. It was observed that glycerol started to be consumed once available sugars were exhausted in the media, demonstrating that cells were able to divert their metabolism towards glycerol consumption. It is interesting to observe that during the period of glycerol metabolism, glucose was also released in the hydrolysates by the slow activity of pectinase on rapeseed meal at 20°C (as opposed to optimum temperature of pectinase activity at 50°C). At the end of the fermentation, glycerol was completely consumed by the yeast and about 3.5 g/l of excess glucose remained in the media. In the pSSF approach with pectinase derived rapeseed meal hydrolysates similar results for astaxanthin production were noted as in batch fermentation with separate hydrolysis and fermentation experiment (~10 mg/l).

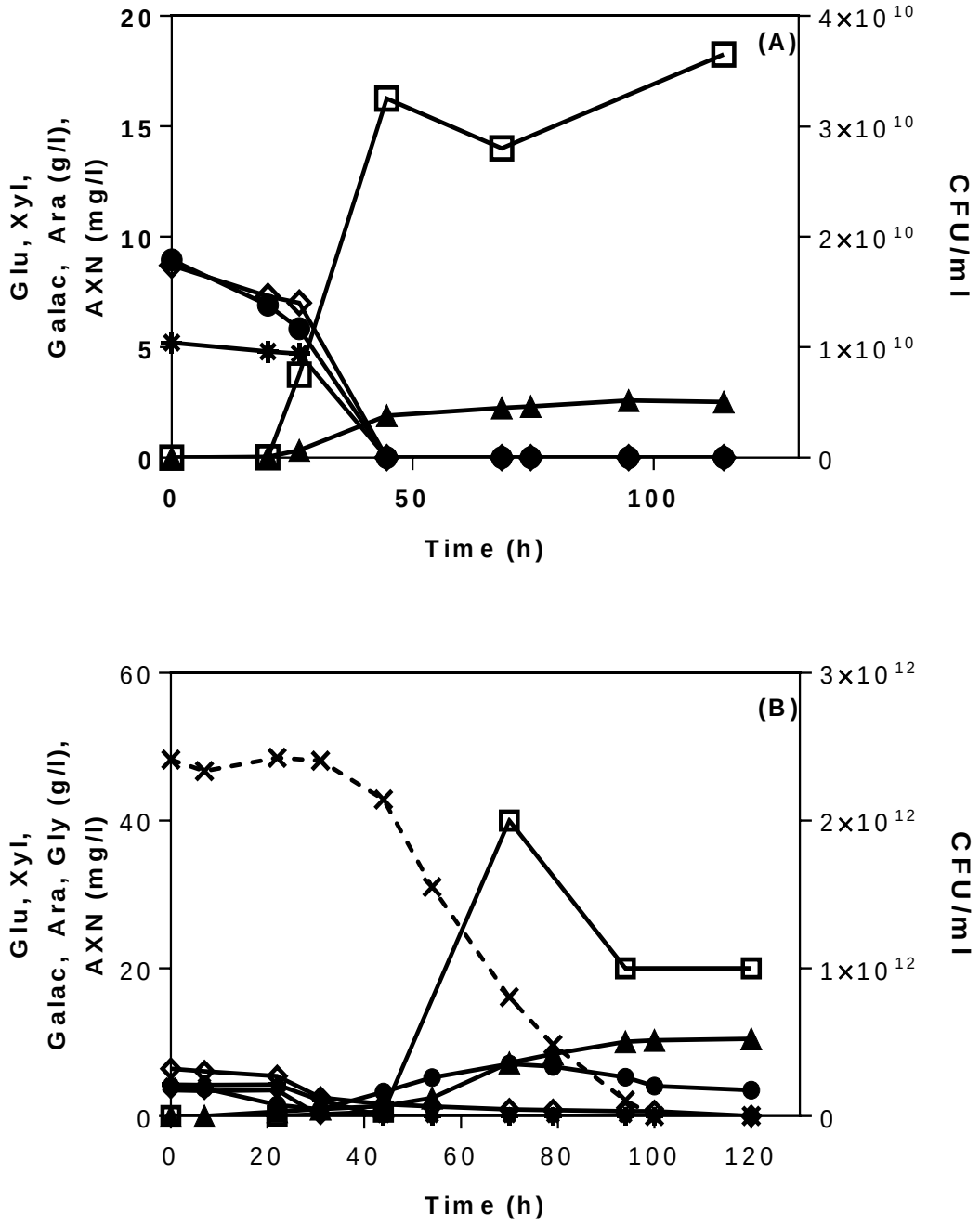

Figure 5-6: Growth profile of *X. dendrorhous* using pSFF approach. (A) 10% (v/v) Accellerase 1500 in 10% (w/v) substrate; (B) 10% (v/v) pectinase in 10% (w/v) substrate. Hydrolysis conditions: agitation, 250 rpm; temperature, 50 °C; time, 24 h. Fermentation conditions: aeration, 1 l/min; agitation, 600 rpm; temperature, 20 °C; pH 6. Symbols represent: ▲- astaxanthin, AXN (µg/ml); ●- glucose, Glu (g/l); ◆- xylose, xyl (g/l), ◇- galactose, galac (g/l), ⁎- Arabinose, Ara (g/l); Δ- dry cell weight, DCW (g/l); ○- ethanol, EtOH (g/l), × - glycerol, Gly (g/l); □ – CFU/ml

5.4 Conclusions

This study demonstrated the suitability of rapeseed meal hydrolysates as substrates for microbial astaxanthin production. Under optimised process conditions (pH 6, 600 rpm, 1 l/min of air), the yeast growth was higher compared to flask fermentations, suggesting the requirement of this yeast species for ample oxygen supply for growth. Pectinase derived rapeseed meal hydrolysates had high capability to support yeast growth and subsequently increased the astaxanthin pigmentation in the cells as compared to Accellerase 1500, mainly due to the presence of glycerol that acted as additional carbon source. Besides that, although in our case the pSSF approach did not significantly increase the yeast performance in terms of biomass and astaxanthin production, it may hold potential for rapeseed meal bioconversion into microbial astaxanthin on a larger scale after further optimisation.

6 Astaxanthin Extraction from *Xanthophyllomyces dendrorhous*: Comparison between Mechanical Abrasion, Enzymatic and CO_2 Supercritical Fluid Extraction

Abstract

This study investigated three different extraction strategies for the extraction of microbially produced astaxanthin from the yeast *Xanthophyllomyces dendrorhous* DSMZ 5626. Firstly, mechanical method (glass beads) accompanied with different types of solvents [ethanol, methanol, acetone, dimethyl sulfoxide, mixture of acetone: DMSO (1:1) and water] was used to measure the astaxanthin extractability (%). Highest astaxanthin extractability was obtained when glass beads disruption coupled with acetone extraction was used to extract astaxanthin in yeast with 95 % extractability. Secondly, enzymatic cell lysis using 2 different enzymes (Accellerase 1500 and Glucanex) on wet and dry cells accompanied with acetone extraction were optimised using central composite design of experiment (DoE) with main variables being pH and reaction temperature. Results showed that Glucanex cell lysis accompanied with acetone extraction resulted in high astaxanthin extractability (>100%) as compared to standard extraction method (DMSO/acetone) under optimised conditions (pH 4.6 at temperature of 30.8 °C). Thirdly, enzyme-assisted supercritical fluid extraction with carbon dioxide (CO_2-SCFE) and ethanol as co-solvent was investigated for its efficiency towards astaxanthin extraction. Increased astaxanthin extractability was observed when the cells were pretreated with Accellerase 1500 and Glucanex as compared to non-treated samples. However, utilising CO_2-SCFE as an extraction strategy was less efficient compared to the glass beads and enzymatic extraction strategies.

Keywords: extraction, glass beads, enzyme, CO_2-SCFE

6.1 Introduction

Astaxanthin found its market in various sector including food, feed, natural colourant and nutraceutical industries due to its highly pigmented colour and high antioxidant capacity (Molino et al., 2018). Microbial astaxanthin needs to be formulated as an active ingredient in various products including animal feed, nutraceuticals and cosmetic products. In some application such as animal feed, whole microbial cells can be formulated as feed ingredient as it is without the need for pigment extrcation. However, in other applications such as cosmetic products and as food ingredients, there is a need to extract the pigment to allow further process to take place. Since microbial astaxanthin is produced intracellularly, there is a need for an efficient extraction strategy, so that the extracted pigment retains its stability, colour and activity. The purity of the extracted astaxanthin pigment will further determines the market price that varies from $2500-7000/kg (Shah et al., 2016). Natural astaxanthin is supplied by microbial fermentation using either yeast, (such as *Xanthophyllomyces dendrorhous*) or microalgae (such as *Haematococcus pluvialis).* The high astaxanthin yields and productivities obtained by *X. dendrorhous,* in fact, make this particular microorganism a promising choice for the potential scaling up and commercialisation of an industrial process (Hu et al., 2007; Monks et al., 2013; Schmidt et al., 2011). The extraction of astaxanthin from cultures of *X. dendrorhous* is one of the most important step, which contributes to the overall complexity and cost of the whole production process.

Considering that astaxanthin accumulates intracellularly in *X. dendrorhous*, cell disruption is the first and most likely the most important step of the separation and purification process. This is attributed to the fact that the yeast cell wall components are composed of polymers of mannose (± 40 % of dry cell mass), β-glucans (± 60 % of dry mass) and small

amounts of chitin (± 2 % of dry cell mass) (Sena, Valasques Junior, Baretto, & Assis, 2012). The complex and rigid structure of yeast cell wall needs to be ruptured prior to solvent extraction of the intracellular carotenoids by commonly used solvents including acetone, petroleum ether, ethyl acetate and hexane (Michelon et al., 2012; Wu & Yu, 2013b; Yang et al., 2011). The various chemical methods act based on the permeability of a particular chemical inside the cell wall and allows periplasmic products to permeate through the yeast cell wall (Liu et al., 2016). Several cell disruption methods have been investigated for astaxanthin extraction from yeast cells including chemical, mechanical and enzymatic methods, either singly or in combination.

The mechanical methods that have been studied for the extraction of bio-products including carotenoids, enzymes and lipid from several yeast cells including *Saccharomyces cerevisiae* and *Xanthophyllomyces dendrorhous*, primarily at small scale, include homogenisation and the utilisation of glass beads (Persike et al., 2002; Reyes, Gomez, & Kao, 2014; Sedmak, Weerasinghe, & Jolly, 1990b). Persike et al. (2002), applied glass beads followed by vigorous vortex for the extraction of invertase and urease from *Xanthophyllmyces dendrorhous* strain 24202. In another study, different mechanical disruption methods were used to extract carotenoids from *X. dendrorhous* cells including maceration with diatomaceous earth, glass beads and ultrasonic waves. The results demonstrated that carotenoids extractability by glass beads in acetone was low (48%) as compared to other disruption methods (51-60%). The disadvantage of the mechanical disruption methods is that they are not product selective as a variety of cellular components are also released along with astaxanthin (Liu et al., 2016).

Enzymatic cell lysis has been extensively applied for the production of yeast extract, mainly from *Saccharomyces cerevisie* (Milic et al., 2007). However, few research works have focused on astaxanthin extraction from *X. dendrorhous* using an enzymatic cell lysis approach. The complex structure of yeast cells requires the synergistic effects of several enzyme activities including chitinase, protease and β-glucanase activities, in order to disrupt the outer layer of their cell wall (Salazar, 2008; Sena et al., 2012), prior to astaxanthin extraction by solvents. The selection of suitable enzymes is critical to ensure a high degree of cell wall disruption that subsequently results in high astaxanthin extractability. This method is considered a much more gentle process than mechanical or chemical disruption strategies. However, the enzymatic method is less cost-effective due to the high cost of the enzymes (Liu et al., 2016).

In addition to the above methods, Supercritical Fluid Extraction (SCFE) has been applied for the extraction of carotenoids from various sources including plants, microalgae and yeast. This method is considered a "green" technology and has the potential to replace the commonly used solvent-based extraction methods for the selective recovery of carotenoids. Carbon dioxide (CO_2) is mostly used as the primary supercritical fluid extraction solvent as its supercritical state has high solvation power, and is non-toxic, non-flammable, non-explosive, cost-effective and can be easily removed from the product (Machmudah et al., , 2006; Wang et al., 2012). The products extracted using this method are free of toxic solvents, in contrast to solvent extraction where the residual solvents might be present in the final products (Jokić et al., 2016). Furthermore, the low critical temperature of CO_2 means that this process could be carried out at moderate temperatures, thus preventing the degradation of carotenoids, which can occur due to their sensitivity to relatively high temperatures (Krichnavaruk et al.,, 2008). This method has been successfully used to extract compounds such as lipid, phenolic compounds and pigments from plants, yeast and bacteria. However, very few research works

have been conducted on astaxanthin extraction from *X. dendrorhous*. In a study by Lim et al. (2002), bead milling was used as the cell disruption strategy prior to astaxanthin extraction by CO_2-SCFE in *X. dendrorhous* cells.

This study is aimed at assessing several methods for extracting astaxanthin from *X. dendrorhous* DSMZ 5626 cells, including the use of glass beads, enzymatic lysis and CO_2 Supercritical Fluid Extraction (CO_2-SCFE). Although previous studies dealing with astaxanthin extraction by various methods have been performed, this study is more focused on comparing between methods. Furthermore, to our knowledge, astaxanthin extraction using CO_2-SCFE is scarce and the enzymatic cell lysis as a pre-treatment strategy prior to CO_2-SCFE has not been investigated before for *X. dendrorhous* cells. A design of experiment (DoE) methodological approach was used to identify the optimal process conditions for astaxanthin extraction in the case of the enzymatic lysis and CO_2-SCFE methods and generate new knowledge on the effect of process conditions on astaxanthin extraction. The efficacy of the above methods was measured in terms of astaxanthin extractability (%) as compared to a standard solvent based extraction procedure, described by Sedmak et al. (1990). The current work thus provides new insights into astaxanthin extraction from yeast cells and can form the basis for developing a scalable and efficient process.

6.2 Materials and Methods

6.2.1 Microorganism and Fermentation Conditions

The yeast strain *Xanthophyllomyces dendrorhous* DSMZ 5626 was used in this study and was purchased from Leibniz Institute DSMZ. The strain proliferation and inoculum preparation were conducted as previously described in Chapter 3. The composition of the semi-defined media used in this study were (in g/l): carbon source (30), yeast extract (2.0), malt extract (2.0), KH_2PO_4 (7.0), $(NH_4)_2SO_4$ (1.0), $MgSO_4.7H_2O$ (1.5), $FeCl_3.6H_20$ (0.15), $ZnSO_4.7H_2O$ (0.02), $MnSO_4.H2O$ (0.06), $CaCl_2.2H_2O$ (0.15).

In this study, a 2-litre stirred tank bioreactor (BIOSTAT B, Sartorious AG, German) was used with a working volume of 1.5 litre within the experimental set up being the same as previously described in Chapter 5. The fermentations were carried out for 5-days under aerobic conditions. The key fermentation parameters that were controlled were: Temperature (20 °C), pH (pH 6), aeration (1 l/min) and agitation (600 rpm); the dissolved oxygen was not controlled. Cell biomass was collected and treated according to the extraction method, as described in the sections below.

6.2.2 Sample Preparation

For the experiments concerning the extraction of astaxanthin by glass beads extraction and enzymatic cell lysis, 2 ml of fresh *X. dendrorhous* were added to 2 ml vials, which were then centrifuged at 10,845 x *g* for 10 min (Multifuge X3R, Fisher Scientific, UK). The pellets (~ 30 mg of cell pellet) were collected and washed twice using distilled water. For the experiment using the wet cells, the fresh cells were kept at 4°C until further use. To prepare

dried samples, the vials containing wet cells were frozen at -20°C and then freeze dried for 24 h (Virtis SP Scientific, UK); these pellet samples corresponded to about 30 mg of dry cells.

In the case of astaxanthin extraction by CO_2-SCFE, samples from 3 bioreactor experiments (of similar conditions) were collected together to ensure that there was enough starting material for the experiment. 1.5 litre of yeast culture medium was collected after 5 days of fermentation and centrifuged at 10845 x *g* for 10 min (Multifuge X3R, Fisher Scientific, UK). The pellet was washed twice with distilled water, kept at -20 °C for 48 hrs, and then freeze dried for 72 hrs (Virtis SP Scientific, UK). The dried cell pellets from the three different fermentation runs were pooled together and thoroughly mixed to ensure a homogenous sample prior to CO_2-SCFE extraction.

6.2.3 Extraction of Astaxanthin

(1) Glass beads / solvent extraction

Freeze dried cell samples (~30 mg of freeze dried *X. dendrorhous* cells), prepared in 2 ml Eppendorf tube as described in section 6.3.2, were used in this study. 0.3 g of glass beads (diameter 1mm) was added to the vials followed by addition of 1 ml organic solvent (list of solvents used provided below). The cells/beads suspensions in the different types of solvent were mixed at 2000 rpm for 10 mins in a ThermoMixer C (Eppendorf). Subsequently, 0.1 ml of NaOH (20% w/v) was added to the mixture, followed by further mixing for 5 mins at 2000 rpm in a ThermoMixer C. The aqueous and organic phases were separated by centrifugation at 5423 x g for 5 min and the organic phase (top layer) was collected for astaxanthin measurement, carried out by a spectrophotometric analysis as described in section 6.2.6. The organic solvents used in this study were: distilled water (dH_20), ethanol (EtOH, 99.8%, MERCK), methanol

MeOH, 100%, Fisher Scientific), acetone (100%, Merck), dimethyl sulfoxide (DMSO, 100% Sigma-Aldrich) and mixture of acetone: DMSO (1:1).

(2) Enzyme hydrolysis / solvent extraction

I. Accellerase 1500 treatment

The enzymatic lysis of the cell wall of *X. dendrorhous* was carried out using the commercial enzyme, Accellerase 1500 (Du Pont) solution, which contained multiple enzymatic activities including cellulase, hemicellulase and β-glucanase. In order to determine the optimum condition for enzymatic hydrolysis a non-factorial 2^2 Central Composite Design of Experiments (DoE), with two factors at three levels was employed. The two independent variables assessed were temperature (30, 40, 50 °C) and pH (4.5, 5.5, 6.5). The dependent variable was astaxanthin extractability (%), calculated by comparing the astaxanthin concentration obtained after each extraction method to the concentration obtained using the standard chemical extraction method by Sedmak et al. (1990), the method is described in detail in section 6.2.6. Thirteen different experiments were carried out in total, which included the low, high and axial points for all the parameters, along with a central point replicated five times to calculate experimental errors. Minitab 17 was used for the experimental design and statistical analysis.

In this experiment, both freeze dried and wet cell samples were used. Sample preparation followed the same method as described in section 6.2.2. The experimental set up was as follows: 0.75 ml of sodium citrate buffer (pH varied according to the DoE - see results sections for details) and 0.25 ml of enzyme (30 % v/v of Accellerase 1500) were added to vials containing wet and freeze-dried cell biomass of *X. dendrorhous*. The mixture was then incubated in a thermomixer (Eppendorf) under agitation at 1400 rpm and the temperature was

controlled according to DoE for 1 hr. After enzyme treatment, samples were centrifuged at 10845 x *g* for 10 mins and the supernatant was discarded. The pellet was washed twice with distilled water to remove excess enzymes and buffer. Subsequently, 1 ml of acetone was added to the pellet and the suspension mixed at 2000 rpm for 10 mins to facilitate the extraction of astaxanthin. 100 µl of NaCl (20% w/v) were then added to the mixture to assist in the formation of the aqueous and organic (solvent) phases. Samples were mixed for another 5 mins and then were centrifuged at 5423 x *g* for 5 mins. The solvent phase (upper layer) was used for astaxanthin analysis, conducted as described in section 6.2.6.

II. Glucanex treatment

Freeze dried Glucanex enzyme (Novozyme, Denmark), containing β-1, 3 glucanase obtained from *Trichoderma harzianum* was used in this experiment. A non-factorial 2^2 Central Composite Design of Experiments (DoE) with two factors at three levels was employed. The two independent variables assessed were temperature (35, 45, 55 ^{o}C) and pH (3.5, 4.5, 5.5). The conditions of the independent variables in this experiment were different compared to the Accellerase 1500 treatment to reflect the optimal activity conditions, recommended by the manufacturer. The dependent variable was the astaxanthin extractability (%), calculated as described in section 6.2.6. Thirteen different experiments were carried out in total, which included the low, high and axial points for all the parameters, along with a central point replicated five times to calculate experimental errors. Minitab 17 was used for the experimental design and statistical analysis.

Initially, a 20 % (w/v) Glucanex stock solution was prepared. The sample preparation followed the same method as that described in section 6.2.2. The experiments set up was as follows: 0.75 of sodium citrate buffer (pH varied according to the DoE - see results section for

details) and 0.25 ml of enzyme (5 % w/v of Glucanex stock solution) were added to vials containing wet and freeze-dried cell biomass of *X. dendrorhous*. The optimisation experiment and the acetone extraction process were conducted following the same procedure to that described above for the Accellerase 1500 treatment.

(3) Enzyme assisted CO_2-supercritical fluid extraction *(SCFE) with ethanol as co- solvent*

In this experiment, *X. dendrorhous* cells were pretreated with Accellerase 1500 (30 % v/v) unless stated otherwise. Subsequently, the pretreated cells were frozen at -20 °C for 24 hrs prior to freeze drying (VirTis Scientific, UK) for 72 hrs. The samples were then ground with a pestle and mortar and kept in amber containers in a desiccator until further use.

Freeze dried samples of *X. dendrorhous* were subjected to carbon dioxide-super critical fluid extraction (CO_2-SCFE) in a CO_2-SCFE rig (SciMed, UK). The apparatus consisted of a recirculating chiller, a CO_2 line, solvent and co-solvent pumps, a heat exchanger, a 200 ml extraction vessel, an automated backpressure vessel, a collection vessel and a controller. For every run, 2.0 g of freeze dried samples and 100 g of inert glass beads (5mm) (Sigma Aldrich) were added to the extraction vessel and submitted to a CO_2 flow rate of 15 g/min.

In order to optimise the extraction process, a non-factorial 2^3 Central Composite Design of Experiments (DoE) method with three factors and 3 levels was employed. The independent variables were: temperature (50, 60, 70°C), co-solvent concentration (EtOH at 5, 10, 15 %) and pressure (150, 250, 350 bar); the run lasted 30 mins. The dependant variable accessed was astaxanthin extractability (%), calculated as described in section 5.2.6. Fourteen experiments which included the low, high and axial points of all parameters were conducted along with a central point, which was replicated three times. At the end of each run, astaxanthin

was obtained in ethanol; the sample was immediately analysed for astaxanthin content using a spectrophotometer (see section 6.2.6).

Response Surface Methodology (RSM) was used to construct a mathematical model describing the effectiveness of astaxanthin extraction; the data were presented in the form of 3-dimensional graphs. All the independent variables of the model equation were tested statistically by the F-test at a 95% interval of confidence. The Coefficient of Variance (CV, %) and the Determination Coefficient (R^2) were used to evaluate the quality of the fitted polynomial model. Finally, the experiments were validated by performing additional experiments (in triplicate) using the optimal conditions for astaxanthin extraction, as suggested by the model. The experimental values from these runs were then compared to the predicted values given by the model to confirm the accuracy of the model.

6.2.4 Testing of Astaxanthin Solubility in Solvents

In order to explain some of the results obtained after extraction of astaxanthin using different solvents, an experiment was conducted to evaluate the solubility of commercial astaxanthin in organic solvents including ethanol (EtOH, 99.8%, MERCK), methanol (MeOH, 100%, Fisher Scientific), acetone (100%, Merck), dimethyl sulfoxide (DMSO, 100% Sigma-Aldrich), mixture of acetone:DMSO (1:1) as well as in water. Approximately 10 mg of pure astaxanthin (Sigma Aldrich) were added to 1 ml of solvent in 2 ml (Eppendorf) tubes and were then mixed at 1200 rpm for 24hrs at 25°C in a thermomixer (Eppendorf). If a clear solution was observed, then additional amounts of astaxanthin (~2 mg) were added to the mixture until there was a visual indication of undissolved material. The obtained solvent/astaxanthin mixtures were diluted 100 times in methanol and were then filtered through a 0.2µm polyvinyl

difluoride (PVDF) membrane filter. Finally, the concentration of astaxanthin in the different solvents was analysed by HPLC to estimate the solubility of astaxanthin in these solvents.

6.2.5 Activity of β-Glucanase

In order to measure the β-glucanase activity of the two enzymes mixtures used in this study (Accellerase 1500 and Glucanex), and calculate the amounts needed for the experiments, the following method was used. 0.25 ml of enzyme solution and 0.25 ml of laminarin solution 1% (w/v) obtained from the seaweed *Laminaria digitata* (Sigma-Aldrich) in sodium acetate buffer (0.1 M, pH 5.5) were mixed, following which the reaction mixture was incubated at 55 °C for 30 mins. The reaction was stopped by heating at 100°C for 5 mins. The reducing sugars were determined by the method of 3, 5-dinitrosalicylic acid (DNS method) using glucose as the standard. For the control, distilled water was used instead of laminarin solution. One activity unit of β-1, 3 glucanase (U) was defined as the release of 1 µmol of glucose/min/ml of enzyme solution.

6.2.6 Determination of Total Carotenoids

i. Standard Chemical Method and Spectrophotometric Analysis

In order to measure the astaxanthin concentration in the different samples (pellets) the standard method of Sedmak et al. (1990) was followed. Briefly, 1 ml of dimethyl sulfoxide (DMSO) was preheated at 55 °C and added to the freeze-dried biomass, followed by vortexing for 30-40 secs. Subsequently, 0.1 ml of 20 % sodium chloride (NaCl) and 1.0 ml of acetone were added to the mixture to extract the intracellular carotenoids. The aqueous and organic phase was separated by centrifugation at 5423 x g for 5 mins. The extraction process was

repeated until a colourless biomass was obtained, and the organic phases were pooled together and their absorbance was measured at 480 nm in a Biomate 3 UV/VIS Spectrophotomer (Thermo Spectronic, NY). The resultant values were then divided by the extinction coefficient of 2150. This spectrophotometric method was also used to determine the astaxanthin concentration in the solvents used for extraction in the glass bead, enzymatic cell lysis and CO_2-SCFE experiments. The equation for the estimation of the total carotenoids concentration is given below:

$$Carotenoids\ content\ (\mu g/g) = \frac{A\ \times V\ (mL) \times\ 10^4}{A_{1cm}^{1\%}\ \times P\ (g)} \qquad \text{Eq. 6-1}$$

Where, A is the absorbance at 480 nm, V is the volume, $A_{1cm}^{1\%}$ is the coefficient (2150) and *P* is the weight of biomass.

Astaxanthin extractability (%) was calculated by Eq. 6-2 (Machado et al., 2016).

$$Astaxanthin\ extractability\ (\%) = \frac{C}{C_{DMSO}} \times\ 100 \qquad \text{Eq. 6-2}$$

Where C is the concentration of astaxanthin extracted from the cells using the different disruption/extraction techniques and C_{DMSO} is the total carotenoids extracted from *X. dendrorhous* using the standard method, i.e. by cell disruption with DMSO/acetone.

ii. High Performance Liquid Chromatography (HPLC)

The quantification of astaxanthin in the experiments assessing the solubility of astaxanthin in different organic solvents (section 01) was performed by HPLC. A HPLC system (Agilent Infinity, 1260 series, Agilent Technology) equipped with a YMC-C30 silica-based reversed-phase column (250 × 4.6 mm, YMC) coupled with diode array detector (DAD, Agilent Infinity 1260 series, Agilent Technology) was used for the identification and quantification of astaxanthin. A gradient method was used, with (A) methanol/ MTBE/ water (82:16:2) and (B) methanol/ MTBE/ water (23:75:2) as the mobile phase. The gradient started at 100% of solvent A. Solvent B was then gradually increased to 50 % (1-45 mins) and further increased to 100% (46–55 mins), where it was held for 5 mins; the duration of each run was of 60 mins in total. The injection volume was 100 µl and the flow rate was kept constant at 1.0 ml/min. For astaxanthin identification and quantification, calibration curves were constructed using commercial astaxanthin standard (Sigma Aldrich). All detected peaks were recorded at 450 nm.

6.2.7 Scanning Electron Microscopy

The morphology of the cell biomass samples after each treatment was analysed using Quanta FEG 600 Environmental Scanning Electron Microscopy instrument (FEI Co. Inc., Hillsboro, Oregon). Samples were mounted onto SEM stubs using carbon tape and then sputter coated with a thin layer of gold to prevent charging during imaging. The parameters used for imaging were: 20 kV of accelerating voltages, 4.0 spot size and a working distance approximately 10 – 12 mm. Images were recorded under vacuum at 6000X magnification.

6.3 Results and Discussion

6.3.1 Glass beads / solvent extraction

Figure 6-1 presents the results from the experiment where astaxanthin extraction was performed with glass beads aiming to disrupt the yeast cell, coupled with different types of solvent to extract astaxanthin simultaneously. The application of glass beads with vigorous shaking at 2000 rpm for cell disruption (freeze-dried samples) accompanied with different types of organic solvents, namely ethanol (EtOH, 99.8%, MERCK), methanol (MeOH, 100%, Fisher Scientific), acetone (100%, Merck), dimethyl sulfoxide (DMSO, 100% Sigma-Aldrich), mixture of acetone:DMSO (1:1) as well as in water, was investigated in this study. Figure 6-1 presents the results from the experiment where astaxanthin extraction was performed with glass beads aiming to disrupt the yeast cell and was coupled with different types of solvent to extract astaxanthin simultaneously. The highest extraction was achieved with acetone (95 %), followed by DMSO: Acetone (84 %). Methanol and ethanol were not significantly different from each other (54-58%), whereas DMSO on its own resulted in low extraction yield (42 %) and water in the lowest (14 %). This might have occurred due to the fact that acetone has good permeability through the cell wall and membrane of *X. dendrorhous* as well as high solubility to astaxanthin (Yin et al.,2013). The difference in the extraction yield obtained with the different solvents could be attributed to the differences in their polarity which were: Ethanol (4.3) < methanol (5.1) < acetone (5.4) < DMSO (7.2) < water (9.00) (Yin et al., 2013). As astaxanthin contains both polar (at the end of the molecule) and non-polar (in the middle of the molecule) components in their chemical structure (Blasko et al., 2008), the use of slightly polar solvent helps to extract astaxanthin from the cell wall/membrane of the yeast cells, which might be attached by the non-covalent binding to specific proteins (Amaro et al., 2015).

In order to understand better the mechanisms of astaxanthin extraction and extractability values (%) obtained, the solubility of astaxanthin in different solvents was tested (Table 6-1). The highest solubility of pure astaxanthin was observed with a combination of DMSO and acetone (1:1), whereas a lower astaxanthin solubility was found in DMSO and acetone alone. The solubility of astaxanthin is highly correlated with the polarity of the solvent used. The highest astaxanthin solubility, i.e. 2.03 mg/ml, was observed when a mixture of DMSO: acetone (1:1) was used. In terms of the astaxanthin solubility in individual solvents, the highest solubility was observed when DMSO was used, a fact that correlates to the high polarity of DMSO. Even though astaxanthin has low solubility in acetone (0.55 mg/ml) as compared to DMSO, the latter demonstrates high permeability through the yeast cell wall and most likely due to this reason it led to higher astaxanthin extractability from the yeast cells (Yin et al., 2013). The mechanism of astaxanthin extraction when using organic solvents is based on the permeation of the solvents through the cell wall and cell membrane, and on its subsequent interaction with astaxanthin using Van der Waals forces. This solvent/astaxanthin complex will diffuse across the cell membrane and remain dissolved in the solvents (Halim et al., 2012).

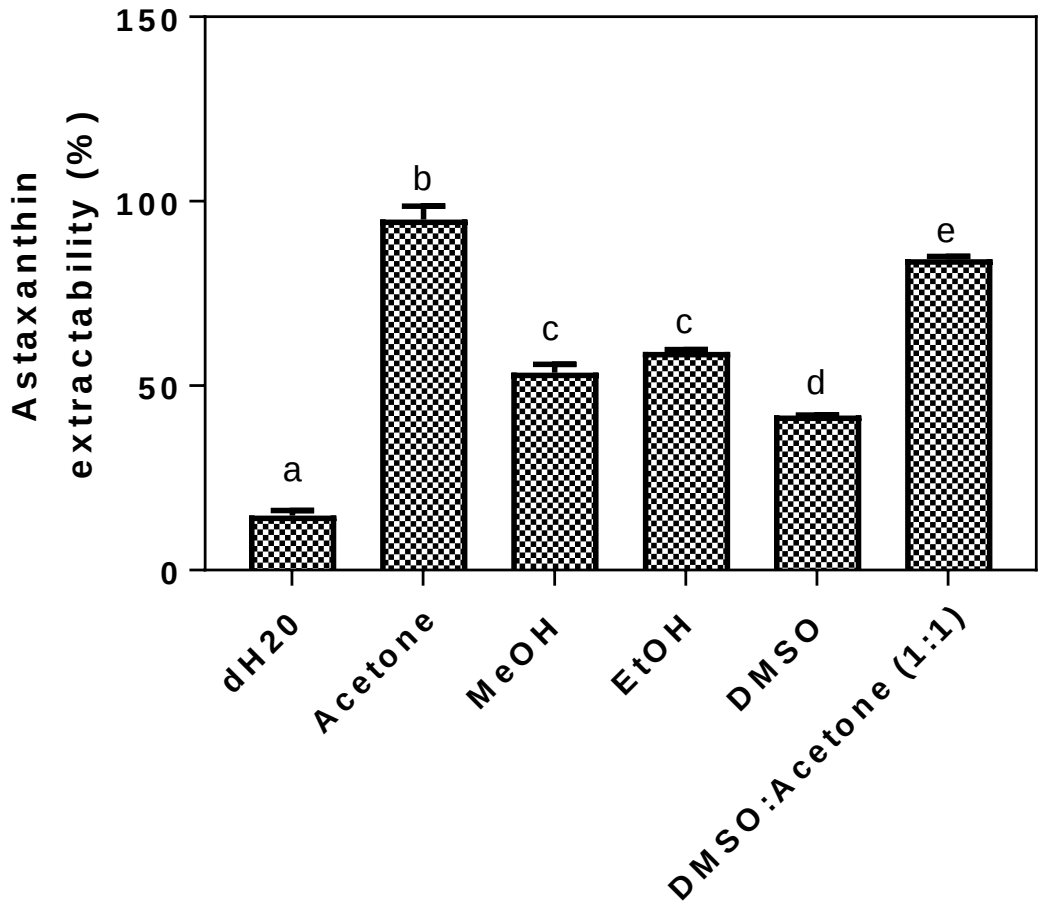

Figure 6-1: Percentage of astaxanthin extractability (%) using glass beads (0.3 g) with different types of solvents. Extraction was performed at room temperature under agitation at 2000 rpm in ThermoMixer (Eppendorf). Different letters show the significant difference between each treatment using Tukey pairwise comparison ($p<0.05$). Abbreviations: dH_20 (distilled water), MeOH (methanol), EtOH (ethanol), DMSO (dimethyl sulfoxide).

Table 6-1: Solubility of astaxanthin in solvents after incubation for 24 h at 25 °C.

Solvent	Concentration (mg/ml)
Water	0
Ethanol	0.038 ± 0.004^{a}
Acetone	0.55 ± 0.02^{b}
Methanol	0.04 ± 0.002^{a}
Dimethyl Sulfoxide (DMSO)	1.64 ± 0.03^{c}
DMSO:Acetone (1:1)	2.03 ± 0.04^{d}

Different letters represent significant difference between solvents by Tukey pairwise comparison ($p<0.05$).

Several works have been published reporting the utilisation of glass beads to extract intracellular products from various yeast species, however there is a very limited number of studies on astaxanthin extraction form *X. dendrorhous*. Glass beads extraction is achieved by disrupting the yeast cells via bead collision zones through compaction or shear force with energy transfer from beads to the cells (Duarte et al., 2017). Previously, glass beads accompanied with acetone were used to rupture *Sporodiobolus pararoseus* and *Rhodotorula mucilaginosa*. Results showed that about 66% and 52% of the carotenoids, were obtained, respectively, compared to the standard DMSO method (Lopes, Remedi, dos Santos Sá, Burkert, & de Medeiros Burkert, 2017). In a study performed by Sedmak et al. (1990), two different methods (glass beads extraction and DMSO/solvent method) were used to extract astaxanthin from four different strains of *Phaffia rhodozyma* cells. Similar results were obtained between these two methods (275 – 276 µg/g), which are also in line with the results obtained in this study. However, the DMSO disruption method is more rapid than the glass bead disruption

process, in which multiple samples can be processed at the same time, the DMSO method is considered more suitable for large nu mber of samples. However, the key drawbacks of the glass bead method is that it is a rather slow method and is also a non-selective extraction process where all polar/non-polar compounds are extracted, a fact that coule potentially create issues during downstream processing and purification of astaxanthin.

The resultant morphology of the *X. dendrorhous* cells after the glass beads treatment was studied by environmental scanning microscopy (Figure 6-2). Before the glass beads treatment, the cells had intact sphere shapes with a smooth surface. After treatment, small particles (< 5 mm) were produced as a result of beads abrasion (Figure 6-2B), as compared with the intact cells before disruption (Figure 6-2A). The small sized cells and debris produced after the glass beads treatment resulted in an increased surface area for subsequent solvent extraction, leading to higher astaxanthin extractability. At this point, astaxanthin extraction was most likely highly dependent on the solubility and polarity of the organic solvents used, as the degree of cell disruption was already high. For example, in the glass beads accompanied with water extraction, the lowest amount of astaxanthin was extracted even though the cells were ruptured. This is attributed to the low ability of the solvent in this case (water) to penetrate the debris/cells as well as the low astaxanthin solubility in water. This experiment demonstrated that the types of organic solvents used after the bead treatment are crucial for astaxanthin extraction and consequently for the efficacy of the overall process.

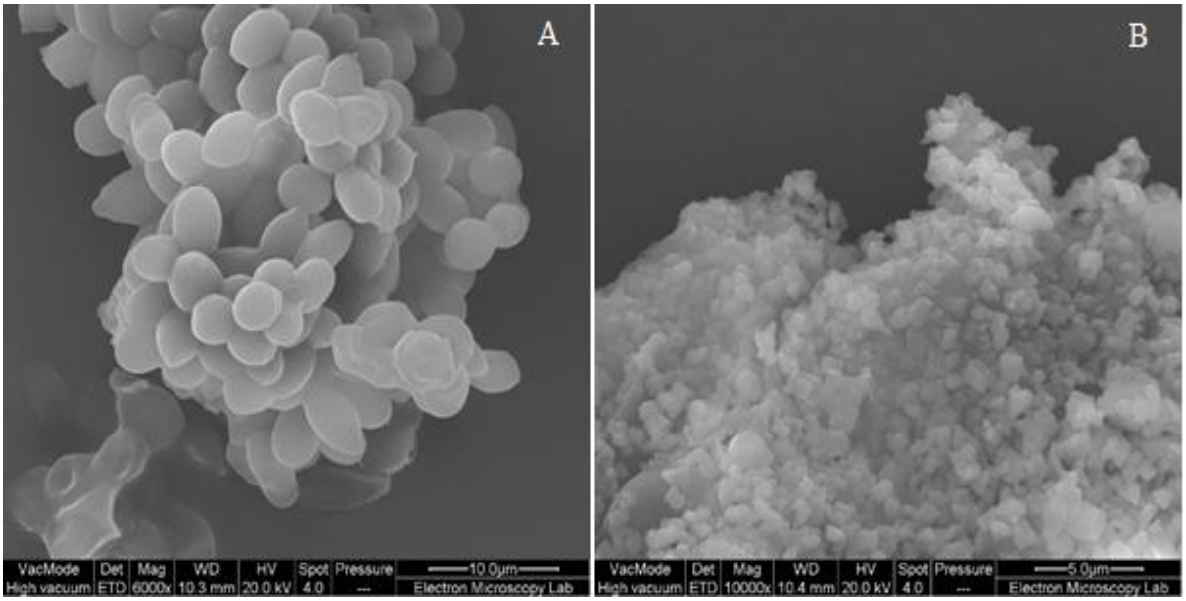

Figure 6-2: Scanning electron microscopy images of *X. dendrorhous* cells A) intact cells, B) cells after treatment with glass beads followed by acetone (100%) extraction. Extraction was performed at room temperature under agitation at 2000 rpm in ThermoMixer (Eppendorf).

6.3.2 Enzymatic Treatment/ Solvent Extraction

i. Accellerase 1500 treatment

In this study, wet and dried *X. dendrorhous* cells were subjected to enzymatic treatment using Accellerase 1500, aiming to disrupt the cell wall, followed by acetone extraction for the extraction of astaxanthin. Accellerase 1500 is an enzyme cocktail that has multiple enzymatic activities including cellulases and hemicellulases, and a measured with β-glucanase activity of 0.03 U/ml. A full factorial central composite design of experiments (CCD) was employed to identify the optimal conditions for astaxanthin extraction. The parameters that were investigated were temperature (30, 40, 50 °C) and pH (4.5, 5.5, 6.5), whereas the treatment time was fixed at 1 hr and the enzyme concentration at 30 % (v/v). The reason for selecting these temperature and pH conditions was that according to the manufacturer, these values are within the range of their optimum enzymatic activity. In total, 12 runs as well as 5 zero points for error estimation, were conducted, and the corresponding results are presented in Table 6-2.

Overall, the results showed that astaxanthin extraction using wet cells resulted in higher extractability compared to freeze dried cells. Possible explanation might be due to the presence of moisture inside the cells, which resulted in a higher water activity and potentially a higher enzymatic activity towards *X. dendrorhous;* such effect of water activity on enzyme reaction has been reviewed by Rezaei, Jenab, & Temelli (2007). On the other hand, in the freeze-dried samples, the moisture levels were much lowed due to the drying process. Amongst the dried samples, the highest astaxanthin extractability (%) was observed for Run 5 (temperature at 40 °C, pH 4.09), whilst the lowest extractability was observed for Run 8 (temperature at 54.1°C, pH 5.51).

Table 6-2: Effects of pH and temperature on astaxanthin extractability (%) of dry and wet samples of *X. dendrorhous* DSMZ 5627 cells after treatment with Accellerase 1500 (30 %, v/v) followed by acetone extraction.

Run	pH	Temperature	Astaxanthin extractability (%)	
			Dry samples	Wet samples
1	4.50 (-1)	30.0 (-1)	6.33	10.59
2	6.50 (+1)	30.0 (-1)	4.91	6.98
3	4.50 (-1)	50.0 (+1)	4.84	8.85
4	6.50 (+1)	50.0 (+1)	4.78	5.30
5	4.09 (-1)	40.0 (0)	8.66	14.08
6	6.91 (+1)	40.0 (0)	4.65	5.81
7	5.50 (0)	25.7 (-1)	4.97	9.30
8	5.50 (0)	54.1 (+1)	2.52	4.00
9	5.50 (0)	40.0 (0)	8.91	12.60
10	5.50 (0)	40.0 (0)	9.12	13.50
11	5.50 (0)	40.0 (0)	7.49	13.11
12	5.50 (0)	40.0 (0)	7.95	12.95
13	5.50 (0)	40.0 (0)	9.43	13.76

The results obtained from the 13 runs were used to construct two quadratic models were constructed, one for dry cells (Eq. 6-3) and one for wet cells (Eq. 6-4), describing the main, interaction and quadratic effects of the independent variables (pH, temperature) on the response (astaxanthin extractability).

$$Astaxanthin\ \ Extractability\ (\%) = -44.1 + 8.29pH + 1.679T - 0.958pH^2 - 0.02412T^2 + 0.0339pH.T \quad \text{Eq. 6-3}$$

$$Astaxanthin\ \ Extractability\ (\%) = -73.7 + 16.43\ pH + 2.544T - 1.714pH^2 - 0.03362T^2 + 0.0016pH.T \quad \text{Eq. 6-4}$$

Where T= temperature and astaxanthin extractability (%) = % astaxanthin extracted from the cells as described in Eq. 6-2.

Table 6-3 presents the regression coefficients of the quadratic equation for describing astaxanthin extractability (%) in the case of Accellerase 1500 treatment. Even though astaxanthin extractability was higher using wet cell as compared to dry, the models generated for both samples demonstrate similar profiles in terms of the significance of the variables tested. With regards to astaxanthin extractability using dry cells, it was observed that both linear and quadratic terms have a statistically significant ($p < 0.05$) effect on the extraction. More specifically, both linear terms of pH and temperature had a negative correlation with astaxanthin extractability, where an increase in temperature and pH resulted in decreased astaxanthin extractability (%). However, the interactions of the linear terms (pH*T) showed no significant effect ($p > 0.05$). Similar results were observed with fresh cells.

Table 6-3: Regression coefficients of the quadratic equation describing astaxanthin extractability as a function of pH and temperature. Astaxanthin extraction was achieved by treating freeze dried *X. dendrorhous* cells with Accellerase 1500, followed by acetone extraction.

Factor	Coefficient	SE	t-value	P-value
Dry cells				
Constant	8.579	0.390	22.02	0.000
pH	-0.894	0.308	-2.09	0.023
T	-0.636	0.308	-2.06	0.078
pH^2	-0.958	0.330	-2.90	0.023
T^2	-2.412	0.330	-7.30	0.000
pH*T	0.339	0.436	0.78	0.460
Wet cells				
Constant	13.191	0.404	32.62	0.000
pH	*-2.358*	0.320	-7.38	0.000
T	*-1.364*	0.320	-4.27	0.004
pH^2	*-1.714*	0.343	-5.00	0.002
T^2	*-3.362*	0.343	-9.81	0.000
pH*T	*0.016*	0.452	0.04	0.973

In order to confirm the accuracy of the model, an analysis of variance (ANOVA) was performed along with a F-test for validation. In terms of the astaxanthin extractability using freeze dried cells, the fit of model, expressed by the coefficient of regression R^2 value, was 0.909. Furthermore, the F-value (14.09) obtained was higher than the tabulated F-value ($F_{5,7}$ = 3.97). In terms of the wet cells, the regression R^2-value, was 0.9631, whereas the F-value (36.57) was also higher than the tabulated F-value ($F_{5,7}$ = 3.97). Hence, it can be deduced that the models for both freeze dried and fresh samples can satisfactorily describe the extraction process.

Figure 6-3 presents the two-dimensional contour surface graphs generated, which provide the model prediction for astaxanthin extractability using Accellerase 1500. The shapes

of the contour plots provide a visual interpretation of the interaction between the two variables and facilitate the location of optimum experimental condition (Shijin, Xiang, Zhihang, Lili, & Jianmeng, 2009). It can be seen that for both wet and dried cells, higher astaxanthin extractability (%), i.e around about 9-14 %, was obtained at an extraction temperature around 35-40 °C and a pH around 4.5-5.5 with. Based on the model prediction, the astaxanthin extractability (%) for both samples could be obtained at a temperature of 38.8°C and pH 4.97. This set of values predicted an astaxanthin extractability of 8.85% (dry cells) and 14.09% (wet cells). The models were subsequently validated by repeating the experiments at these critical parameters (temperature at 38.8 °C and pH 4.97) and were compared with the predicted values. The results showed that the astaxanthin extractability values were similar, although slightly higher than the prediceted values, i.e. with 10.25 % for the dry cells and 14.99 % for the wet cells, which demonstrated that the generated models can be considered valid to describe the extraction process.

Overall, this experiment demonstrated that the astaxanthin extraction was much lower in the case of Accellerase 1500 treatment compared with the previous results with glass beads extraction. Enzymatic cell lysis in yeast occurs as a synergistic effect of protease and glucanase activities (Michelon et al., 2012). Generally, enzymatic cell lysis in yeast starts with the binding of the lytic protease to the outer mannoprotein layer of the cell wall, which results in exposure of the protein structure and releases the cell wall protein and mannan while exposing the glucan surface. Subsequently, the glucanase enzyme attacks the inner cell wall and solubilises the glucans (Salazar, 2008). The soluble structure of yeast allows then acetone to permeate through the cell wall and solubilise astaxanthin. The action of acetone on astaxanthin were discussed in detail in section 6.3.1.

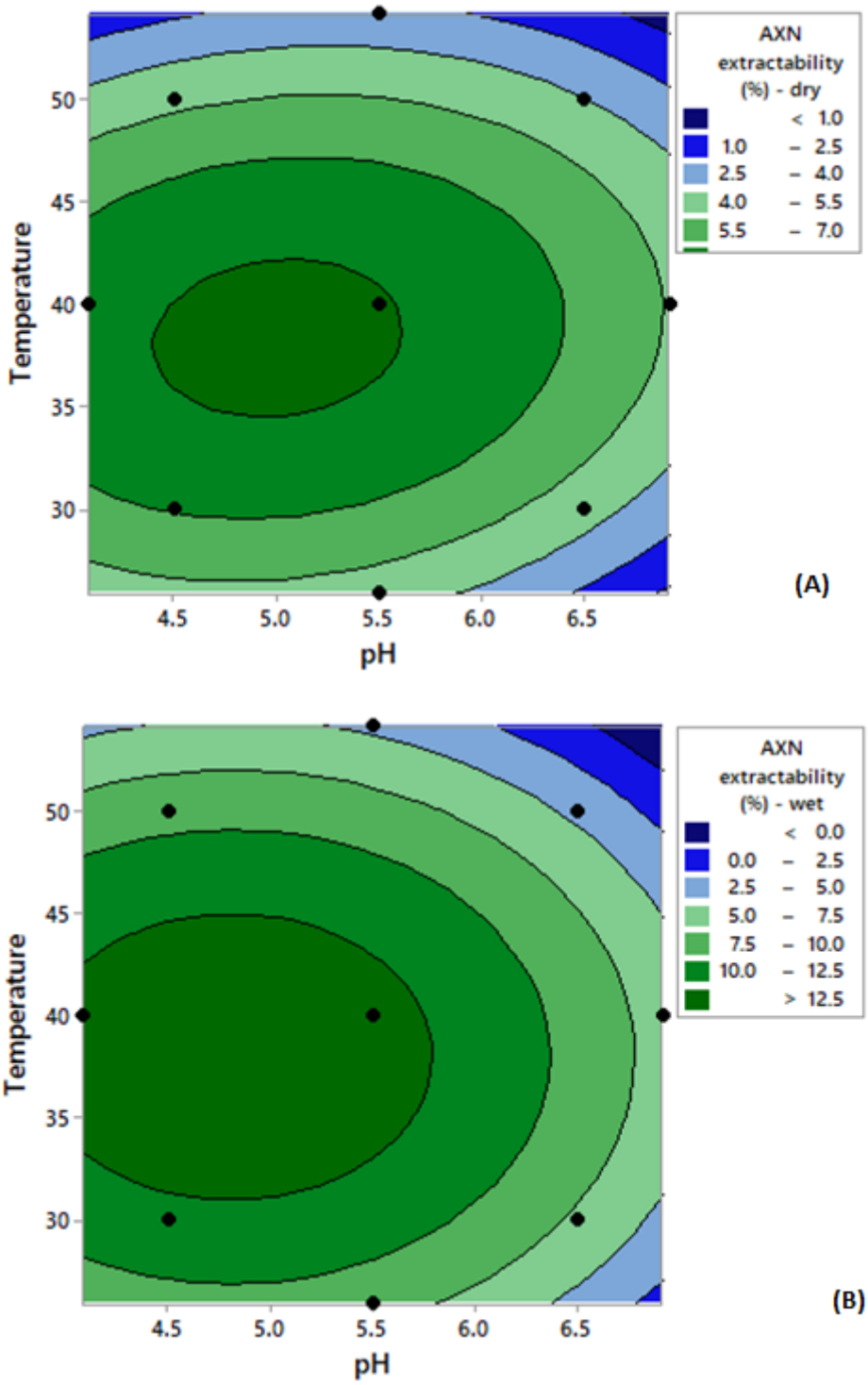

Figure 6-3: Contour plots depicting the effects of temperature and pH on astaxanthin extractability (%) of *X. dendrorhous* cells. Astaxanthin extraction was achieved by treating the cells with Accellerase 1500 (30 % v/v) for 1 h. (A) dry cells (B) wet cells. * The black dots on the contour plot represent the experimental points. Abbereviationn: AXN - astaxanthin

Scanning electron microscopy images (Figure 6-4) show that after enzymatic treatment, the yeast had an irregular shape as compared to the intact cells. A portion of the cells still maintained their cell integrity, however most of them lost their intact shape. The enzymes attack specific regions of the cell wall, hence the effect is not as damaging as in mechanical abrasion using the glass beads as observed in Figure 6-2. During enzymatic lysis, no astaxanthin was secreted into the liquid, due to its insolubility in water. The astaxanthin extraction only occurred in the presence of solvents (acetone). The activity of Accellerase 1500 on the yeast disruption was specific and mild in and hydrolysed the yeast cell wall in a more selective manner, as compared to the mechanical and chemical extraction, which were non-selective. This approach of using Accellerase 1500 to hydrolyse the *X. dendrorhous* cell wall has not been reported before. Although the process is not as effective as the glasss beads method, the results show indicate that this method could potentially be used as a pretreatment strategy prior to mechanical or chemical treatments such as ultrasonication, CO_2-SCFE or solvent extraction, to enhance the extraction selectivity (Liu et al., 2016). However, further optimisation work is needed to achieve this.

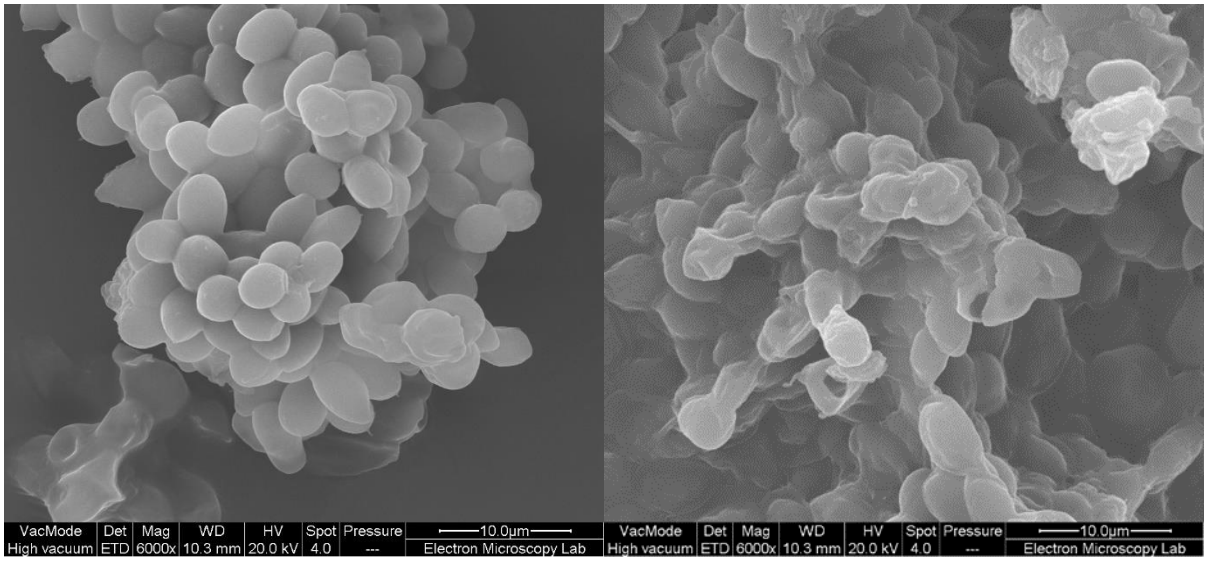

Figure 6-4: Scanning electron microscopy images of *X. dendrorhous* cells before and after treatment (A) intact cells, B) cells after treatment with Accellerase 1500 (30 % v/v) followed by acetone (100 %) extraction.

ii. Glucanex treatment

Beside Accellerase 1500, the effects of the Glucanex enzyme was also investigated in this study. Glucanex is a lytic enzyme from *Trichoderma harzianum* with a measured β-glucanase activity of 0.4 U/ml. A response surface design was also used in this study with 2 variables being investigated (temperature and pH). A full factorial CCD was applied for this purpose. The parameters that were investigated were temperature (35, 45, 55 °C) and pH (3.5, 4.5, 5.5), whereas the treatment time was fixed at 1 hr and the enzyme concentration at 5 % (w/v). The reason for selecting these temperature and pH conditions was that according to manufacturer, these values were in range of optimum enzymatic activity. In total, 12 factorial runs as well as 5 zero points, for error estimation, were conducted and the results are presented in Table 6-4.

Similarly to the case of Accellerase 1500 treatment, in the case of Glucanex treatment the wet cells resulted in significantly higher astaxanthin extractability as compared to the dry cells. However, overall astaxanthin extractability was much higher in the case of Glucanex than Accellesae 1500. The highest astaxanthin extractability (%) was observed at run 5 (temperature at 30.9 °C and pH 4.5) (115%) whilst the lowest extractability was observed at run 4 (temperature at 55°C and pH 5.5) (43%). Overall, the results indicated that both pH and temperature play a critical role in determining the extraction of astaxanthin by *X. dendrorhous* using Glucanex.

Table 6-4: Effects of pH and temperature on astaxanthin extractability (%) of dry and wet samples of *X. dendrorhous* DSMZ 5627 cells. Astaxanthin extraction was achieved using Glucanex (5 %, w/v) for 1 hour, followed by acetone extraction.

Run	pH	Temperature	Astaxanthin extractability (%)	
			Wet cells	Dry cells
1	3.5 (-1)	35.0 (-1)	91.80	79.80
2	3.5 (-1)	55.0 (+1)	39.95	22.04
3	5.50 (+1)	35.0 (-1)	93.33	76.91
4	5.50 (+1)	55.0 (+1)	43.00	18.09
5	4.5 (0)	30.9 (-1)	114.83	84.36
6	4.5 (0)	59.1 (+1)	47.58	25.99
7	3.09 (-1)	45.0 (0)	44.83	22.04
8	5.91 (+1)	45.0 (0)	60.24	35.57
9	4.50 (0)	45.0 (0)	88.91	61.41
10	4.50 (0)	45.0 (0)	90.89	66.73
11	4.50 (0)	45.0 (0)	95.16	63.23
12	4.50 (0)	45.0 (0)	84.94	61.26
13	4.50 (0)	45.0 (0)	90.89	65.36

The results obtained from the 13 runs were used to construct two quadratic models were constructed, one for dry cells (Eq. 6-5) and one for wet cells (Eq. 6-6), describing the the main, interaction and quadratic effects of the independent variables (pH, temperature) on the response (astaxanthin extractability).

$$Astaxanthin\ Extractability\ (\%) = -201 - 0.20T + 143.1pH - 0.0241T^2 - 15.59pH^2 - 0.026pH.T \quad \text{Eq. 6-5}$$

$$Astaxanthin\ Extractability\ (\%) = -275.1 + 170pH + 1.35T - 18.71pH^2 - 0.0442T^2 + 0.038pH.T \quad \text{Eq. 6-6}$$

Where T= temperature and astaxanthin extractability (%) = % astaxanthin extracted from the cells as described in Eq. 6-2

To confirm the accuracy of the models, a variance analysis of (ANOVA) was performed along with a F-test for validation (Table 6-5). In the case of the freeze-dried cells, the coefficient of regression R^2-value was 0.9492, whereas the F-value (26.17) obtained was higher than the tabulated F-value ($F_{5,7}$ = 3.97), indicating the good fit of the model. It was observed that only the linear term of temperature and the quadratic term of pH had a statistically significant ($p < 0.005$) influence on the extraction, whereas the linear term of temperature, the quadratic term of pH and the interaction between pH and temperature did have a significant effect ($p > 0.05$). In the case of the wet cells, the coefficient of regression R^2-value was 0.9870, whereas The F-value (106.11) obtained was also higher than the tabulated F-value ($F_{5,7}$ = 3.97), indicating the good fit of the model. Similarly to the dried cells, the interaction term between pH and temperature did not have a significant effect ($p > 0.05$) on astaxanthin extractability.

Table 6-5: Regression coefficients of the quadratic equation describing astaxanthin extractability as a function of pH and temperature. Astaxanthin extraction was achieved using Glucanex (5 %) for 1 h, followed by acetone extraction.

Factor	Coefficient	SE	t-value	P-value
Dry cells				
Constant	63.60	3.19	19.92	0.000
pH	1.54	2.52	0.61	0.562
T	-24.89	2.52	-9.86	0.000
pH^2	-15.59	2.71	-5.76	0.001
T^2	-2.41	2.71	-0.89	0.403
pH*T	-0.27	3.57	-0.07	0.943
Wet cells				
Constant	89.86	1.67	53.86	0.000
pH	3.28	1.32	2.49	0.000
T	-24.58	1.32	-18.64	0.042
pH^2	-18.71	1.41	-3.13	0.017
T^2	-4.42	1.41	-13.23	0.000
pH*T	0.38	1.87	0.20	0.844

The model prediction is presented in the two-dimensional contour surface graphs generated (Figure 6-5). It can be seen that high astaxanthin extractability was obtained at extraction temperature lower than 35 °C and a pH of approximately 4.5. According to the model, the highest astaxanthin extractability (%) could be obtained at a temperature of 30.8 °C and at pH 4.6. This set of values predicted an astaxanthin extractability of 94 % (dry cells) and 116 % (wet cells). The models were then validated by repeating the experiments using the critical parameters (temperature at 30.8 °C and pH 4.6) and the experimental data were compared to the predicted results. A slight increased value was obtained for dried cells (105 %) and slightly decreased for wet cells (108%), however it can be overall deduced that the models prediction were satisfactory.

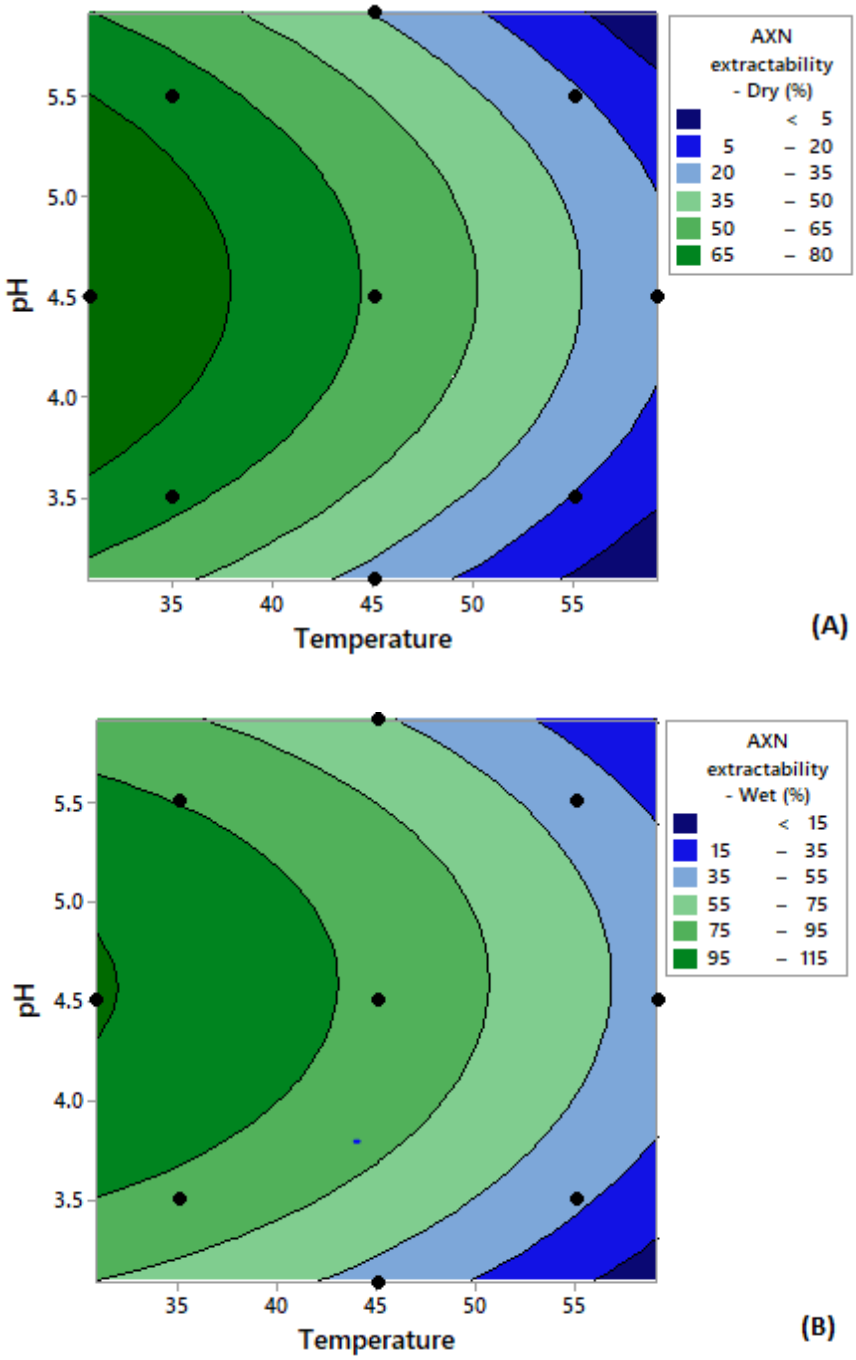

Figure 6-5: Contour plots depicting the effects of temperature and pH on astaxanthin extractability (%) of *X. dendrorhous* cells. Astaxanthin extraction was achieved using Glucanex (5 % w/v) for 1 h. (A) dry cells (B) wet cells. *The black dots on the contour plot represent the experimental points. Abbreviation: AXN = astaxanthin

This study demonstrated that astaxanthin extractability (%) using Glucanex (5 % w/v) was considerably higher than using Accellerase 1500. This probably occurred due to the differences in the activities of these two commercial formulations, which inlcuded β-glucanase, cellulase, protease and chitinase; as demonstrated experimentally, the β-glucanase activity of Glucanex was significantly higher than that of Accelerase 1500. The high β-glucanase activity most likely led to cell lysis through the hydrolysis of the structure of (1-3)-glucose of the yeast cell wall glucans. As a result, the hydrolysed cell walls allowed for acetone to permeate into the cells and extract astaxanthin. The high astaxanthin extractability values obtained when using Glucanex was supported by the SEM images before and after extraction (Figure 6-6). The images show that after Glucanex treatment, the cells had irregular shapes and shrinked in size as compared to the intact cells. However, some of the cells still retained their intact shapes. It can be deduced that the difference in the yeast morphology between Glucanex treatment and glass beads treatment was significant, as in the latter the cells were ruptured and formed small particles.

Enzymatic cell lysis as the means for extracting bioproducts from yeast cells has gained wide interest among researchers and has beed used to produce yeast extract from *Saccharomyces cerevisiae* (Milic et al., 2007). In terms of astaxanthin extraction from yeast cells, Michelon et al. (2012) combined maceration with diatomaceous earth and Glucanex lysis, and this process resulted in the extraction of 122% extractability of carotenoids from *Phaffia rhodozyma*, compared to the standard chemical method. In a different study, enzymatic cell lysis using different types of enzymes (Glucanex, Lyticase and Driselase) was combined with ultrasound pretreatment to extract astaxanthin inform the microalgae *Haematococcus pluvialis* (Machado et al., 2016). This strategy resulted in 84% of astaxanthin extractability compared to the standard chemical method. Overall, these works indicate that there is considerable

postential in using enzymatic cell lysis with other cell disruption methods to increase astaxanthin extractability from yeast cells.

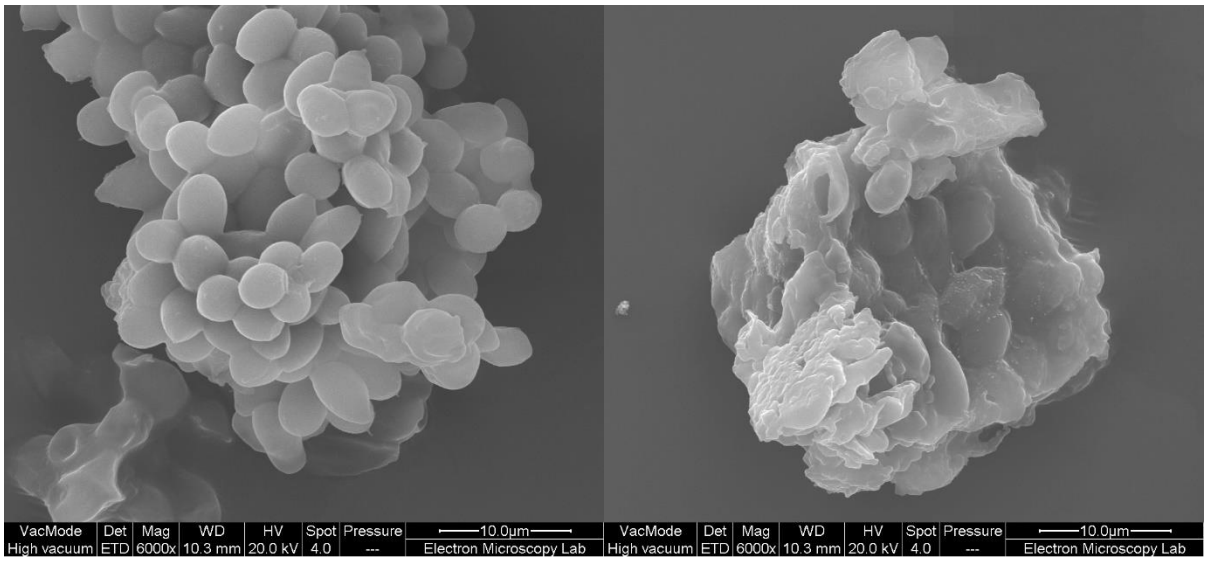

Figure 6-6: SEM images depicting *X. dendrorhous* cells before and after pre-treatment (A) intact cells, B) cells after treatment with Glucanex (5% w/v) for 1 hour, followed by acetone (100 %) extraction

6.4 Enzyme assisted CO_2-Supercritical Fluid Extraction (SCFE) with ethanol as co-solvent

The aim of this work was to extract astaxanthin from *X. dendrorhous* by using CO_2-supercritical fluid extraction (SCFE) with ethanol as co-solvent. A preliminary experiment was conducted using freeze-dried cells without prior enzymatic pretreatment. However, low astaxanthin extractability was obtained (data not shown). Therefore, it was decided to incorporate a yeast cell pretreatment step using an enzyme (Accellerase 1500), aiming to disrupt the cell wall and increase subsequent astaxanthin extraction by CO_2-SCFE. The reason for selecting Accelerase 1500 was to evaluate whether the combination of the two treatments would increase astaxanthin extractability.

A non-factorial Central Composite Design of Experiments (DoE), which included 12 factorial runs and 5 were zero-point runs; Table 6-6 details the experimental conditions and the results. The astaxanthin extractability (%) values ranged from 0.04 % (run 11) to 23.0 1% (run 8).

Table 6-6: Effects of temperature, pressure and co-solvent concentration (ethanol) on astaxanthin extractability (%). Astaxanthin extraction was achieved by treatment of freeze dried *X. dendrorhous* cells with Accellerase 1500 (30 % v/v) for 3 hour, followed by CO_2-SCFE extraction.

Run	Temperature (°C)	Pressure (bar)	EtOH (%)	Astaxanthin extractability (%)
01	50.0 (-1)	150.0 (-1)	5.0 (-1)	7.78
02	70.0 (+1)	150.0 c	5.0 (-1)	1.62
03	50.0 (-1)	350.0 (+1)	5.0 (-1)	16.37
04	70.0 (+1)	350.0 (+1)	5.0 (-1)	17.94
05	50.0 (-1)	150.0 (-1)	15.0 (+1)	20.33
06	70.0 (+1)	150.0 (-1)	15.0 (+1)	14.50
07	50.0 (-1)	350.0 (+1)	15.0 (+1)	21.70
08	70.0 (+1)	350.0 (+1)	15.0 (+1)	23.01
09	43.2 (-1)	250.0 (0)	10.0 (0)	13.40
10	76.8 (+1)	250.0 (0)	10.0 (0)	14.82
11	60.0 (0)	81.8 (-1)	10.0 (0)	0.04
12	60.0 (0)	418.2 (+1)	10.0 (0)	13.38
13	60.0 (0)	250.0 (0)	1.6 (0)	4.24
14	60.0 (0)	250.0 (0)	18.4 (+1)	19.64
15	60.0 (0)	250.0 (0)	10.0 (0)	14.30
16	60.0 (0)	250.0 (0)	10.0 (0)	14.32
17	60.0 (0)	250.0 (0)	10.0 (0)	16.52

In order to develop a model that predicts the influence of temperature, pressure and ethanol concentration on astaxanthin extraction by CO_2-SCFE, the results were used to compute the main interaction and quadratic effects of the independent variables on astaxanthin extractability (Table 6-7); the equation of the quadratic model is given in Eq. 6-7. It was observed that only the linear term of pressure and ethanol concentration demonstrated a statistically significant ($p < 0.005$) effect. The linear term of temperature, the quadratic term of temperature, pressure and ethanol concentration as well as the interaction terms between these variables did not have a significant effect ($p > 0.05$).

Both pressure and co-solvent concentrations significantly influenced astaxanthin extraction. Co-solvent (ethanol) acts by increasing the polarity of CO_2, allowing the dissolution of polar compounds. Even though astaxanthin has low polarity, it has a high molecular weight (MW= 596.8). Therefore, the presence of ethanol facilitates the extraction process as it can aid the dissolution of heavier substances in CO_2 (Andrade-Lima, Charalampopoulos, & Chatzifragkou, 2018). It was observed that increasing the ethanol concentration presented a positive correlation with astaxanthin extractability (%). This can be observed in run 3 and run 7, as an increase in EtOH from 5 % to 15% resulted in increased astaxanthin extractability, i.e. from 16.4 % to 21.7 %. In terms of the influence of pressure, a similar trend was observed, as an increase in pressure positively affected astaxanthin extractability. This phenomenon can be observed in run 11 and run 12, where a significant increase of pressure from 82 bar to 418 bar (at the same temperature, i.e. 60^{o}C, and ethanol concertation, i.e. 10 %) resulted in a significant increase in astaxanthin extractability (from ~ 0 % to ~13%). The higher pressure most likely led to a higher disruption of the yeast cell wall and causing the release of the pigment, as also been shown with plant cellular systems previously (Andrade-Lima et al., 2018).

Table 6-7: Regression coefficients of the quadratic equation describing astaxanthin extractability (%) as a function of temperature, pressure and ethanol concentration. Astaxanthin extraction was achieved by treatment of freeze dried *X. dendrorhous* cells with Accellerase 1500 (30 % v/v) for 3 hour, followed by CO_2-SCFE extraction.

Factor	Coefficient	SE	t-value	P-value
Constant	8.90	1.20	7.41	0.000
T	-0.298	0.564	-0.53	0.613
P	2.528	0.564	4.49	0.003
E	2.728	0.564	4.84	0.002
T^2	0.434	0.620	0.70	0.506
P^2	-1.145	0.620	-1.85	0.107
E^2	-0.028	0.620	-0.05	0.965
TP	1.121	0.736	1.52	0.172
TE	0.006	0.736	0.01	0.994
PE	-1.134	0.736	-1.54	0.167

$$Astaxanthin\ Extractability\ (\%) = 18.5 - 0.832T + 0.0379P + 1.13E + 0.00434\,T^2 - 0.000114P^2 - 0.0011E^2 + 0.001121TP + 0.0001TE - 0.00227PE \quad \text{Eq. 6-7}$$

Where *T*= temperature, *P* = pressure, *E* = Ethanol and astaxanthin extractability (%) = % astaxanthin extracted from the cells as described in Eq. 6-2.

An ANOVA test was performed to confirm the accuracy of the models, along with an F-test. The coefficient of regression (R^2) was 0.8846 and the the F-value (5.96) was higher than the tabulated F-value ($F_{9,7}$=2.72), indicating that the model gave a good fit and was able to describe satisfactorily the astaxanthin extraction process from yeast cells using CO_2-SCFE. Figure 6-7 shows the response surface graphs; according to the model the maximum predicted maximum astaxanthin extractability (25.7%), was obtained at 76.8°C temperature, 360 bar pressure and 18% (v/v) ethanol concentration. The model was then validated by repeating the experiments using these critical parameters and the experimental data were compared to the

predicted results. An astaxanthin extractability value of 22% was obtained which demonstrates that the model can predict satisfactorily the extraction process.

Figure 6-8 shows the difference in the appearance of the *X. dendrorhous* cells before and after CO_2-SCFE. The discolouration of the cells occurs after CO_2-SCFE treatment resulted from astaxanthin being extracted from the cell wall. The results obtained in this study are in line with previous works in this area, concerning the extraction of pigments and lipids. Previous research incorporated a cell pretreatment step using a bead mill to disrupt the *Phaffia rhodozyma* cells prior to CO_2-SCFE extraction (Lim et al., 2002). This strategy was found to increase astaxanthin extractability ~90% under optimised conditions (temperature = 40 °C, pressure = 500 bar). In another study, Duarte et al. (2017) investigated the use of CO_2-SCFE in combination with ultrasound treatment, to extract intracellular lipids from the yeast *Candida* sp. LEB-M3. They found that pretreatment with ultrasonication followed by CO_2-SCFE resulted in a relatively low lipid extractability (20 %) as compared to the conventional chemical extraction method, indicating that the ultrasonication method was not able promote significant cell rupture. Besides that, the effect of the pre-treatment step, the type of microorganism is likely to affect considerably the performance of the SCFE process. For example, an SCFE method was used to extract carotenoids from microalgae species, in particular *Haematococcus Pluvialis*. Under optimised conditions (Temperature = 55 °C, pressure = 20 MPa and EtOH = 13 %), 84% of astaxanthin extractability was obtained using CO_2-SCFE, using disrupted, freeze dried cells (Reyes et al., 2014). Overall, the present work as well as previous works demonstrate that there is significant scope for further research combining different pre-treatment methods (i.e. enzymatic, chemical, physical) with SCFE to maximise the extraction of astaxanthin from yeast cells.

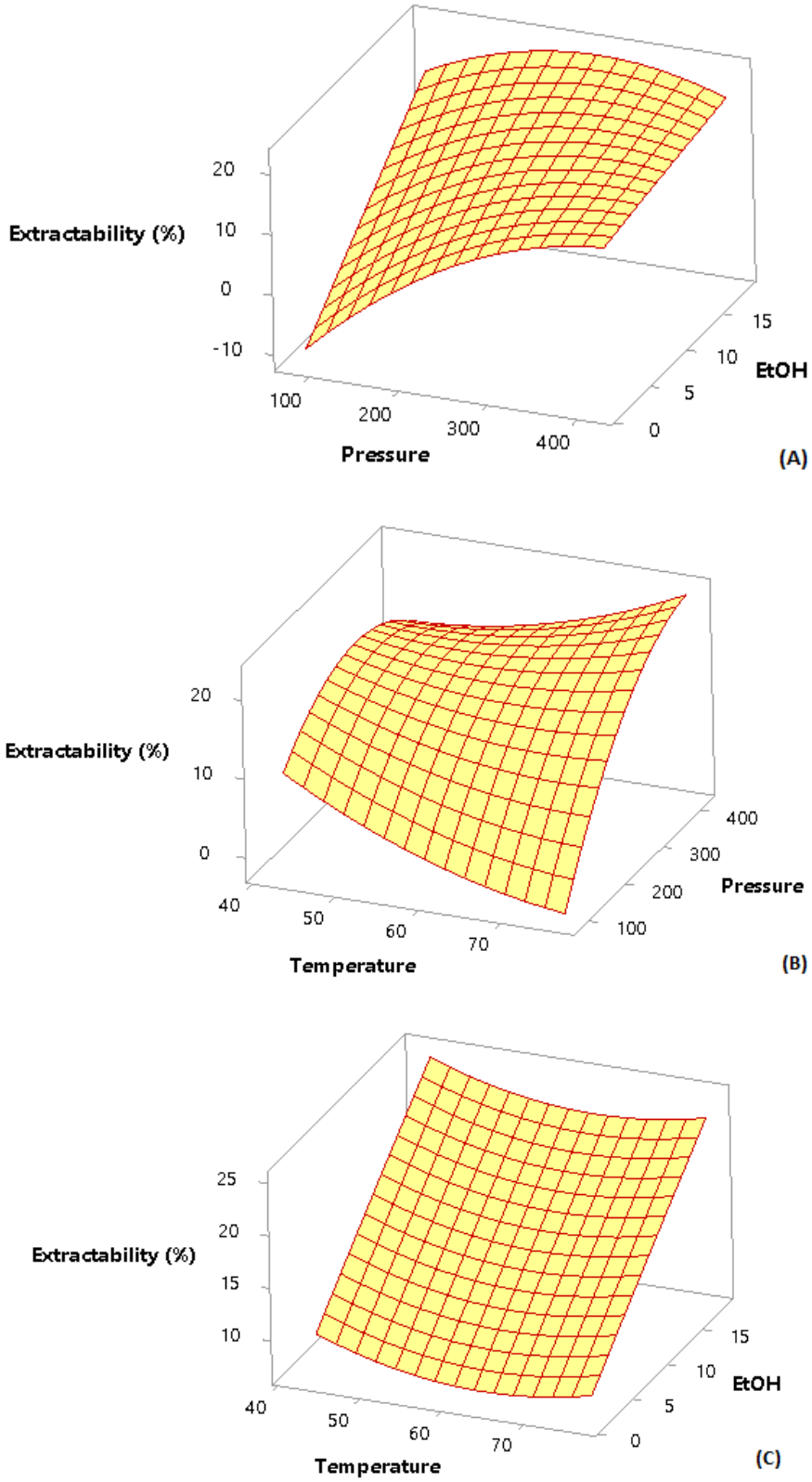

Figure 6-7: Response surface plots depicting the effects of temperature, pressure and ethanol concentration astaxanthin extractability (%): (A) Effect of pressure and ethanol, (B) effect of temperature and pressure and (C) effect of temperature and ethanol. Astaxanthin extraction was achieved by treatment of freeze dried *X. dendrorhous* cells with Accellerase 1500 (30 %) for 3 hours, followed by CO_2-SCFE extraction.

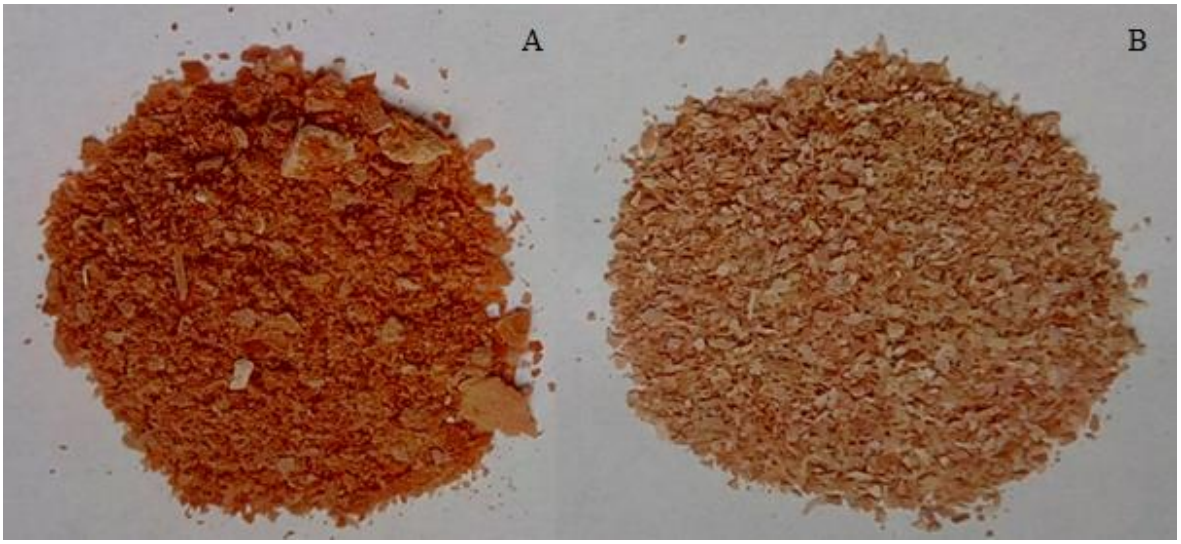

Figure 6-8: Differences in the freeze-dried *X. dendrorhous* cells: (A) cells treated with Accellerase 1500 (30% v/v) for 3 h before being subjected to CO_2-SCFE extraction; (B) after extraction with CO_2-SCFE using ethanol (15 %) as co-solvent.

6.4.1 SCFE Extraction of Astaxanthin after Using Different Enzymes for Pretreatment of Yeast Cells

The aim of this experiment was to investigate in more depth the effect of treatment, used as a pre-treatment step for improving CO_2-SCFE extraction of astaxanthin from *X. dendrorhous* cells. Two different concentrations of Accellerase 1500 were tested, i.e. 30 and 70 % (v/v), as well as Glucanex (5% v/v). Figure 6-9 depicts the kinetics of astaxanthin extraction during the CO_2-SCFE process. The CO_2-SCFE process was conducted for a longer period (80 min) than in the previous experiment in order to investigate the kinetics of astaxanthin extraction at the optimised condition identified previously (360 bar, 76.8°C and 18% EtOH). It was decided not to extend the extraction process for longer than 80 minutes as this would require additional amounts of solvent and energy rendering the process not economically viable (Andrade-Lima et al., 2018).

In the case of freeze dried cells without any pre-treatment, the maximum astaxanthin extractability obtained was 14% (Figure 6-9). In comparison, about 3.5 times higher astaxanthin extractability (~45%) was obtained when Accellerase 1500 (70%) was used as pretreatment step. It seems that by increasing the Accellerase 1500 concentration, a higher degree of cell wall rupture occurred, which allowed higher extraction of astaxanthin. Figure 6-10 shows the scanning electron microscopy images of intact *X. dendrorhous* cells before and after CO_2-SCFE extraction. It can be observed that during CO_2-SCFE extraction the cells' morphology changed significantly, particularly after enzyme pre-treatment (Figure 6-10 C, D) where a considerable level of rupture and shrinkage can be observed; the latter probably due to the release of intracellular components.

From the results in Figure 6-9, it can be observed that astaxanthin was extracted mostly at the beginning of the extraction (before 20 mins) process. At this stage, there did not seem to be considerable differences in rate of astaxanthin extraction regardless of the enzyme used. This phase is known as the Constant Extraction Rate (CER), a condition where easily accessible solute is dissolved and extracted. This process was facilitated by a mass transfer mechanism (convection) from the solid phase to the fluid phase, and extraction was limited by astaxanthin solubility in the CO_2-SCFE (Silva et al., 2016). After 20 min, the rate of astaxanthin extraction started to decrease, most likely due to the decrease in the concentration of available astaxanthin within the cells, as the process continued. This is a slow process which is driven by diffusion and convention and is known as the Falling Extraction Period (FER). In this region, once the easily available pigments are depleted, CO_2 has to be diffused into the cell wall, dissolve the pigments and diffuse out. In the final stage of the extraction process is known as the Low Extraction Rate (LER) or diffusion-controlled phase (DC); during this stage the extraction rate is even lower than during the FER phase, and mass transfer occurs mainly by diffusion inside

the yeast cells (Silva et al., 2016; Andrade-Lima et al., 2018). This stationary phase. Overall, the optimum time for astaxanthin extraction was found to be between 60-70 mins.

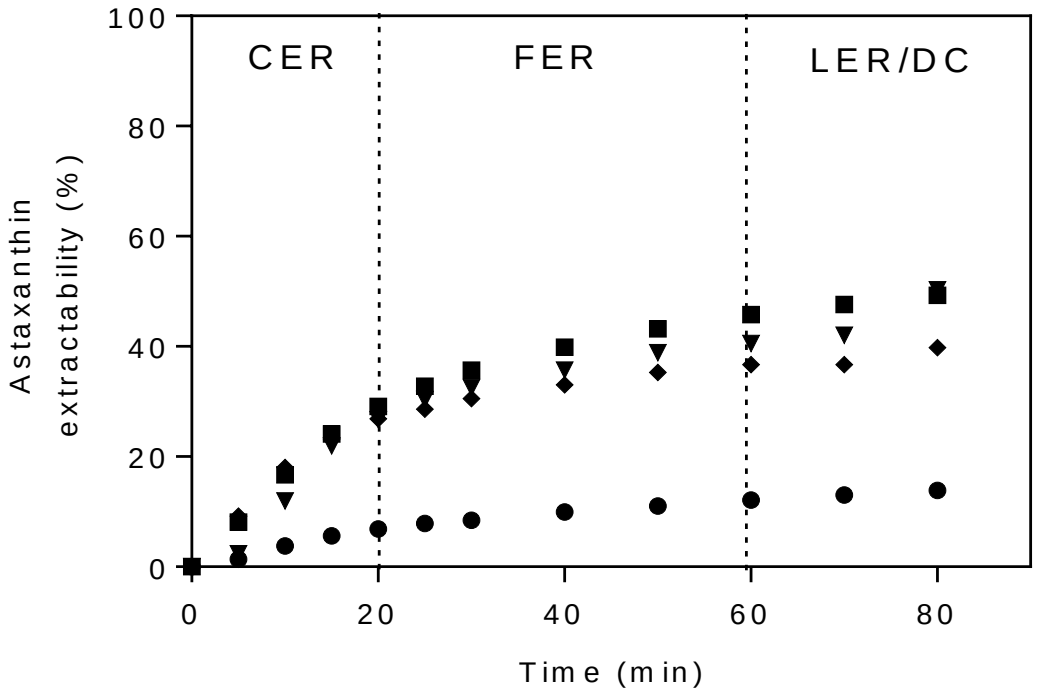

Figure 6-9: Kinetics of CO_2-SCFE extraction of freeze-dried *X. dendrorhous* cells, pre-treated with different enzymes. Symbols represent (■) 70% Accellerase, (▼) 30% Accellerase, (◆) 5% Glucanex (●) control- no pretreatment. CER = constant extraction rate, FER = falling extraction period and LER = low extraction rate and DC = diffusion-controlled phase

Comparing the results from this CO_2-SCFE experiment to the previous experiment where Glucanex treatment was used followed by acetone extraction (section 5.3.2), it can be observed that lower astaxanthin extractability was obtained in the former case (~45% vs ~100%, respectively). Theoretically, once the cells were ruptured, the permeability of the solvent through the cell wall as well as the solubility of astaxanthin in this particular solvent should dictate to a large extent the astaxanthin extractability values obtained. The lower astaxanthin extractability (%) obtained in the case of the CO_2-SCFE experiment might be due to the lower solubility of astaxanthin in ethanol that was used as co-solvent, compared to acetone. Nevertheless, astaxanthin extraction by CO_2-SCFE has a lot of potential for large-scale operations taking into account the potential environmental advantages (less solvent) of such green technology as well as the lower processing time compared to other methods, e.g. the glass beads treatment followed by acetone extraction or the enzyme treatment (e.g. Glucanex) followed by acetone extraction. Another advantages of this process is that the processing conditions (e.g. pressure, temperature, co-solvent type and concentration) can be tailored for the extraction of targeted pigments present in complex mixtures, such as in microbial cells or plant materials. This would simplify the subsequent purification process.

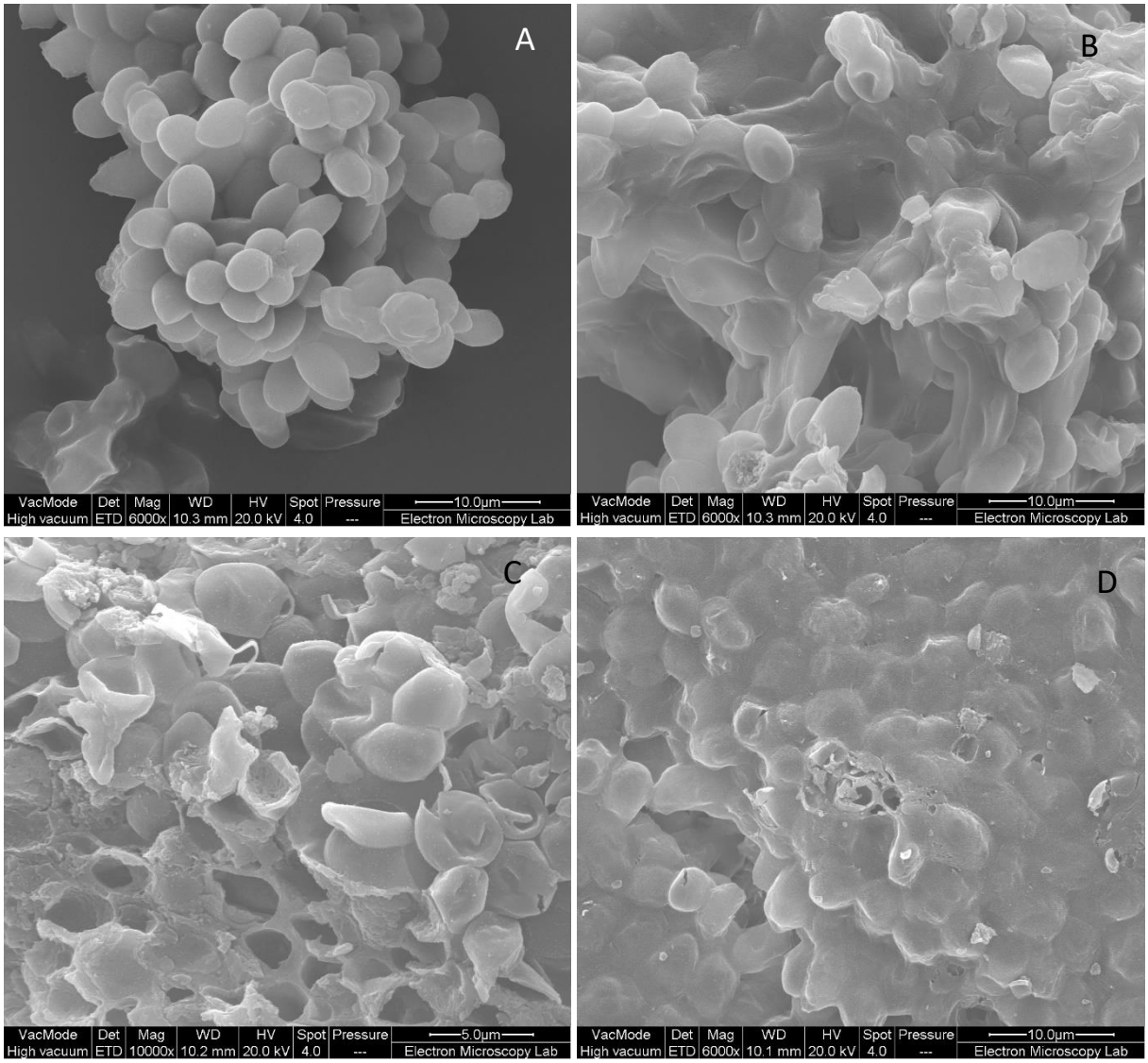

Figure 6-10: SEM images depicting *X. dendrorhous* cells before and after pre-treatment: (A): Freeze dried *X. dendrorhous* cells, (B): Freeze dried *X. dendrorhous* cells (no pretreatment) after CO_2-SCFE extraction (control cells), (C): Freeze dried *X. dendrorhous* cells (pretreated with Accellerase, 30 % v/v) after CO_2-SCFE extraction , (D): Freeze dried *X. dendrorhous* cells (pretreated with Glucanex, 5 % v/v) after CO_2-SCFE extraction.

6.5 Conclusions

The results presented in this study allowed an assessment of a number of strategies for the extraction of astaxanthin from *X. dendrorhous* cells. The best extraction strategy involved the use of Glucanex (5% w/v) followed by acetone extraction; this resulted in even higher astaxanthin extraction, compared to the standard extraction method (DMSO/acetone). The use of Accellerase (30% v/v) to disrupt the cell wall did not help in extracting astaxanthin (using acetone as the solvent) mainly due to the different enzyme activities present in the two enzymes, and particularly the low β-1, 3 glucanase activity of Accellerase 1500. Glass beads accompanied with acetone extraction was a simple and effective method to rupture the cells and extract astaxanthin, as resulted in extractability of higher than 80%. CO_2-SCFE was also evaluated as it is a much more environmentally friendly method due to the considerably lower amounts of solvent used compared to the above methods. The method showed potential although an enzymatic pre-treatment step was deemed necessary to rupture the cell was structure and reach extractability values of ~ 45%. Further studies are needed to optimise the method and increase the astaxanthin extractability.

7 General discussion and recommendations for future studies

7.1 General discussion

One of the challenges in natural astaxanthin production is associated with its expensive production route compared to synthetically produced astaxanthin, as it involves a series of processes including fermentation and downstream processing. It is uncertain whether natural astaxanthin can be produced at a low cost. Therefore, this current work aims to investigate an alternative route for the sustainable production of microbial astaxanthin that can compete with the synthetically produced astaxanthin by investigating three main key process stages, including: (i) the lignocellulosic hydrolysis, (ii) fermentation process and (iii) pigment extraction (downstream processing). This thesis generated new scientific knowledge relevant to both academic and scientific communities, and more specifically in (i) identifying a potential processing route for rapeseed meal hydrolysis into fermentation nutrients using enzyme technology; (ii) demonstrating that rapeseed meal is suitable to be valorised as substrate for astaxanthin production by the yeast *X. dendrorhous* DSMZ 5626; and (iii) investigating different methods for maximising astaxanthin extraction yield from yeast cells.

Generally, renewable resources such as rapeseed meal, a by-product of rapeseed processing industry hold a potential to serve as cheap and sustainable substrates for microbial conversion into astaxanthin by yeast species. The selection of rapeseed meal as a starting material in this study was based on the abundance of rapeseed meal that is generated annually in the United Kingdom. Currently, rapeseed meal is used as a feed for livestock as it is high in protein. In order to increase the value of rapeseed meal, research has been carried out worldwide, mainly focusing on valorisation of its protein content. On the contrary, in this current study, we aim to explore the potential of rapeseed meal as sole fermentation substrate

for natural astaxanthin production by yeast. To our knowledge, the utilisation of rapeseed meal as carbon source for microbial astaxanthin production has not been reported elsewhere. Based on the findings of this current study, we aim to be able to propose a strategy for the exploitation of rapeseed meal as raw material for natural astaxanthin bioconversion process.

One of the major tasks carried out in this study was the enzymatic hydrolysis of rapeseed meal into nutrient-rich hydrolysates that could be used as sole nutrient source for *X. dendrorhous*. One of the challenges in this work was to select suitable enzymes that can hydrolyse the rapeseed structure and lead into a liquid nutrient rich medium that could further support yeast growth as well as intracellular astaxanthin pigmentation. It was proved that the selection of enzymes for rapeseed meal hydrolysis had its impact on supporting the yeast growth and astaxanthin pigmentation. The use of Viscozyme L (an enzyme cocktail that has multiple activities) on rapeseed meal resulted in sufficient hydrolysis yield but the subsequent hydrolysate was not suitable to support yeast growth and astaxanthin pigmentation in *X. dendrorhous*, possibly due to the presence of growth inhibitors that were released during hydrolysis stage as well as due to Crabtree effect during fermentation. Further investigation on biomass pretreatment focusing on thermal pretreatment prior to enzyme hydrolysis was performed to increase the cellulose hydrolysis rate. This thermal pretreatment using autoclave is considered as a simple and mild pretreatment strategy to adopt as it does not require special reactor and exclude the use of acid or alkali that could influence the hydrolysates produced in later stage. The thermal pretreatment acts by disrupting the hydrogen bonds that hold the crystalline structure of cellulose and lignin matrices together and subsequently causes a swelling in the biomass and a disruption of the cellulose structure. The increased solubility of the lignin will then allow a better accessibility of the enzymes towards its specific site (Brodeur et al., 2011).

To further investigate the scalability of astaxanthin production in *X. dendrorhous* with rapeseed meal hydrolysates, bioreactor studies are important as the output will determine the feasibility of this process for scaling up towards commercialisation. The key parameters investigated included pH and agitation, which were proved to play major role in maximising astaxanthin production in yeast. Higher agitation rate was required to provide enough oxygen as well as ensuring better heat and mass transfer and subsequently increase yeast growth and astaxanthin production. The pSSF approach is an improvement of simultaneous saccharification and fermentation (SSF), which was not found suitable in this study due to large differences between optimal temperatures for yeast growth and enzymatic activity. The objective of this study was to provide a new approach for a simplified fermentation process. This pSSF approach for astaxanthin production has not been reported before. However, results showed that this approach was not suitable for astaxanthin production in yeast as it leads to additional hurdles in the intracellular astaxanthin extraction process, as the separation between rapeseed meal biomass and yeast was difficult to be carried out.

The last part of this current study focused on downstream processing for the extraction of intracellular astaxanthin from yeast cells. Yeast derived astaxanthin is characterised as natural and can be used as an ingredient in products formulation including feed, food and as a colourant. The experiments demonstrated that the application of mechanical extraction (glass beads) gave high degree of cell disruption and subsequently increased astaxanthin extraction by solvent. This is considered as a feasible method to be used in large scale as it is a simple, cheap and reliable and does not require special instrument. One disadvantage of glass beads cell disruption is that it requires longer time when processing a high density of cells. As for enzymatic cell lysis, two different enzymes (Acellerase 1500 and Glucanex) were investigated for their efficacy to hydrolyse the yeast cell wall glucans. The application of enzyme cell lysis

with high β-glucanase activity managed to successfully lyse the yeast cell wall and subsequently increase the permeability of acetone for astaxanthin extraction. The setback that hindered the use of enzyme for scaling up process is the high costs that are associated with the provision of the selected enzyme.

On the other hand, the use of CO_2-SCFE for astaxanthin extraction from *X. dendrorhous* failed to meet expectations. Even with modification by utilising ethanol as co-solvent and yeast pretreatment prior to extraction, the astaxanthin extractability was low compared to other strategies previously mentioned. The complex and rigid structure of yeast cell wall that is composed of mannose, β-glucans and chitin hinders the effectiveness of CO_2-SCFE strategy. However, there are possible improvements that can be performed to increase the astaxanthin extractability in CO_2-SCFE, such as selection of suitable pretreatment method to adequately disrupt the cell wall structure of *X. dendrorhous* prior to CO_2-SCFE such as glass beads and ultrasonication.

The schematic detailing of unit operations for the proposed astaxanthin production in *X. dendrorhous* DSMZ 5626 as discussed in this study is outlined in Figure 7.1. This diagram also points out the possible improvements that can be performed to increase the feasibility of the overall process (shown in dotted line). Generally, the results obtained in this study suggested that the wild strain of *X. dendrorhous* DSMZ 5626 used was able to produce astaxanthin to satisfactory levels (240-250 µg/g of yeast dry weight) in a 2-litre stirred tank bioreactor, using solely the enzymatically produced rapeseed meal hydrolysates. However, there is a room for improvement in order to increase astaxanthin production using rapeseed meal hydrolysates in this particular process.

The first improvement that can be performed is to separate between the hull and seed meal using mechanical sieve prior to milling process. In this study, it was observed the leftover debris after enzymatic hydrolysis in the reaction vial consists of the hull part rather than the seed part. It shows that the hull part is more difficult to be hydrolysed as compared to seed meal. By separating the seed and hull part, different approach can be performed on each material. The seed part that composed of radicle and cotyledon can be directly subjected towards mild enzymatic hydrolysis, whereby the hull part is subjected towards pretreatment process. As the composition of the hull part is hard and complex as it composed of very strongly associated cell wall polysaccharide, harsh pretreatment strategies such as steam explosion could be applied to break down or solubilize the structure prior to enzyme hydrolysis, to maximise sugar recovery from the hull part. As for the seed part, mild enzymatic treatment is able to breakdown its structure into sugars and amino acids. Therefore, by separating these two components of rapeseed meal, higher sugar yield could be obtained whilst maintaining the quality of hydrolysates produced as not the whole rapeseed meal are exposed to harsh conditions during pretreatment, that could lead to production of HMF and furfural. On the downstream processing stage, further studies on the astaxanthin extract could be performed to investigate the stability of the extract in different types of solvents. Investigating the performance of the extracted astaxanthin in product formulation such as in feed and colourant, is a key next step for this research work.

The economic feasibility of the overall process of astaxanthin production using the rapeseed meal hydrolysates depends on various factors. In 2017, the price of rapeseed meal was about 225 USD per tonne, which is cheaper than soybean cake (336 USD per tonne) (FAO, 2018). This raw material is generally cheaper than the cost of the chemicals required for the semi-defined media formulation. Even though cheap substrate is used to replace refined sugars

during fermentation, but the enzymes used in this study are costly. The enzymatic hydrolysis step contributes to the major operational cost involved in astaxanthin production in *X. dendrorhous* as compared to the fermentation and the extraction process. As an example, the cost of the Pectinase enzyme itself is about 1080 USD per litre. Moreover, the hydrolysis yield of rapeseed meal into monomeric sugars is at minimal values with < 55% glucose yield. Hence, improvement of the process need to be performed to maximise the sugars hydrolysed, subsequently higher astaxanthin yield could be obtained, making the overall process more valuable. Besides that, life cycle assessment analysis (LCA) is necessary to be conducted to measure the impacts of every step in the life cycle of a product, of the whole process starting with the extraction of the raw material up to disposal of the product. LCA is known the common decision support tool for the industry to assess the impacts and viability of a process (McIntosh and Pontius, 2017).

After considering all the aspects investigated, the commercialisation of astaxanthin production rom *X. dendrorhous* DSMZ 5626 using the rapeseed meal is not an economically feasible to be commercialised unless improvement on the process is carried out. There are several aspects that needs to be focused, especially on minimising the cost of overall process prior to potential commercialisation. However, the general process involves in this study provides a scientific knowledge on the possible route for the sustainable production of natural astaxanthin in yeast. Thus, it can be concluded that this current study serves as a preliminary investigation towards the commercialisation of natural astaxanthin produced from *X. dendrorhous* using rapeseed meal hydrolysates as the fermentation substrate.

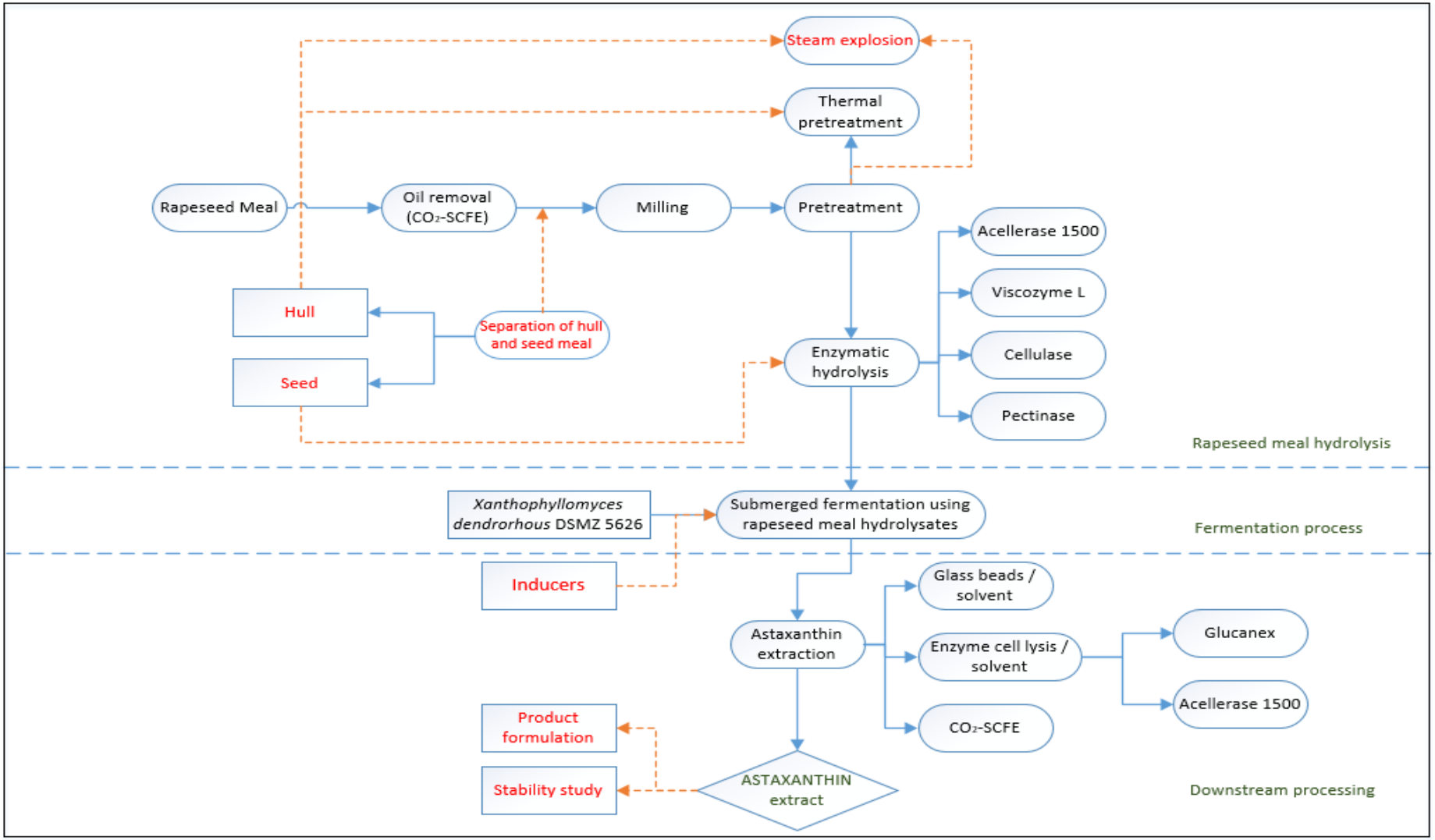

Figure 7-1: The schematic detailing of unit operations for the proposed astaxanthin production in *X. dendrorhous* DSMZ 5626 using rapeseed meal as substrate as discussed in this study. The diagram contains proposed improvements that could be performed to increase the process efficiency and are presented in the dotted line.

7.2 Recommendations for further studies

Although the study presented in this thesis has established the potential for utilising rapeseed meal for the microbial production of astaxanthin by *X. dendrorhous*, it only represents a preliminary investigation. Some limitations were identified in this study that could potentially lead to future research. A Crabtree effect in this yeast was observed to occur during the fermentation. However, the mechanism of the Crabtree effect in *X. dendrorhous* needs to be fully characterized, including the determination of the critical threshold limit and the key enzymes involved during the yeast metabolism. A complete understanding of the genes responsible for the induction of this effect will lead to opportunities for process optimisation that can completely eliminate this effect.

Addition of inducers (acetic acid, hydrogen peroxide and ethanol) was found to increase astaxanthin pigmentation in the cells. However, the mechanism behind this induction process was not investigated in this work. A study involving investigation at the gene level will give more insight into potential mechanism and understanding of the induction process. Besides that, strain improvements such as using chemical mutagenesis or genetic engineering to produce a yeast strain that has a higher capability to produce the astaxanthin could be performed. The improved strain could make the overall fermentation process more cost effective along with a higher productivity. Nangia et al., (2016) investigated the use of ultraviolet (UV) as a mutagen to improve astaxanthin production in *Phaffia rhodozyma*.

In the current study, the thermal pre-treatment step (126 ^{o}C, 30 min) was conducted as a simple pre-treatment strategy aiming at a subsequent increase in the enzymatic hydrolysis by the Accelerase 1500. However, this pre-treatment strategy resulted in only a 25% increment as compared to control (no-pretreatment), giving a maximum glucose yield of 40 % (w/w). This

value is considered to be low, and the yield can be increased by utilising a more suitable pre-treatment strategy. Therefore, other pre-treatment strategies such as steam explosion, acid or alkali pre-treatment, as well as biological pre-treatment by fungi can be explored for their feasibility to increase the hydrolysis rate of the rapeseed meal and subsequently be used for the microbial astaxanthin production.

Moreover, an improvement in the astaxanthin accumulation in the *X. dendrorhous* using the rapeseed meal hydrolysates can be performed by using a fed-batch or continuous fermentation approach. In these approaches, different variations of the feeding solutions can be studied. In addition to the carbon these feeding solutions should contain nitrogen sources as well so as to sustain the growth and the astaxanthin production in the yeast.

Utilising the CO_2-SCFE as a green technology for astaxanthin extraction on its own is not a feasible strategy as demonstrated in this study. However, there is a lot of potential for this method and should be further investigated. In the current study, the astaxanthin extractability (%) was improved when the enzyme-assisted CO_2-SCFE was applied. Further work on different yeast pre-treatment strategies prior to the CO_2-SCFE should be carried out. Different cell disruption strategies such as glass beads, ultrasonication or thermal pre-treatment prior to the CO_2-SCFE can be applied as pre-treatment strategies to improve astaxanthin extraction during CO_2-SCFE. Besides that, the extracted astaxanthin should also be assessed for its stability in various solvents.

Future studies should aim to improve the biotechnological route for the production of astaxanthin by *X. dendrorhous* DSMZ 5626. Starting right from the selection of the raw materials to the astaxanthin extraction process, each step needs to be carefully examined in order to provide a sustainable route for the microbial production of astaxanthin that can compete with synthetically produced astaxanthin. As seen in this study, the rapeseed meal hydrolysate

has been confirmed as a feasible feedstock containing all the essential nutrients including carbohydrates, proteins and amino acids that are able to support the *X. dendrorhous* growth and support astaxanthin production as well.

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