Rapeseed meal hydrolysate as substrate for microbial astaxanthin production

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Abstract

Rapeseed meal, a by-product of oil processing industry, was evaluated as a substrate for astaxanthin production by the yeast *Xanthophyllomyces dendrorhous* DSMZ 5626. Four commercial enzymes were tested at different concentrations (1 – 15 %, v/v) for their ability to break down the cellulosic and hemicellulosic compounds of rapeseed meal into fermentable sugars. Viscozyme® L and cellulase demonstrated the highest glucose recovery yields (47 – 52%, w/w for 15 % (v/v) of enzyme loading) with 7- 11 g/l of net glucose released in the hydrolysates. Pectinase and Accellerase® hydrolysates supported the best cell growth and astaxanthin production in batch shake flask cultures, with maximum biomass of 26 g/l and 15 g/l, respectively, and astaxanthin yields ($Y_{P/X}$) of 258 to 332 µg per g of biomass. In batch bioreactor trials, pectinase hydrolysates resulted in high biomass (42 g/l) and astaxanthin production (11 mg/l) aided by the presence of glycerol (originating from the enzyme formulation) which served as additional energy and carbon source. Finally, simple glass beads disruption lead into satisfactory astaxanthin extraction (95 %, w/w) in acetone. The findings of this study generate knowledge towards scale-up potential of microbial astaxanthin production using rapeseed meal hydrolysate as fermentation feedstock.

Keywords: Astaxanthin, rapeseed meal, fermentation, hydrolysate, extraction, *Xanthophyllomyces dendrorhous*

1 Introduction

Oilseed crops, such as rapeseed, sunflower and soybean, are extensively cultivated for their oil. 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 [1]. 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 [2].

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 [3]. The world production of rapeseed meal in 2016/17 was estimated at 40 million tonnes, with 13.9 million tonnes generated in the EU [1], a rise linked with intense rapeseed farming to meet the increasing demand for vegetable oils. This in turn suggests that opportunities still exist for developing alternative uses for rapeseed meal beyond animal feed [4]. Currently, rapeseed meal is used as an organic fertiliser and as a supplement in livestock feed due to its high protein content (26-31%) [5,6]. However, its 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 [7]. Moreover, rapeseed meal may contain anti-nutritional compounds such as glucosinolates, erucic acid and phenols, thus making it less favourable for livestock [4,8].

There is significant potential for the valorisation of rapeseed meal, particularly as a substrate for microbial bioconversions, since besides protein, it also contains large amounts of carbohydrates (36-36%, w/w) [6,9] as well as minerals such as calcium, phosphorous and iron [6,10]. The utilisation of rapeseed meal in microbial bioconversions is not a single step process, since most microorganisms lack the necessary enzymes for cellulose/hemicellulose 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 [7,10]. The rapeseed meal hydrolysates can be potentially used for several microbial conversions, including the production of platform and speciality chemicals such as succinic acid [11], 1,3-propanediol [10], biopolymers (poly-hydroxy-alkanoates)[12] and lipids [7].

Astaxanthin belongs to the xanthophyll group of carotenoids and is a powerful antioxidant, which acts by donating 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 organisms [13]. The application of an external source of antioxidants such as astaxanthin is necessary for human body to regulate the free radicals and suppress the occurrence of oxidative stress which subsequently can alter lipids, proteins and DNA, as well as trigger human diseases [14]. Astaxanthin also possesses anti-inflammatory activities and has potential as a therapeutic agent against cardiovascular diseases in humans [15]. Additionally, astaxanthin is used in aquaculture 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 [16,17]. Currently, the commercial demand for astaxanthin is met by its synthetic production (petroleum derived) via the Wittig reaction and Grignard condensation methods [18]. 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 exhibits prolonged shelf life [19]. 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 their complex production process. The value of natural astaxanthin is estimated around USD 2500–7000 per kg, depending on its purity, as opposed to the value of synthetically-produced astaxanthin, which is estimated around USD 2000 per kg [20,21].

Studies aiming to extract rapeseed protein have been conducted using various methods such as commercial cellulolytic enzymes [22] as well as the use of the proteolytic fungus, *Aspergillus oryzae* [10]. However, studies involving carbohydrates hydrolysis from rapeseed meal are scarce in

comparison to protein hydrolysis [22,23]. Thus, the aim of this study was to enzymatically hydrolyse the lignocellulosic components of rapeseed meal and produce a nutrient-rich hydrolysate, which was subsequently evaluated as a fermentation substrate for astaxanthin production by the yeast *X. dendrorhous* DSMZ 5626. In addition, the application of glass beads was investigated as a simple method for the extraction of intracellular astaxanthin from yeast cells.

2 Materials and Methodology

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).

2.2 Materials

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

2.3 Enzymatic Hydrolysis

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 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). Distilled water was used to make up the volume to 1.5 ml. 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 h to 24 h and the reactions were terminated by incubation at 95 °C for 10 min. The mixtures were then centrifuged at 10 845 x *g* (Heraeus Multifuge X3R, Thermo Fisher, USA) for 10 min and the supernatants were collected and analysed for sugar composition using HPLC as described in section 2.7. The schematic diagram of the experimental procedure is illustrated in Figure 1.

2.4 Rapeseed meal pre-treatment

The thermal pre-treatment of rapeseed meal was carried out prior to enzymatic hydrolysis using the commercial enzyme Accellerase 1500®. 10% (w/v) of rapeseed meal were added to 30 ml of distilled water and subjected to autoclaving at different temperatures (126 and 135 °C, pressure ~2 bar) for 15, 30 or 60 min. Once the heat treatment was completed, 10% (v/v) of enzyme was added to the mixture and hydrolysis was carried out at 50 °C for 24 h under continuous stirring (250 rpm) in an orbital shaker (GFL, 3015, SciQuip, UK).

2.5 Microbial Fermentations

2.5.1 Shake flask fermentation

In order to investigate the suitability of rapeseed meal hydrolysates as substrates for *X. dendrorhous* growth, batch shake flask experiments were conducted. Rapeseed meal hydrolysates were prepared using different enzymes under identified optimised conditions. In one case, rapeseed meal was thermally pre-treated, by autoclaving at 126°C for 30 min prior to enzyme addition (10 %, v/v of Acellerase® 1500). 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 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.

2.5.2 Batch fermentation (Separate hydrolysis and fermentation)

150 g of defatted rapeseed meal 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, 150 ml of enzyme (10% v/v) were added to the vessel (giving a total of solids content 10% w/v). 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 aseptically transferred to a 2-litre bioreactor (BIOSTAT B, Sartorious AG, German) with a working volume of 1.5 litres, previously autoclaved at 121°C for 20 min. Fermentation conditions were as follows; temperature, 20 °C; agitation speed, 600 rpm; aeration, 1 l/min. The pH of the hydrolysates was maintained at pH 6 throughout the fermentation by automatic additions of NaOH (2 M) via a peristaltic pump.

2.5.3 Batch fermentation (Pre-hydrolysis and fermentation)

In pre-hydrolysis and fermentation (pSSF) approach, enzymatic hydrolysis and subsequent fermentation of rapeseed meal 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 were added to the reactor with 1.35 litre of distilled water. The bioreactor was then subjected to autoclaving at 126 °C for 30 min. After cooling, 150 ml of enzyme were added to the fermentation vessel (giving a total of solids content 10% w/v) 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* pre-culture suspension. Fermentation conditions were as follows; temperature, 20 °C; pH 6; agitation speed, 600 rpm; aeration, 1 l/min.

2.6 Glass bead / solvent extraction

Approximately 30 mg freeze dried *X. dendrorhous* cells were prepared in 2 ml Eppendorf tubes. 0.3 g of glass bead (diameter 1 mm) were added to the vials followed by addition of 1 ml organic solvent. The yeast and beads suspension were mixed at 2000 rpm for 10 min in a ThermoMixer C (Eppendorf, Germany) to ensure similar rate of mixing for each sample. The extraction temperature was controlled at 25 °C. Subsequently, 0.1 ml of NaOH (20% w/v) was added to the mixture, followed by further mixing for 5 min at 2000 rpm in a ThermoMixer C. The aqueous and organic phases were separated by centrifugation at 5423 x g for 5 min and organic phase (top layer) was collected for astaxanthin measurement by spectrophotometer. The organic solvents evaluated in this experiment were 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); distilled water was used as control/

2.7 Solubility of Astaxanthin in Solvents

The solubility of astaxanthin in organic solvents was tested. As such, 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 water were used as solvents. Approximately 10 mg of pure astaxanthin (Sigma Aldrich) were added to 1 ml of solvent in 2 ml (Eppendorf) tubes and were then agitated at 1200 rpm for 24 h 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 filtered through a 0.2µm Polyvinyl Difluoride (PVDF) membrane filter. Finally, the concentration of astaxanthin in different types of solvents was determined using HPLC analysis.

2.8 Analytical Methods

The carbohydrate content of the rapeseed meal was modified based on analytical procedure provided by National Renewable Energy Laboratory (NREL/TP-510-42618) [24]. Briefly, 300 mg of rapeseed meal was pre-hydrolysed with 3 ml of 72 % (v/v) H_2SO_4 at 30 °C for 1 h. Subsequently, 84 ml of distilled water were added to the mixture in order to dilute the sulphuric acid content to 4 % (v/v) and hydrolysis was carried out at 121 °C for 30 min. The obtained mixtures were neutralised with calcium carbonate to pH 5-6 and were analysed using a High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) system (Dionex, Thermo, UK). 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: 25°C; flow rate: 1 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 Kjeldahl and Soxhlet method, respectively (AOAC, 1990) and the lignin content was determined according to NREL protocol [24]. After hydrolysis with 72 % (w/w) H_2SO_4 for 1 h at 30°C, samples were hydrolysed with 1.0 M H_2SO_4 at 121 °C for 30 min 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 equation 1 (*Eq 1*). The washed residue was dried at 100 °C for 18 h. Subsequently, the dried samples were placed in a furnace (500 °C; 5 h) and the residue was weighed and classified as acid insoluble lignin. Total lignin was calculated as the sum of acid soluble and acid insoluble lignin.

$$\% \, ASL = \frac{A \times DF \times V(ml)}{\varepsilon \times P \, (g)} \times 100 \qquad Eq\ 1$$

Where, *A* is the absorbance at 240 nm, DF is the dilution factor, *V* is the volume of filtrate, ε is the coefficient at 30 and *P* is the weight of biomass.

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-weight 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 frozen at -20 °C prior to freeze drying (Virtis Sentry 2.0, UK). Biomass was measured by weight difference. In bioreactor experiments that involved pre-hydrolysis and fermentation steps, cell growth was monitored by total plate count method. Hence, 100 µl of serially diluted samples were plated on Yeast and Mold agar plates (YM agar), incubated at 20°C for 5 days and biomass was expressed in CFU/ ml. 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. Quantification 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 was quantified using Dimethyl Sulfoxide (DMSO) disruption method [26]. 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 after centrifugation at 5423 x *g* for 5 min. The extraction process was repeated until a colourless biomass was obtained. The organic phases were then pooled together, and their absorbance was 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 in equation 2 (*Eq 2*) [26].

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

Where, *A* is the absorbance at 480 nm, *V* is the volume, and *P* is the weight of biomass and $A_{1cm}^{1\%}$ is the coefficient of 2150 (based on determination using synthetic astaxanthin) [26].

2.9 Scanning Electron Microscopy

The morphology of the cell biomass samples prior and after extraction were 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 voltage, 4.0 spot size and a working distance approximately 10 – 12 mm. Images were recorded under vacuum at 6000 x magnification.

3 Results and Discussion

3.1 Compositional analysis of rapeseed meal

During 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 correspondence [27], the rapeseed meal used in this study contained substantial amounts of oil (16.7 ± 0.1 %). Table 2 shows the composition of 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 in the literature [6] (i.e., 30 %, w/w), possibly due to differences among rapeseed cultivars as well as due to differences in the oil extraction process [28].

The carbohydrate content was similar to that reported in the literature accounting for 36 % (w/w) [6]. 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. It was reported that arabinose is the major hemicellulosic sugar present in rapeseed meal, suggesting the presence of arabinans, which might be attached in the side chains of pectin [6]. Besides, the presence of xylose and arabinose probably originates from arabinoxylan structure in rapeseed meal. Arabinoxylan is a hemicellulose consisting of a linear backbone of β-1,4 xylose residues with arabinose substitution [29].

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 [6, 30]. Small amounts of fructose (0.3 % w/w) were detected in the acid treated fraction of the rapeseed meal, in agreement with previously reported data in the literature [4,30]. In the water soluble fractions of rapeseed meal, small amounts of sucrose were also detected (data not shown). Sucrose and fructose have been previously reported in the water soluble fractions of rapeseed meal and normally act as an energy reserve in plants [4,6].

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 those (~16 %) reported by studies investigating the production of biodiesel from rapeseed meal [31,32].

3.2 Enzymatic hydrolysis of non-treated and thermally pre-treated rapeseed meal

Rapeseed meal contained high amounts of carbohydrates and protein, however, most microorganisms cannot assimilate these compounds directly as they usually lack of cellulolytic and proteolytic enzymes in their cluster [10]. Therefore, commercial enzymes were employed, targeting the conversion of cellulose and hemicellulose into 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 2 shows the effect of different enzyme concentrations on the release of monomeric sugars after 24 h of hydrolysis using four different commercial enzymes (cellulase, pectinase, Viscozyme® L and Accellerase® 1500). The sugar yield was defined as g of sugars released after 24 h of hydrolysis per g of sugar originally present in the rapeseed meal. The highest glucose yield was observed by Viscozyme® L (53 %, w/w), followed by cellulase (47 %, w/w), pectinase (41.8 %, w/w) and Accellerase® 1500 (30 %, w/w). Viscozyme® L is a multi-enzyme complex containing mixtures of cellulases, hemicellulases and xylanase and has also a proteolytic activity [22,33,34]. Hence, the synergistic effect of these enzymes rendered the rapeseed meal structure more accessible to enzymatic attack, 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 2A).

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 [35,36]. Endo-cellulases attack the β-1,4-glycosidic bonds randomly, targeting the amorphous region along the cellulose structure and produce either cellobiose or glucose. On the other hand, exo-cellulases act on the linkages from the non-reducing end of cellulose releasing cellobiose as the main reaction product [36]. Similar patterns were observed for

the hemicellulose-derived monosaccharides in this study. 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 produced by cellulase (Figure 2C).

In terms of sugar concentrations in the hydrolysates, glucose and galactose were mainly present. In the case of Viscozyme® L hydrolysates (Figure 2A), 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 in enzyme formulations. In the case of pectinase (Figure 2B), 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 2C), 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 treated hydrolysates, 11 g/l of total sugars were present, with glucose (~ 6 g/l) and galactose (~4 g/l) being the most prominent sugars. The synergistic action of enzyme mixtures assists in further break down of carbohydrate polymers, as indicated by the action of pectinase on rapeseed meal. This was demonstrated by a greater hydrolysis yield of rapeseed meal, as 41 % (w/w) of glucose was extracted using pectinase enzyme, which contained a mixture of side activities including cellulase and hemicellulase. In contrast, Accellerase® 1500 treatment that did not exert pectinase activity, resulted in lower glucose yields (30 %, w/w). The enzymatic hydrolysis of rapeseed meal has been widely reported on the rapeseed protein extraction rather than on carbohydrates. Three different enzymes (Celluclast, Viscozyme® L and Pectinase G) were tested, both singularly and in combination, in order to hydrolyse dilute acid pretreated rapeseed meal that was subsequently used for succinic acid production by *Actinobacillus succinogenes* [11]. Around 28.6 g/l of total sugars

were released from rapeseed meal after Pectinase G (2 %, w/w) hydrolysis that consisted of sucrose, glucose, fructose and arabinose [11]. In another study, Viscozyme® L was found to assist in protein hydrolysis from rapeseed meal with high protein yield (68 %) and total carbohydrates yield (80 % of total reducing sugars) [22].

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 rapeseed meal derived sugars. As such, a thermal pre-treatment step was carried out to evaluate its effectiveness towards enhancing the enzymatic hydrolysis of rapeseed meal. Table 3 presents the composition of rapeseed meal hydrolysates following heat pre-treatment and enzymatic digestion by Accellerase 1500 (10 % v/v). Heat pre-treatment at 126 °C for 30 min was found to significantly increase ($p < 0.05$) glucose hydrolysis yield by 17 % compared to control (no pre-treatment). However, higher temperature conditions (135 °C for 30 min) 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 higher temperature in the presence of mild acid conditions. The acidic environment is 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 [54]. Similar to glucose, the recovery of the hemicellulosic-derived sugars also decreased when higher pre-treatment temperatures were applied.

3.3 Microbial Production of Astaxanthin in Rapeseed Meal Hydrolysates

3.3.1 Batch Shake flask fermentations

A key objective of this study was to investigate the suitability of rapeseed meal hydrolysates as 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, significantly different cell biomass and astaxanthin production values were obtained depending upon the hydrolysate. In terms of total biomass and astaxanthin production, the best substrate was the pectinase hydrolysate (~26 g/l of cell biomass, 6.7 mg/l of astaxanthin), whereas Viscozyme® hydrolysate had the poorest performance (~3 g/l of cell biomass, 0.4 mg/l of astaxanthin). However, in terms of biomass yield on substrate consumption ($Y_{X/S}$) and astaxanthin yield on cell biomass ($Y_{P/X}$), the highest values (0.84 g/g and 332 µg/g, respectively) were 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 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 [37–39]. 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 initial glucose concentration 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); correspondingly, a similar trend was observed in the latter.

However, in the case of the pectinase hydrolysate, no ethanol was detected, a fact which indirectly suggested that the Crabtree effect was suppressed as glycerol was the predominant carbon source in the medium rather than hexose sugars. As for Accellerase® 1500 hydrolysis, two types of rapeseed meal samples were used (crude samples and thermal pretreated samples). Between these two samples, different fermentation results were obtained where pretreated samples resulted in

higher biomass (12 g/l) and astaxanthin production (3.2 mg/l). It is interesting to note that ethanol was detected in this fermentation only in trace amounts (< 1 g/l).

The involvement of two possible mechanisms in the induction of pigmentation by ethanol has been proposed before [40]. 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 [40,41]. 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 mevalonate pathway [40]. 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 **Error! Reference source not found.**3 depicts the kinetic profile of *X. dendrorhous* cultivated in the pectinase hydrolysate in batch flasks fermentations. The pectinase 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 hemicellulose derived sugars. Once all sugars were exhausted from the hydrolysate, glycerol was utilised as an energy and carbon source for biomass production and astaxanthin accumulation. In contrast to the Accellerase® 1500 hydrolysate, the cells did not experience a lag phase in the pectinase hydrolysate. The cells continued to grow throughout the fermentation period (1 h - 120 h) and showed a growth pattern similar to diauxic growth, reflecting the sequential consumption of sugars and glycerol (Fig. 3B). 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 the glycerol, which was present in substantial amounts does not initiate a Crabtree effect. Astaxanthin was produced throughout the fermentation (including during the glycerol consumption phase) reaching 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 (Table 4). 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 reason for the lower astaxanthin yield could be the absence of certain minerals in the pectinase hydrolysate, which are important for carotenoid production [42]. 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* [43]. Moreover, high levels of antinutritional components such as glucosinolates and phenols, which are present in rapeseed meal might also inhibit carotenoid synthesis [44].

To our knowledge, no studies 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 [45]. In this study, the rapeseed meal hydrolysates were able to support higher astaxanthin production and high astaxanthin yields, indicating that this medium contained sufficient nutrients 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. The use of molasses demonstrated positive effects of yeast growth (3.5 g/l), but not on the astaxanthin formation (105 µg/g) [46]. Besides that, a mutant strain of *Xanthophyllomyces dendrorhous* was cultivated in a Yucca plant-based medium supplemented with date juice in 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) [47]. 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.

3.3.2 Batch fermentations (Separate hydrolysis and fermentation)

Fermentations were carried out in a 2-l stirred tank bioreactor to investigate the yeast growth and astaxanthin production under controlled conditions. In addition, 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 treated by different enzymes (Accellerase® 1500 and pectinase) were used.

Figure 4 shows the growth profile of *X. dendrorhous* in two different rapeseed meal hydrolysates and Table 5 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 14.3 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. The astaxanthin yield on cell biomass ($Y_{P/X}$), was equal to 250 µg/g of produced biomass. These values were higher compared to those obtained in shake flask fermentations utilising rapeseed meal hydrolysates as substrate (12 g/l of biomass and 3.2 g/l of astaxanthin). 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.

Batch bioreactor fermentations using pectinase derived hydrolysates were also carried out, with the hydrolysates consisting of 20 g/l of total monomeric sugars and glycerol (52 g/l) that was sourced from the enzyme formulation as previously mentioned. *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 5B).

Total biomass production was positively correlated with increased sugars concentration in all tested hydrolysates. However, in terms of biomass yield ($Y_{X/S}$), it was found out that Accellerase® treated hydrolysates best supported biomass production (Table 5). Furthermore, the biomass yield was higher under controlled environment (bioreactor) as compared to shake flasks fermentation using similar hydrolysates. In terms of 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 treated 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 fermentations. This suggests the ability of the particular yeast to produce higher intracellular astaxanthin 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, composed of mixtures of sugars, protein and other nutrients, seems to promote astaxanthin production in *X. dendrorhous.*

3.3.3 Batch fermentation (Pre-hydrolysis and fermentation)

The possibility of conducting pre-hydrolysis and fermentation (pSSF) strategy was investigated, 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* [35]. In SSF strategy, enzymatic hydrolysis of cellulosic material and microbial fermentation is

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.

Figure 5 5 depicts the growth profile of *X. dendrorhous* cultivated in a 2-litre bioreactor using pSSF approach in Accellerase® 1500 hydrolysates (A) and pectinase hydrolysates (B). 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).

3.4 Glass beads / solvent extraction

Figure 6 presents the results from the astaxanthin extraction with glass beads aiming to disrupt the yeast cell, coupled with different types of solvent to extract astaxanthin simultaneously. The highest extraction was achieved with acetone (95 %, w/w), followed by DMSO: Acetone (84 %, w/w). Methanol and ethanol were not significantly different from each other (54-58 %, w/w), whereas DMSO on its own resulted in low extraction yield (42 %, w/w) and water in the lowest (14 %, w/w). 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 [48]. The difference in the extraction yield obtained with the different solvents could be attributes to the differences in their polarity which were: Ethanol (4.3) < methanol (5.1) < acetone (5.4) < DMSO (7.2) < water (9.00) [48]. As astaxanthin contains both polar (at the end of the molecule) and non-polar moieties (in the middle of the molecule) in its chemical structure [49], the use of slightly polar solvent helps to extract astaxanthin from the cell wall membrane of the yeast that might be attached by the non-covalent binding to specific protein [50].

In order to understand better the mechanisms of astaxanthin extractability, the solubility of astaxanthin in different solvents were tested (Table 6). 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 polarity of the solvent used. The highest astaxanthin solubility was observed a when mixture of DMSO: Acetone (1:1) was used with 2.03 mg/ml. As for astaxanthin solubility in individual solvent, highest solubility of astaxanthin was observed when DMSO was used, that correlated to the high polarity of DMSO. Even though astaxanthin has low solubility in acetone (0.55 mg/ml) as compared to DMSO, it has high permeability through the yeast cell wall and resulted in higher astaxanthin extractability from the yeast cells [48]. The mechanism of astaxanthin extraction with organic solvents occur by permeation of solvents through the cell wall and cell membrane, and subsequently interact with astaxanthin compound using Van der Waals forces. This

solvent/astaxanthin complex will diffuse across the cell membrane and remain dissolve in solvents [51].

Several studies report the utilisation of glass beads to extract intracellular products from various yeast species using beads extraction on wider aspects but are limited with regards to astaxanthin extraction form *X. dendrorhous*. Glass beads extraction was achieved by disrupting the yeast cells via bead collision zones by compaction or shear force with energy transfer from beads to the cells [52]. Glass beads accompanied with acetone were used to rupture *Sporodiobolus pararoseus* and *Rhodotorula mucilaginosa*. Results showed that about 66 % (w/w) and 52 % (w/w) of carotenoids extractability was obtained as compared to standard DMSO method [53]. In different study, two different methods (glass beads in bead beater and DMSO/solvent) of astaxanthin extraction in four different strains of *Phaffia rhodozyma* cells was investigated [26]. Similar results were obtained between these methods (275 – 276 μg/g). However, the DMSO disruption method is more rapid than glass bead disruption process, in which multiple samples can be processed at the same time, the DMSO method is considered more suitable for large number of samples. However, the drawbacks of this process are that it is a non-selective extraction process where all polar/non-polar compounds were extracted depending on the solvents used during the process.

The resultant morphology of the *X. dendrorhous* cells after glass beads treatment was studied by environmental scanning microscopy (Figure 7). Before the glass beads treatment, cells were in intact sphere shapes with a smooth surface. After treatment, small particles (< 5 mm) were produced as a result of beads abrasion (Figure 7B), as compared with intact cells before disruption (Figure 7A). The small sized cells and debris produced after glass beads treatment resulted in an increased surface area for subsequent solvent extraction, leading to higher astaxanthin extractability. Apparently, at this point, astaxanthin extraction was highly dependent on the solubility and polarity of organic solvents used, as the degree of cell disruption was already high. For example, in glass beads accompanied with water extraction, minimum astaxanthin pigments were extracted even though the cells were ruptured. This is attributed to the ability of the solvent

used to penetrate the debris/cells to extract the pigments as well as astaxanthin was not soluble in water. It can be deduced that the types of organic solvents used in astaxanthin extraction are very crucial for the success rate of the process.

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 simple, cheap and reliable and does not require special instrumentation. Moreover, further studies on the astaxanthin extract are required 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 future step for the biotechnological production of astaxantin.

4 Conclusion

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. Improvement of the process should target high astaxanthin yields in order to render the overall process more valuable and allow for microbial astaxanthin to compete with synthetically produced astaxanthin. Rapeseed meal holds potential as substrate for the microbial production of astaxanthin. This work demonstrated a process for rapeseed meal bioconversion into astaxanthin by *X. dendrorhous* DSMZ 5626. The process flow starts from screening commercial enzymes for their potential as biocatalysts for rapeseed meal conversion into carbon source rich hydrolysate, down to fermentation optimisation and extraction process. This approach could offer a sustainable alternative to chemically synthesised astaxanthin and can stimulate the circular bioeconomy through the utilisation of low value, abundant resources.

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Tables

Table 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

Table 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
Glucoronic acid	0.4 ± 0.3
Galacturonic acid	2.8 ± 0.1

Table 3: Effect of thermal pre-treatment (at 126 ºC and 135 °C) of rapeseed meal suspension (10 %, w/v) followed by 15% (v/v) of Accellerase 1500 treatment for 24 h 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 ($p<0.05$)

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

Rapeseed meal hydrolysates	Time (h)	Biomass (g/l)	*P* (mg/l)	$Y_{P/X}$ (µg/g)	$Y_{X/S}$ (g/g)	$EtOH_{max}$ (g/l)
Viscozyme® L	115	3.07 ± 0.07	0.4 ± 0.03	156 ± 11.75	0.07 ± 0.02	3.8 ± 0.13
Cellulase	115	14.63 ± 0.64	1.10 ± 0.18	71 ± 11.4	0.25 ± 0.01	6.92 ± 0.00
Pectinase	120	25.83 ± 1.52	6.71 ± 0.44	258 ± 1.83	0.46 ± 0.03	-
Accellerase® 1500	118	8.2 ± 0.01	2.69 ± 0.05	332 ± 12	0.84 ± 0.01	2.02 ± 0.15
Thermal treated plus Accellerase® 1500	140	11.95 ± 0.35	3.2 ± 0.4	271 ± 26.4	0.81 ± 0.05	0.5 ± 0.01

$Y_{P/X}$: Yield of astaxanthin on biomass produced.

$Y_{P/S}$: Yield of astaxanthin on substrate used.$EtOH_{max}$: Maximum ethanol concentration produced during the fermentation

P: Astaxanthin production

X: Dry cell weight

Table 5: 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)	$Y_{x/s}$ (g/g)
RSM+10% Accellerase®	112	17.66 ± 0.2	14.3 ± 0.9	3.6 ± 0.1	251 ± 5	0.81 ± 0.05
RSM + 10% Pectinase	113	72.1 ± 4.3	42.0 ± 0.4	10.2 ± 0.2	242 ± 6	0.58 ± 0.06
Semi – defined media (30 g/l glucose)	123	27.6 ± 1.1	16.3 ± 0.4	3.6 ± 0.1	221 ± 8	0.59 ± 0.01

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

$Y_{P/X}$: Yield of astaxanthin on biomass produced.

$Y_{P/S}$: Yield of astaxanthin on substrate used.$EtOH_{max}$: Maximum ethanol concentration produced during the fermentation

P: Astaxanthin production

X: Dry cell weight

Table 6: 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 ($p<0.05$).

Figures

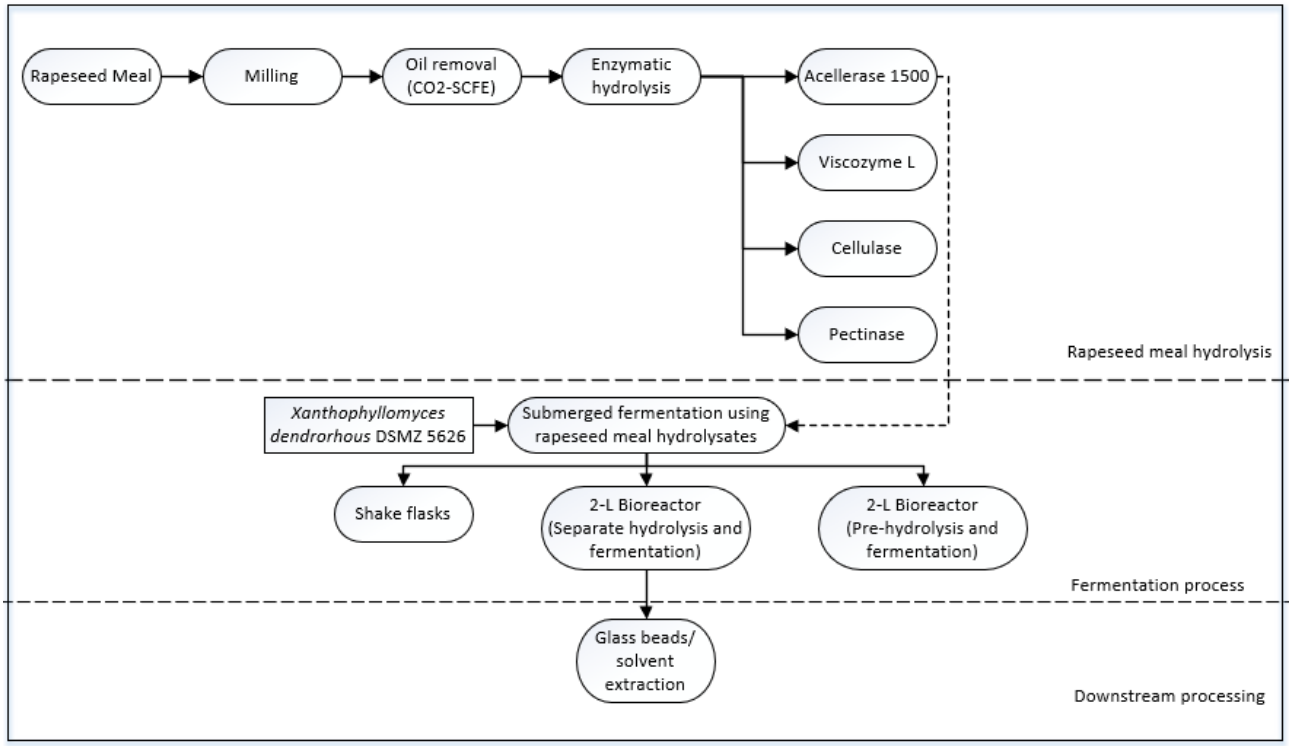

Figure 1: Schematic diagram of experimental procedure for astaxanthin production from rapeseed meal in X. dendrorhous.

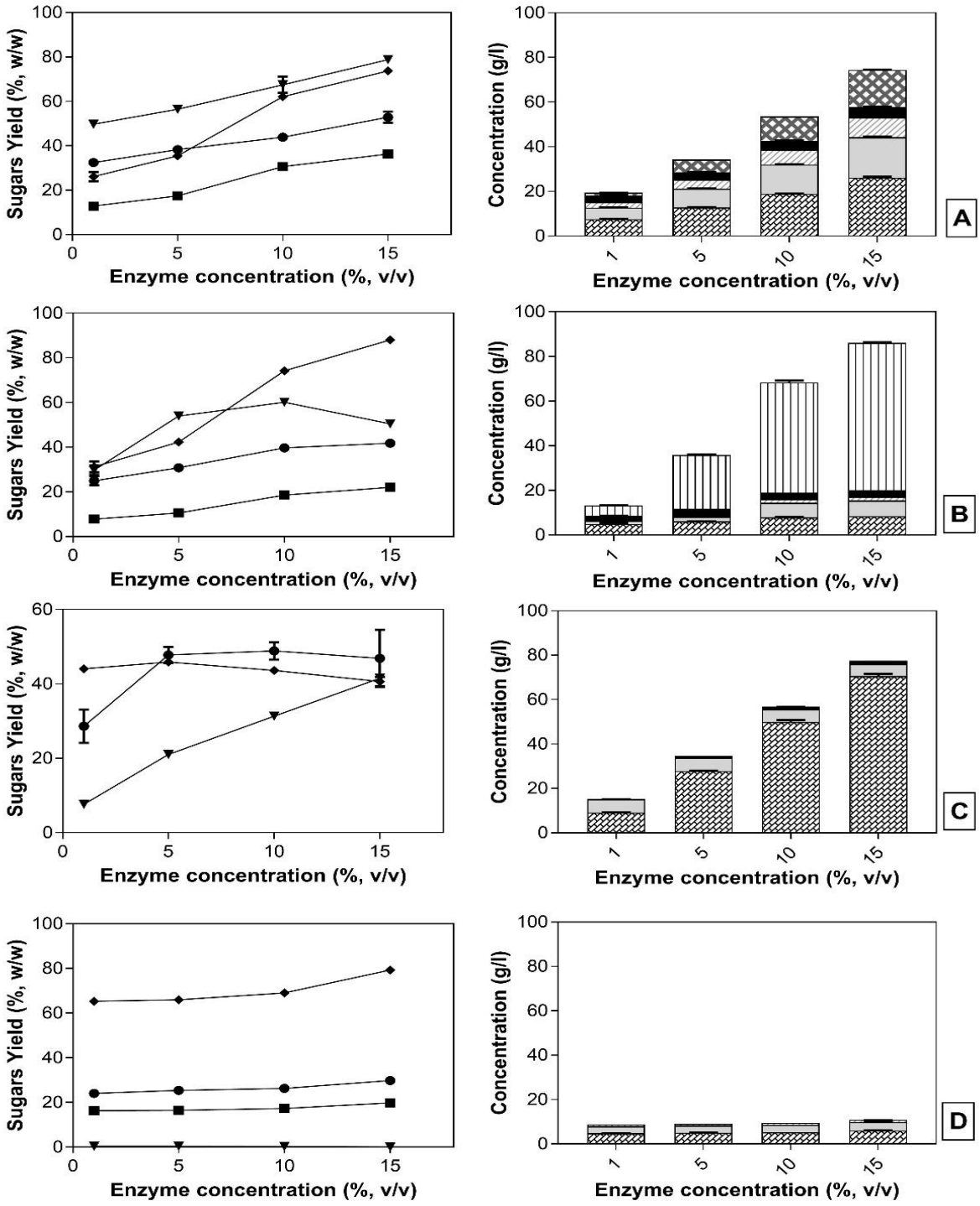

Figure 2: Effect of different enzyme concentrations on the total sugars released after 24 hrs of hydrolysis of 10% (w/v) rapeseed meal using commercial enzymes [Viscozyme® (A), pectinase (B), cellulase (C) and Accellerase® 1500 (D)]. Line graph represents the sugars yield (%), whereas the bar graph represents 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), - 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*

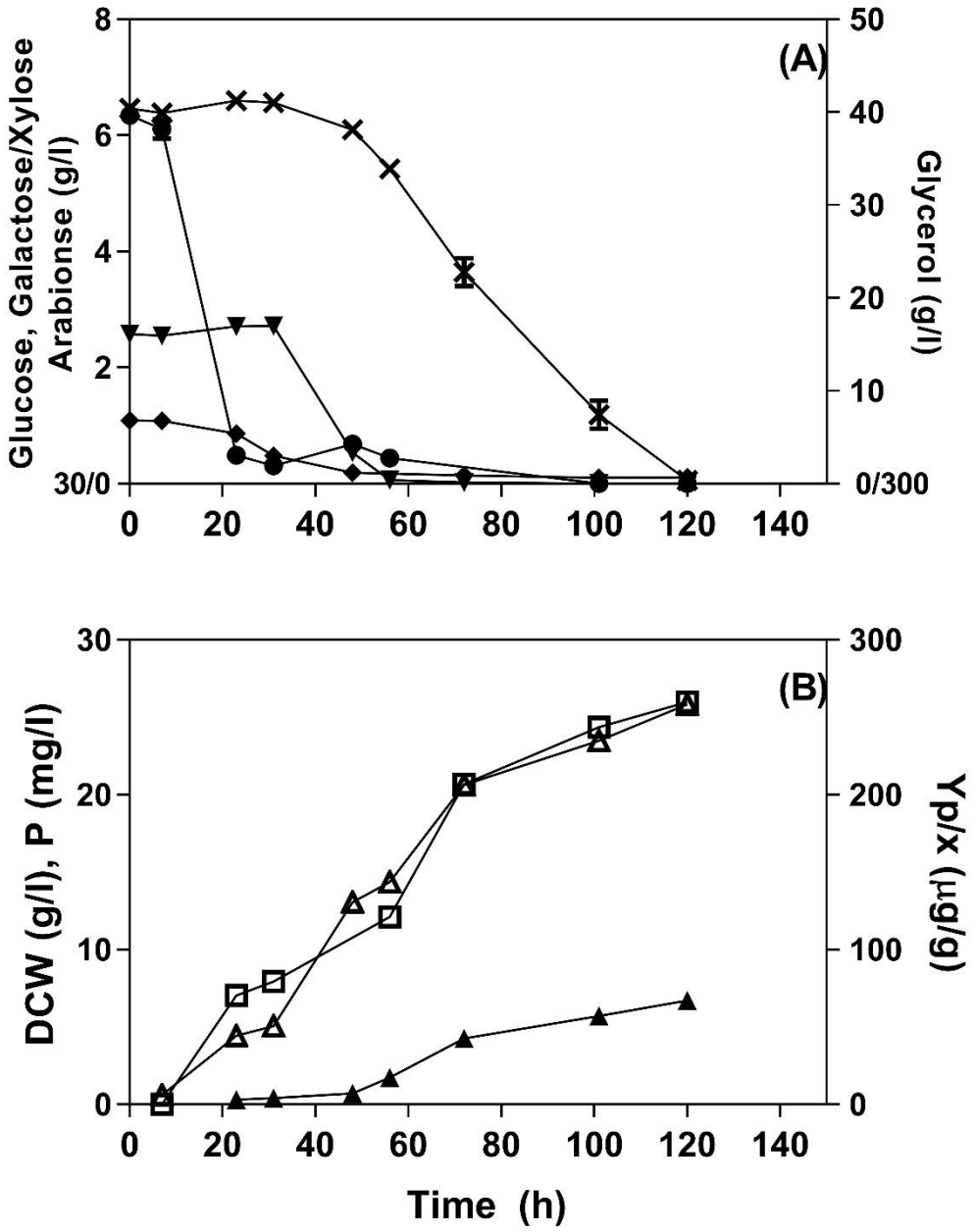

Figure 3:Kinetic profile of (A) carbon sources consumption and (B) product formation during X. dendrorhous growth in pectinase rapeseed meal hydrolysate in shake flasks.

Symbols represent: Glucose (●, g/l), arabinose (◆, g/l), galactose and xylose (▼, g/l), glycerol (×, g/l), yield of astaxanthin on biomass, Yp/x (□, µg/g), astaxanthin production P (▲, mg/l), dry cell weight (Δ, g/l).

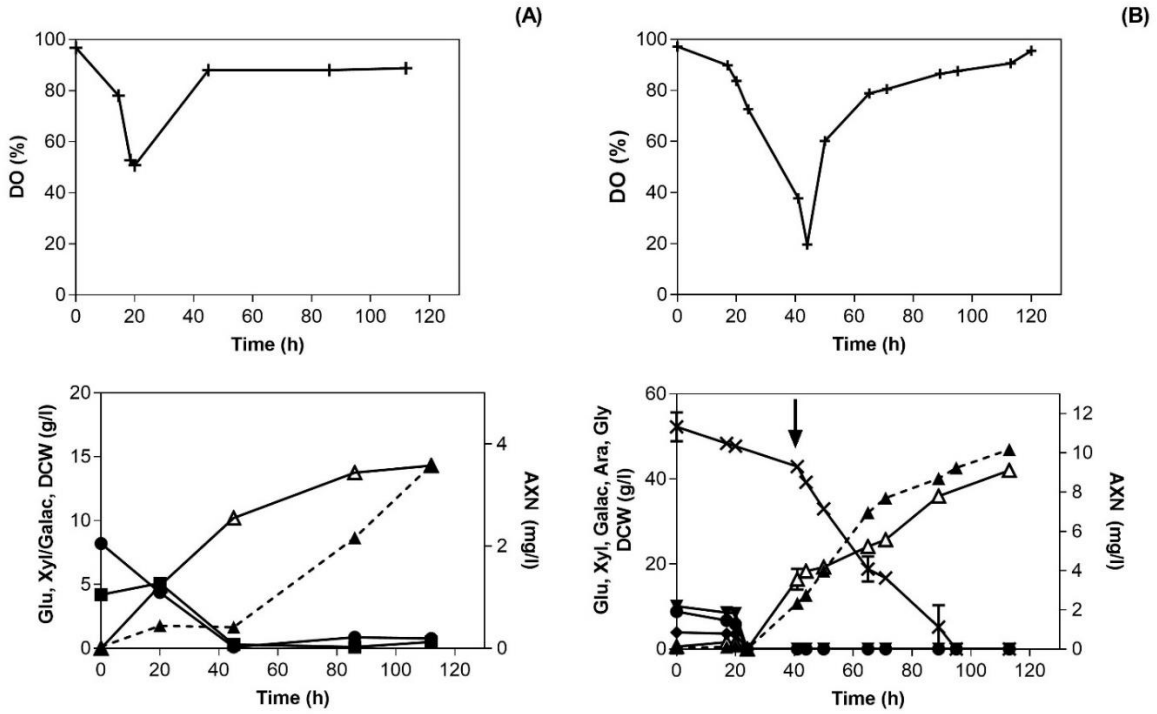

Figure 4: Growth profile of X. dendrorhous cultivated in 2-L stirred tank bioreactor with rapeseed meal hydrolysates as sole fermentation substrate with separate hydrolysis and fermentation strategy. A) Accellerase® 1500 treated hydrolysates (15% v/v of enzyme and 10% w/v of substrate); B) pectinase treated 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/ galactose, Xyl, Galac (▼, g/l), arabinose, Ara (◆, g/l); dry cell weight, DCW (Δ, g/l); glycerol, Gly (×, g/l); dissolved oxygen, DO (+, %).

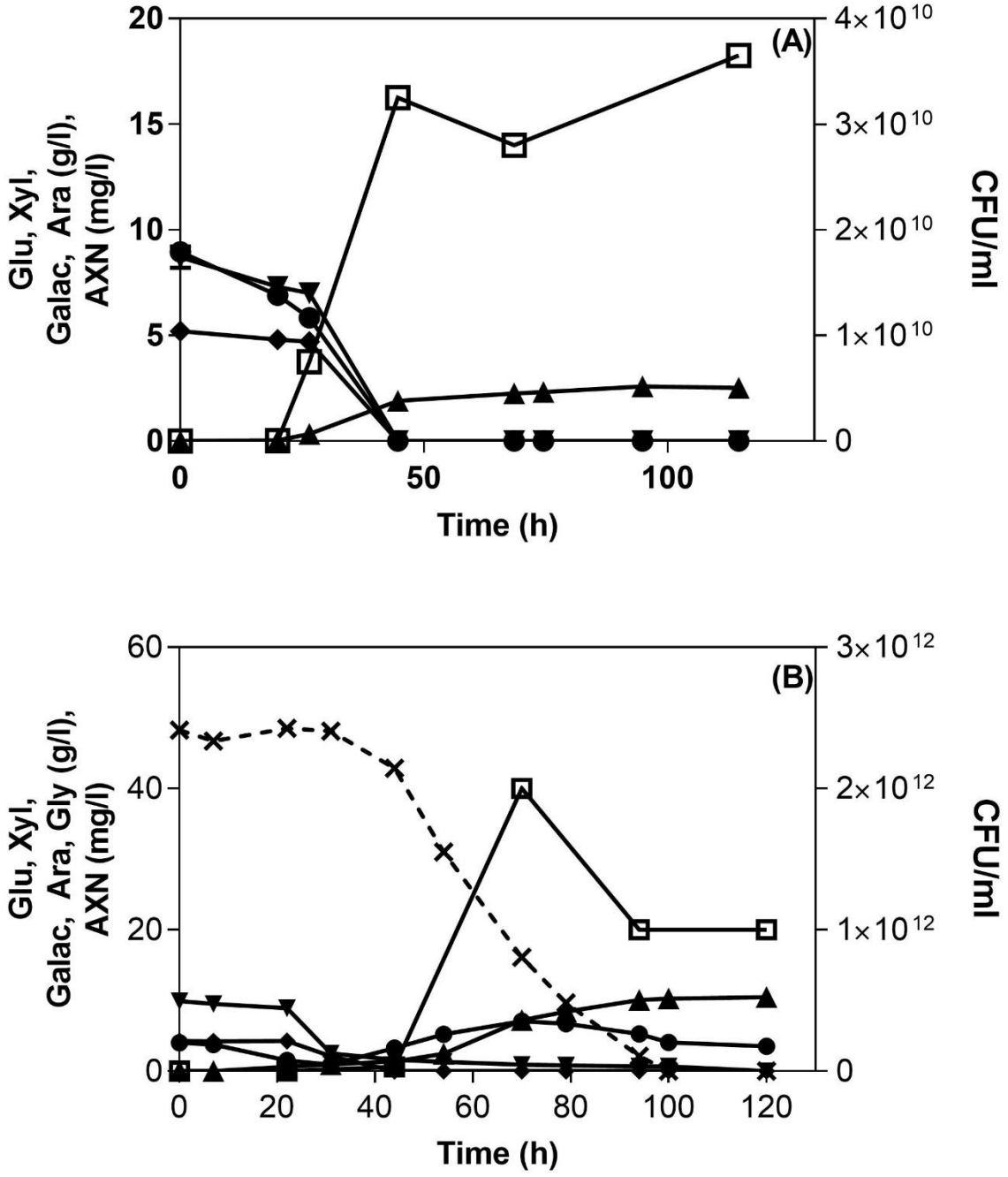

Figure 5: Growth profile of X. dendrorhous using prehydrolysis and fermentation approach. (A) 10% (v/v) Accellerase® ® Accellerase® ® 1500 in 10% (w/v) substrate; (B) 10% (v/v) pectinase in 10% (w/v) substrate

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/ galactose, Xyl, Galac (▼, g/l); glycerol, Gly (×, g/l); CFU (□, CFU/ml)

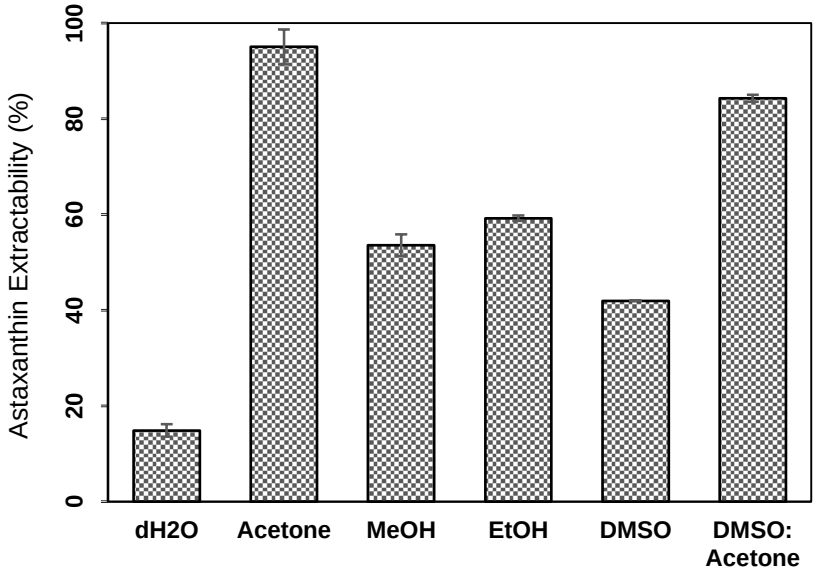

Figure 6: Percentage of astaxanthin extractability (%) using glass beads (0.3 g) with different types of solvents. Extraction was performed at room temperature under agitation at 2000 rpm

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)

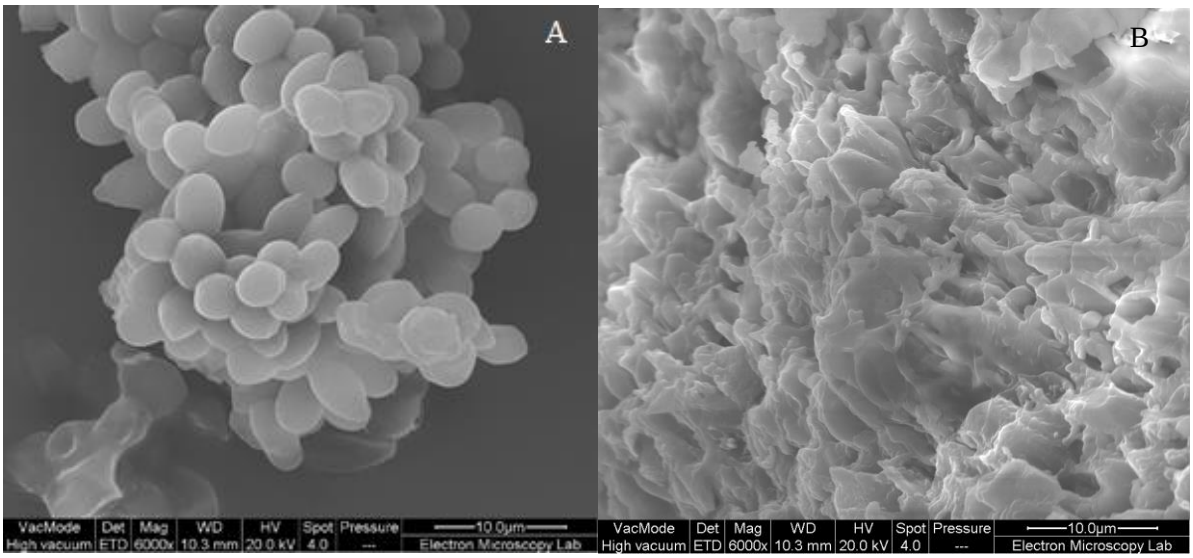

Figure 7: Scanning electron microscopy images X. dendrorhous cells A) intact cells, B) cells after treatment with glass beads (0.3 g) followed by acetone (100%) extraction.