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Effect of Different Sugar Sources on *P. rhodozyma* Y1654 Growth and Astaxanthin Production

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Abstract. *Phaffia rhodozyma* (also known as *Xanthophyllomyces dendrorhous*) is one of the most promising natural sources of commercial astaxanthin. It has high growth rates, easy cultivation conditions and able to utilize different carbon substrates. This provides an opportunity to further lower production cost by using industrial waste such as molasses. This research therefore evaluates the growth dynamic and astaxanthin production of *P. rhodozyma* Y1654 growing on soy and sugar beet molasses-based media. Liquid growth media based on soy molasses (SM), sugar beet molasses (SBM) and glucose (control) as main sugar source with peptone and yeast extract supplementation were inoculated with 48 h old seed culture (grown in standard glucose media: 2.0% glucose, 1.0% peptone, 0.2% yeast extract) and incubated at 20 °C with stirring speed of 180 rpm for 7 days. Samples were taken daily throughout the study period to assess; cell count, dry cell weight (DCW) and amount of astaxanthin. Soy molasses-based media resulted in the highest biomass yield (7.7 g L^{-1}) followed by SBM (5.8 g L^{-1}). Generally, more than 90% of initial fermentable sugar was consumed at the end of the study. However, about 40% of total sugar in SM was unassimilable by *P. rhodozyma* Y1654. The highest astaxanthin yield was observed in the control media (77 µg g^{-1} of DCW). Cultivation of *P. rhodozyma* Y1654 in SBM resulted in as much as twice (32.8 µg g^{-1} of DCW) the astaxanthin yield of SM (12.4 µg g^{-1} DCW). Molasses-based media are good for growth of *P. rhodozyma* Y1654 but for astaxanthin production, they need further optimization.

Key words: astaxanthin, carotenoids, molasses, *Xanthophyllomyces dendrorhous*, agric-food wastes, byproducts, microbial pigment.

INTRODUCTION

Astaxanthin (3,3'-dihydroxy-β,β'-carotene-4,4'-dione) is a red-orange pigment belonging to the group of oxygenated carotenoids, the xanthophylls. Mainly found in marine organisms, it has been an outstanding colorant with immense importance in the aquaculture feed industry (Fakhri et al., 2018). Over the past two decades, there has been an increased interest in astaxanthin as a potent and promising agent in human health and nutrition due to the biological activities it exhibit, most notably its overwhelming

antioxidant activity (Fang et al., 2019). Its extended structure, with polar regions at each end and a nonpolar middle section, allows for optimal orientation in the lipid bilayer of cell membranes, offering protection against oxidative assaults with its conjugated double bonds (Satoh, 2016). Aside its prominent antioxidant activity, it has been reported to exhibit anti-diabetic, anti-inflammatory, anti-cancer, neuroprotective, cardioprotective and immune-modulative activities. It has also been said to improve skin and ocular health, as well as fertility and reproduction (Fakhri et al., 2018). As a result, it is widely used in the pharmaceutical, nutraceutical and cosmetics industries, in addition to its age-old use as a pigment in aquaculture. It is currently regarded as the second most important carotenoid in the global market, after β-carotene, with a global market size exceeding $ 600 million in 2018 and a projected value in billions by 2026 (Ahuja & Rawat, 2019).

Astaxanthin can be obtained from both natural sources and chemical synthesis, with the latter being the major source representing about 97% of commercially available astaxanthin (Schmidt et al., 2011). The growing demand for products with natural additives due to rising concerns about the harmful and potentially harmful effects of chemical additives coupled with the fact that synthetic astaxanthin has lower biological activity, has led to a wide exploitation of natural sources of astaxanthin with potential for industrialization (Higuera-Ciapara et al., 2006; Stachowiak, 2012). Next to *H. pluvialis,* the red yeast *P. rhodozyma* is one of the most promising sources of natural astaxanthin for the commercial market, capable of biosynthesizing astaxanthin in quantities up to 84% of its total carotenoid composition output (Stachowiak, 2014; Stoklosa et al., 2018). Although astaxanthin yield from *P. rhodozyma* is lower compared to *H. pluvialis*, the former is preferred due to higher growth rates and easier cultivation conditions that might decrease the production time at industrial scale (Amado & Vazquez, 2015). Despite this, natural astaxanthin is unable to compete with synthetic astaxanthin due to its high cost of production. Consequently, many studies have set out to find ways to improve natural astaxanthin production at low cost by searching for hyper-producing strains, optimizing growth conditions and of much interest, finding low-cost media alternatives. There has therefore been heightened interest in the use of low-cost raw materials primarily byproducts of the agro-food industry for the microbial production of astaxanthin. The use of waste/byproducts from the agriculture and food industries is of immense interest because it does not only provide a way to lower production cost of astaxanthin, it as well offers an opportunity to minimize environmental and energetic problems related to the disposal of these wastes (Frengova & Beshkova, 2009). Stimulatingly, *P. rhodozyma* may utilize different carbon substrates: glucose, maltose, sucrose, cellobiose, xylose, arabinose, lactose and many other (Stachowiak, 2014). Due to this ability, *P. rhodozyma* offers the possibility to increase production and lower production cost of natural astaxanthin by the use of new culture medium especially natural complex media based on plant extracts or wastes produced by the agri-food industry (Stoklosa et al., 2018).

To meet the food and nutritional needs of the ever-growing population of the world, the food and agriculture industries have increased their production capacities. This, consequently, has led to the generation of a significant amount of waste which constitute a huge environmental problem in terms of their disposal. Currently, most of these wastes are either incinerated, dumped on landfills, used as animal feed or feedstock for the production of bioenergy (Spalvins & Blumberga, 2019; Tamelová et al., 2018). There is, however, a rising interest in valorization of these wastes into value-added products

including enzymes, single cell oil, enzyme inhibitors, among others (Gruduls et al., 2018; Eveleva et al., 2019; Spalvins & Blumberga, 2019; Vybornova et al., 2019). Prior researches have demonstrated the use of various agricultural feedstock and industrial byproducts for the cultivation of *P. rhodozyma* including sweet sorghum Juice (Stoklosa et al., 2018), corn steep liquor (Urnau et al., 2019), residual pineapple juice (Jirasripongpun et al., 2008), *Yucca fillifera* date juice (Luna-Flores et al., 2010), corn fiber hydrolysate (Nghiem et al., 2009), residual coconut milk (Dominguez-Bocanegra et al., 2007), Jerusalem artichoke (Jiang et al., 2017), hydrolysate of barley straw and sugarcane bagasse (Montanti et al., 2011), mussel processing wastewater (Amado & Vazquez, 2015), cane molasses (Haard, 1988) etc. Nevertheless, little to no information is available regarding exploitation of soy or sugar beet molasses in producing astaxanthin.

In Russia, molasses of soy and sugar beet represent a considerable amount of industrial byproducts generated from the soya processing and sugar industries respectively. Generally, sugar beet molasses contains up to 80% total solids (TS) with > 50% of this representing total sugar (mainly sucrose – 60% of TS; glucose and fructose – < 1% of TS), in addition to nitrogenous compounds (> 2% of TS), minerals and vitamins (Acan et al., 2020; Scoma et al., 2016; Sjölin et al., 2020). On the other hand, soy molasses usually contains not less than 30% (w/w) of total sugar, majority (61%) of which represents carbohydrates. A considerable amount (27% of TS) of this represents raffinose-family oligosaccharides (stachyose – 15% of TS; raffinose – 12% of TS) with just a little over 30% of TS being fermentable sugars (mainly sucrose – 26% of TS; glucose – 4.6% of TS; fructose – 2.9% of TS). Soy molasses as well contains substantial amounts of lipids (15.6% of TS), proteins (6.4% of TS) and some minerals (Romão et al., 2012; Wang et al., 2019). Despite the high sugar content and rich composition of sugar beet and soy molasses, they are primarily used just as animal feed in this part of the world, without any added value. Nevertheless, the use of molasses as an inexpensive fermentation feedstock for production of yeast biomass, biofuels, organic acids, vitamins enzymes, etc. has widely reported. It is of this accord and interest that this study explored soy and sugar beet molasses as cheap carbon sources for the growth of *P. rhodozyma* and production of astaxanthin. In this study, the P. rhodozyma growth and astaxanthin production was evaluated in media of soy or sugar beet molasses supplemented with peptone and yeast extract.

MATERIALS AND METHODS

Microorganism

Lyophilized cells of *Phaffia rhodozyma* Y1654 were purchased from National Bioresource Center, All-Russian Collection of Industrial Microorganisms, Research Institute of Genetics and Selection of Industrial Microorganisms, Moscow, Russian Federation. Cells were revived and grown on solid Yeast Extract–Peptone–Glucose (YPG) media (containing per liter, 20 g glucose, 10 g peptone, 2 g yeast extract, and 9 g agar) at 20 °C. After 3 days of growth, agar plates were then stored at 4 °C for further use.

Feedstock and Chemicals

Soy Molasses (Mass fraction of dry matter – 75%; Mass fraction of sucrose by direct polarization – 60%; Fats – 5% of dry weight; Proteins – 5% of dry weight; pH – 7.7) was provided by Joint-Stock Company 'Agroproduct', Kaliningrad region, Svetly, Russian Federation, Sugar beet molasses (Total solids – 82%; Sucrose – 47%, Reducing sugars – 0.44%; pH – 7.0) was obtained from Krasnodarsky Sugar Plant, Russian Federation, HPLC-grade solvents were supplied by HIMMED Inc., Russia while rest of solvents used in this study were supplied by EKOS-1 Inc., Russia.

Preparation of seed culture

Prior to preparation of seed culture, cells were sub-cultured on fresh solid media. Two loopful of yeast cell from a fresh culture on agar plate was suspended in 5 mL of sterile distilled water. Following aseptic procedures 4 mL of the suspension was transferred into a 250 mL Erlenmeyer flask containing 200 mL of YPG broth (containing per liter, 2.0% glucose, 1.0% peptone, 0.2% yeast extract), and incubated at 20°C and shaking speed of 180 rpm, for 48 h under constant illumination in an incubator equipped with an orbital shaker (Yihder Orbital Shaking incubator LM 570RD).

Inoculation and Cultivation

Fresh sterilized culture media (200 mL) in 500 mL Erlenmeyer flask was inoculated with the seed culture at 10% v/v and incubated in the same conditions as the seed culture for 168 h. All experiments were carried out in triplicates. Three different media based on the source of sugar were used: Yeast Extract–Peptone–Glucose (YPG) medium as control, Soy molasses-based medium (SM) and Sugar beet molasses-based medium (SBM). Each growth medium contained per liter 2.0% sugar, 1.0% peptone, 0.2% yeast extract.

Cell Yield Determination

Cell density was determined by counting with the aid of a Neubauer chamber under a light microscope (Altami Bio 8). For dry cell weight (DCW) determination, 5 mL samples of culture were measured into pre-weighed (W1) 15 mL centrifuge tubes and centrifuged for 10 min at 4,000 × g. The supernatant was reserved for measurement of sugar content while the cell pellet was washed twice with 5 mL of distilled water. The biomass after washing was dried together with the container at 50 °C until a constant weight (W2) was obtained when measure. The DCW was quantified as weight difference between W2 and W1.

Determination of Sugar Content

Sugar content was determined using the phenol-sulfuric acid method as described by Nielsen (2010) with some modifications. Molasses were diluted according to the ratio 1:5,000 while for supernatants a ratio ranging from 1:1,000 (on the first day) to 1:50 (on the last day) was used. Absorbance was measured at 487 nm (the Abs_{max} observed in preliminary studies) with a Shimadzu 1800-UV spectrophotometer. The initial sugar concentration of each media was as well measured.

Extraction of Astaxanthin

For the determination of astaxanthin content, 10 mL aliquot of culture was collected and centrifuged at 4,000 × g for 10 min to separate cells. The supernatant was discarded, and cell pellet washed twice with 5 mL of distilled and then stored in the freezer until needed. Cell disruption was carried out as described by Cheng and Yang (2016) with some modifications. The frozen cells were thawed and suspended in 2 mL of DMSO then subject to an ultrasonic bath (Bandelin Sonorex Digitec DT 31 H) at 35 °C for 10 min. Subsequently, 5 mL of petroleum ether and 1 mL of 20% w/v NaCl were added then extracted for 10 min with frequent rigorous shaking. The resultant was then centrifuged at 3,500 × *g* for 5 min to separate the petroleum ether containing pigment. Petroleum ether was evaporated on a rotary evaporator (Heidolph Hei-VAP) at 35 °C and astaxanthin re-dissolved in 1 mL HPLC-grade acetonitrile and then quantified by HPLC as described as below.

Quantification of Astaxanthin

Extract (100 µL) was manually injected into the HPLC system (Shimadzu LC-20AD HPLC device equipped with a Shimadzu SPD-20AUV/Vis detector). Separation was done on a PerfectSil® Target ODS-3 HD 5µm 150×4.6 mm column with isocratic flow, 95% acetonitrile as the mobile phase at rate of 0.8 $mL \cdot min^{-1}$. Astaxanthin was detected and measured at 474 nm. Quantification was done using an astaxanthin (synthetic) standard calibration curve.

Statistical Analysis

All experiments were conducted in triplicates. Experimental results are expressed as mean values. Data were analyzed by one-way *ANOVA* or *t-test* at a 95% confidence level. A *P-value* < 0.05 was considered statistically significant. Statistical analyses were done using graphPad Prism 8.

RESULTS AND DISCUSSION

Sugar content of molasses

Prior to being used for the preparation of growth medium, the sugar content of both soy and sugar beet molasses were assessed. It was shown that sugar beet molasses had an average sugar content of 53% per gram, > 1.5 times higher than that of soy molasses (29%) (Table 1). It has been reported sugar beet molasses mostly has no less than 48% of total sugar despite various optimization at the industrial level (Duraisam et al., 2017). A large part (> 97%) of this as reported by Scoma et al. (2016) consist of sucrose, a fermentable disaccharide which is easily converted and utilized by several yeasts including *P. rhodozyma*. The remainder comprises simple sugars (glucose and fructose), however minute concentrations of oligo- and polysaccharides (raffinose and starch) have also been reported (Sjölin et al., 2020). In fact, it is largely due to the high residual sugar that sugar beet molasses is considered as alternative cheap sugar/carbon source for thecultivation and production of

Table 1. Sugar content of Molasses

Description	Average Sugar content (% per gram ± SEM)
Soy Molasses	29.30 ± 0.38
Sugar Beet Molasses	53.60 ± 0.40

SEM = Standard Error of Mean.

microbial biomass as well as their associated products. In many parts of Europe, sugar beet molasses is used as a cheap carbon-rich source for bioethanol production (Akbas & Stark, 2016). On the other, the lower total sugar content of soy molasses (compared to sugar beet molasses) plumbs further as more than 40% of this is reported to comprise of raffinose-family oligosaccharides (Romão et al., 2012). The presence of these complex oligosaccharides limits the application of soy molasses in industrial biotechnology as most of industrial microorganisms including yeasts, lactic acid bacteria, etc. cannot utilize these sugars unless they are pre-hydrolyzed to monomer (Yang et al., 2018). Pre-hydrolysis is nonetheless not attractive as it significantly adds to cost of production.

The low fermentable sugars in soy molasses is, however, augmented by its rich lipid content (an alternative source of carbon which represents up to 15% of total solids), proteins (> 6% of total solids) and other components essential for microbial growth (Romão et al., 2012) – making it as well a suitable inexpensive alternative nutrient source for cultivation of microorganisms to produce value-added compounds.

Sugar consumption by *P. rhodozyma* in different media

P. rhodozyma exhibited fairly the same sugar consumption pattern in all three media, with more than 80% of initial fermentable sugar consumed by the first 24 h (Fig. 1). Nevertheless, not all the sugar in media was consumed at the end of the study; the amount of residual sugar in the control media (YPG) was the lowest followed by SBM with SM having the highest amount of residual sugar. A similar but less rapid sugar consumption pattern was reported by Stoklosa et al. (2018) when *P. rhodozyma* was cultivated using sweet sorghum juice (containing mainly sucrose). The authors also observed that increasing yeast extract concentration in addition to nitrogen availability significantly promoted sugar consumption leading to complete utilization of fermentable sugars by 168 h. Approximately 40–43% of the total sugar in soy molasses was left at the end of the study as residual sugar, unassimilable by the yeast. This most-likely comprise of the complex oligosaccharides (like stachyose and raffinose, which reportedly represent more than 25% of total carbohydrates present in soy molasses (Rodrigues et al., 2017). However, available literature indicates that *P. rhodozyma* is able to metabolism oligosaccharides (Stachowiak, 2012) and even polysaccharides (Schmidt et al., 2011). Notwithstanding this, it is evident that the strain of *P. rhodozyma* used in this study is unable to metabolize some of sugars present in soy molasses. Adjusting the total sugar in SM to compensate for the unassimilable sugar (by increasing the amount of molasses added) did not in any way affect the sugar consumption pattern in soy molasses-based media, just that both the total amount of sugar consumed, and the residual sugar were accordingly increased (Fig. 1, B). Thus, to maximize the utilization of sugar in SM it is necessary to pre-hydrolyze complex sugars. This mostly down using acids or enzymes (Romão et al., 2012). All the same, the results show that *P. rhodozyma* is able to effectively utilize the principal sugar (sucrose) which is present in both molasses. *P. rhodozyma* have long to been reported to produce invertase, which gives it the ability to utilize sucrose (Stoklosa et al., 2019). In the presence sucrose, glucose and fructose, *P. rhodozyma* is reported to rapidly degrade sucrose leading to a lag in monomer assimilation by increasing concentration of both glucose and fructose. Nonetheless, *P. rhodozyma* is known to assimilate and metabolize glucose prior to utilization of any other simple sugar; glucose is considered a priority carbon source for these yeasts and its presence especially at high concentrations repress the utilization of

other sugars (Stachowiak, 2012). This probably accounts for the reason SBM has a slightly higher amount of residual sugar (most of which is suspected to be fructose) when compared to YPG (control).

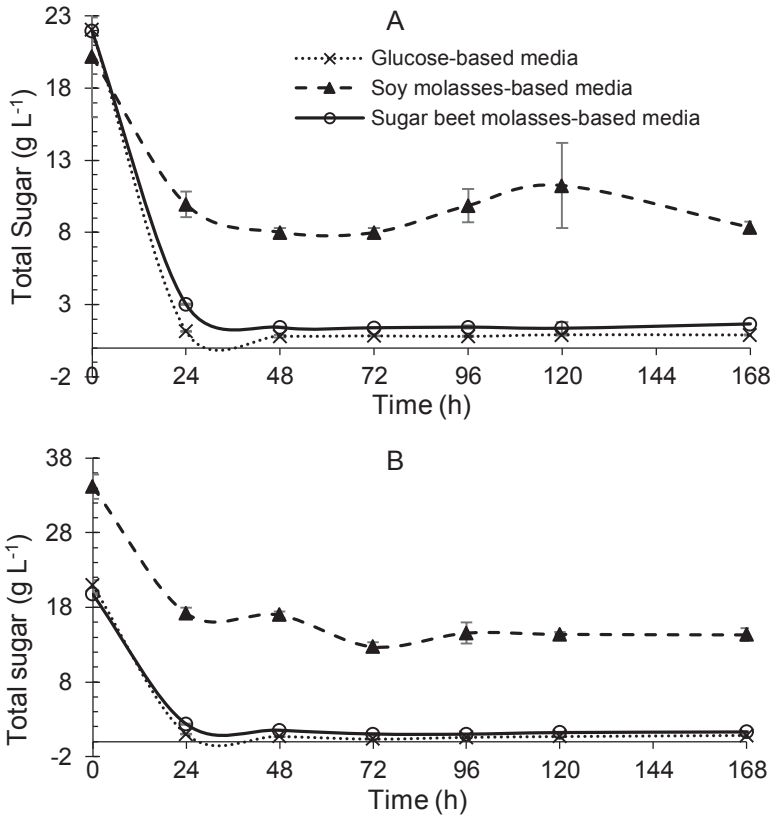

Figure 1. Sugar consumption of different Media (A – when unassimilable sugar was not considered; B – after adjusting sugar concentration in SM).

Effects of different sugar sources on cell growth and biomass production

Given that raw materials for culture media represent from 30 to 70% of bioprocess cost, development of low-cost media, using by-products and residues of agro-industrial origin, for cultivation of microbes have been under intense focus (Villegas-Méndez et al., 2019). As such one of the main objectives of this study was to evaluate how well soy and sugar beet molasses could support the grow of *P. rhodozyma* Y1654. Generally, cells grown on molasses media were somehow larger than those of the control when observed under a microscope. Cell size is an important characteristic that significantly influences nearly all aspects of cellular physiology and thus, a key indicator of overall physiological state of cells. Though under a given condition cells of a given species typically vary less about their mean size, it is well known that extracellular conditions (including nutrient availability) can drastically alter cell size – under nutrient limitation cells tend to have a reduced size and budding yeast produce daughter cells less than 20% of the mother cell size (Turner et al., 2012). In addition, cells grown on molasses media exhibited active budding, even on the last day of the study (Fig. 2).

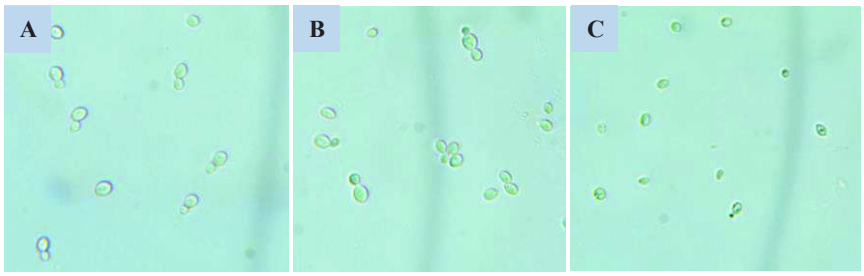

Figure 2. Appearance of cells (400X) at the end of study (A – in soy molasses-based media; B – in sugar beet molasses-based media; C – in glucose based-media).

The presence of digestible nitrogen as well as other essential nutrients like vitamins and minerals in molasses could be one of the major factors accounting for the promotion of cell growth by molasses-based media. Moreover, yeast are known to adjust their growth in response to their nutritional environment, such that depletion of essential nutrients halts cell growth (including proliferation) until nutrients are replenished then cell reenters the cell cycle and begin to proliferate (Gurvich et al., 2017). It could therefore be inferred that the limited budding as seen in the control at the end of the study (Fig. 2, C) is probably as a result of nutrient limitation. In terms of growth profile, a similar growth curve is observed irrespective of the initial sugar source, exponential growth was observed within the first 24 h afterwards only slight changes in cell density was observed (Fig. 3). Nonetheless, the lowest biomass yield was recorded for SM in the first experiment when unassimilable sugar was not considered (Fig. 4, a). Despite this, *ANOVA* analysis of the maximum biomass yield for each media suggested that the difference in biomass among the groups was not statistically significant ($P = 0.167$).

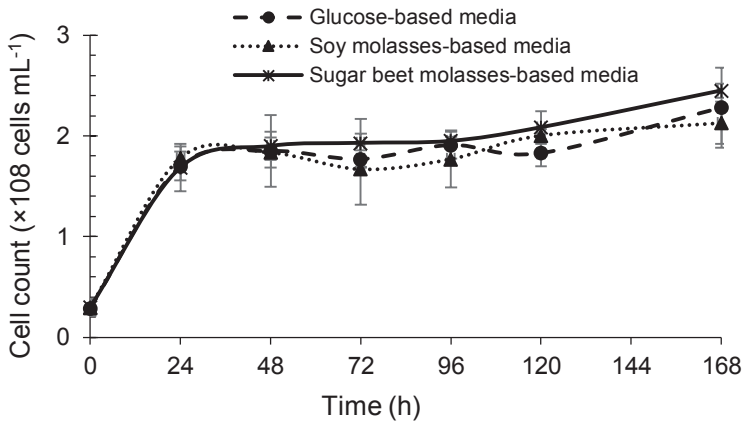

Figure 3. Kinetic curve of *P. rhodozyma* growth in different media.

However, when the total sugar in SM was adjusted to compensate for the unassimilable sugar, such that the total assimilable sugar in all media where similar, the biomass yield for SM increased significantly ($P = 0.008$) recording the highest biomass yield in the present study (Fig. 4, B). In an earlier study, Jirasripongpun et al. (2007)

similarly recorded increase in biomass production in accordance with increasing molasses concentration in the range of 2–8% v/v. Regardless, SBM always recorded a higher biomass yield than YPG. The highest biomass yield in each case was recorded at the end of 168 h of incubation. Similar observation in biomass accumulation are have been report in other studies, in these studies however, steep rise in biomass (characteristic of the exponential growth phase) is observed after 24 h (Jiang et al., 2017; Stoklosa et al., 2018).

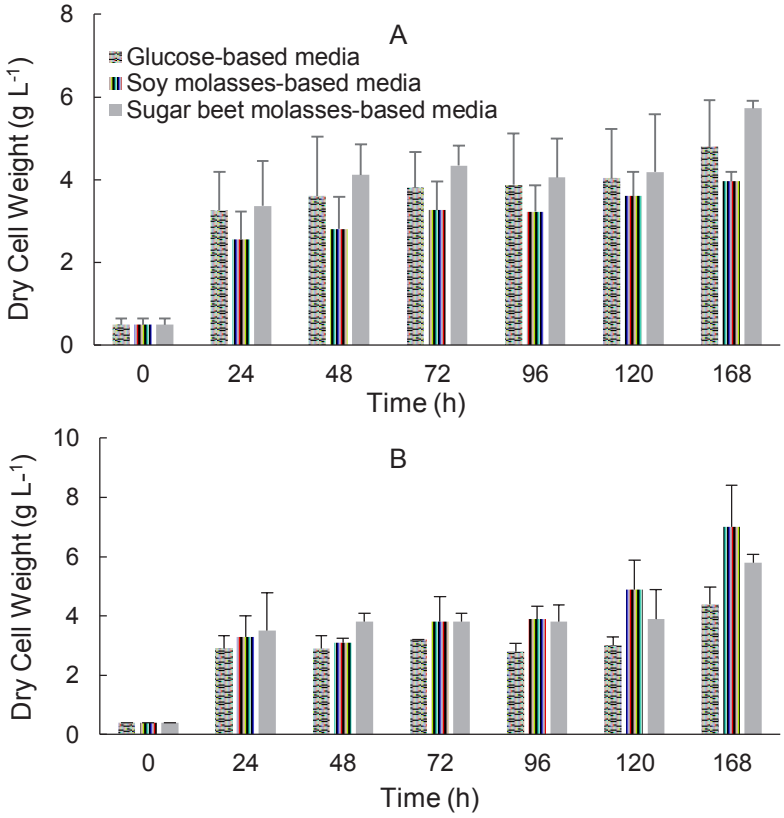

Figure 4. Biomass yield in different media (A – when unassimilable sugar was not considered; B – after compensating for unassimilable sugar in SM).

With regards to specific biomass yield, thus amount of biomass produced (in this case the highest biomass yield for each media) per gram of sugar consumed, SM recorded the highest notwithstanding the fact that in the first experiment the total assimilable sugar in SM lower (just a little over half that in SBM and YPG) while the lowest was observed in YPG (Table 2). When the sugar concentration of SM was increased to compensate for unassimilable sugar a corresponding increase in specific biomass yield was observed. Nitrogen source is one of the indispensable components of a fermentation medium, and the sources as well as concentration affects cell growth and also products produced by these cells (Ni et al., 2007). Although all media were supplemented with peptone and yeast extract, soy molasses is known to contain a substantial amount of nitrogenous substances (up to approximately 9–10% of total solids

(Zhong & Zhao, 2015; Caldeirao et al., 2016)), these nitrogenous sources to some extent might be one of the influencing factors making SM good for biomass production. As observed by Vustin et al. (2004) large amounts of nitrogen in the culture medium or low C/N ratio stimulates active biomass growth. It is therefore plausible assumed that the ratio of C and T is lower in media with molasses media compared to glucose, which is why when cells are grown using media with molasses the biomass content is higher and the cell size is larger. Over the past years, several researchers have evaluated different agro-industrial wastes for cultivation of *P. rhodozyma*. Shake flask cultures in these studies have resulted in varying biomass yields. As much as 4.3 g L^{-1} of biomass was obtained cotton husk hydrolysate, 4.7 g L^{-1} with corncob hydrolysate, and 6.6 g L^{-1} with mesquite pods extract (Villegas-Méndez et al., 2019). Also, 4.9 g L^{-1} biomass yield was obtained with a combination of corn steep liquor, parboiled rice water and glycerol (Urnau et al., 2019). The results of the present study are comparable to those of the above studies. However, much higher biomass yields have also been reported with other agro-industrial byproducts: 17 g L^{-1} with sweet sorghum juice (Stoklosa et al., 2018), 15.6 g L^{-1} with sweet sorghum bagasse hydrolysate (Stoklosa et al., 2019), 12.8 g L^{-1} with barley straw hydrolysate (Montanti et al., 2011). These varying yields in biomass corresponds to the diverse nutritional composition of byproducts from the agrio-food industry. In addition, byproducts from industries come along with different unfavorable compounds, originating from different points during the processing of raw material, these compounds sometimes have growth inhibitory effect on cultivated microbes. In this current study, the chemical composition of molasses was not analyzed to ascertain the presences of possible inhibitors, however as the biomass yield for molasses-based were higher than that of the control it is probable that possible inhibitory compounds were either absent or present in concentration not potent enough to stall growth. Largely, molasses from different sources have demonstrated, in several studies as well as in the present study, to be an excellent and efficient carbon source for large-scale production of various industrial microorganisms. However, the efficiency of use of molasses, like other complex heterogenous industrial waste, for industrial fermentation is highly dependent on intrinsic properties of microbe like nutrient requirements (e.g., flexibility or specificity on carbon source utilization, organic or inorganic nitrogen requirements, mineral and vitamins requirements, etc) (Koutinas et al., 2014; Papizadeh et al., 2020).

Table 2. Summary data for sugar consumption and biomass production

Media	Experiment	Total Sugar (g L^{-1})	Initial Assimilable Sugar (g L^{-1})	Residual Sugar (g L^{-1})	Maximum DCW (g L^{-1})	*P*-value	Specific Biomass Yield (g g^{-1})
YPG	1st	22.063	22.063	0.780	4.800	0.699	0.225
	2nd	20.546	20.546	0.689	4.400		0.222
SM	1st	20.197	12.000	8.130	3.960	**0.008***	0.328
	2nd	34.568	20.000	14.010	7.700		0.375
SBM	1st	21.957	21.957	1.250	5.730	0.797	0.276
	2nd	19.700	19.700	1.194	5.800		0.313

*statistically significant at 95% confidence level; 1st – When unassimilable sugar was not considered; 2nd – After adjusting sugar concentration in SM to compensate for unassimilable sugar.

Effect of different sugar sources on astaxanthin production

A typical chromatogram obtained from *P. rhodozyama* extracts is presented in Fig. 5. Seven (7) major peaks were observed, 6 within the span of 20 min and one after 35 min. Since the extraction method used was selective for carotenoids, these major peaks most-likely correspond to various carotenoids or their derivatives. The peak around 9.5–9.8 min was identified to correspond to astaxanthin. As astaxanthin represents more than 80% of total carotenoids in *P. rhodozyma* (Schmidt et al., 2011), it is not surprising that it's corresponding peak is the highest. Though molasses-based media as reported earlier were superior in terms of biomass production, this wasn't the case when astaxanthin production was considered. As reported by Villegas-Méndez et al. (2019), one of the most significant factors affecting the production of carotenoids was the carbon source with a contribution of more than 60%. In the present study, the control (YPG) was supreme in astaxanthin production (reaching a maximum of 0.499 mg L^{-1}) followed by SBM (0.237 mg L^{-1}) then SM (0.059 mg L^{-1}). The maximum accumulation of astaxanthin was recorded on the last day of the experiment (after 168 h) for all media. The accumulation of astaxanthin by cells was time dependent and generally associated with the stationary phase, with a sharp increase after 72 h and reaching a maximum at the end of the study (Fig. 6), typical pattern exhibited by secondary metabolites; this pattern of accumulation of astaxanthin is similar to that reported by de la Fuente et al. (2010) and Amado & Vazquez (2015).

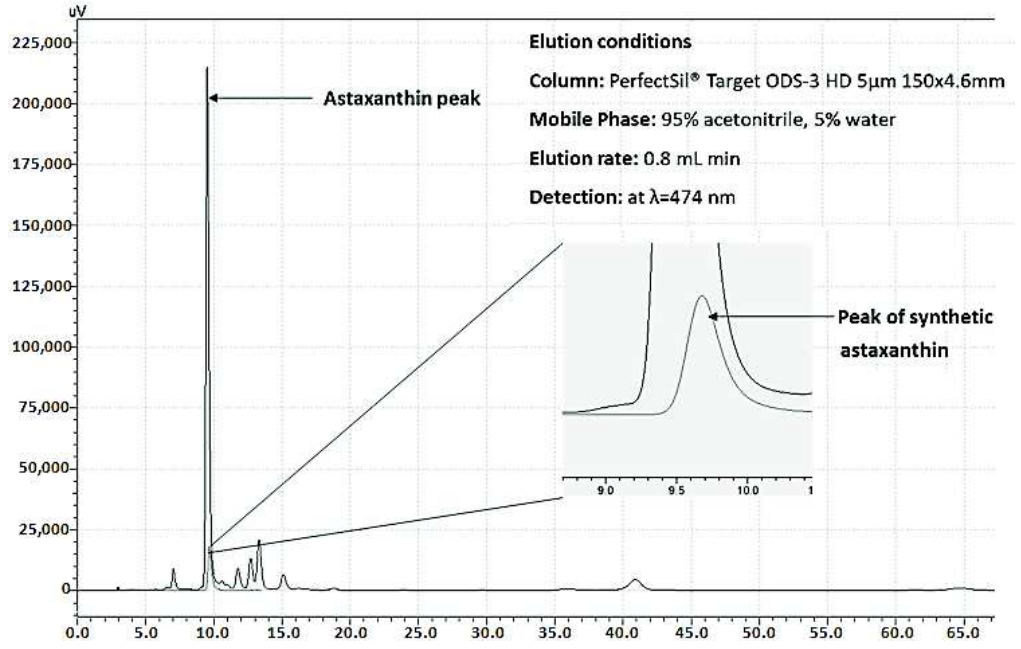

Figure 5. Typical chromatogram of astaxanthin-containing extracts from *P. rhodozyma*.

Carbon to nitrogen ratio (C/N) is another crucial factor that affect the production of microbial carotenoids. Both high and low nitrogen concentration in media affects carotenogenesis, while in the former excess nitrogen exerts its effect by inhibiting gene expression, the latter impact the output of carotenoids due to nitrogen limitation

(Stoklosa et al., 2019). In *P. rhodozyma* astaxanthin accumulation is reported to be greatly enhanced by higher C/N ratio while lower C/N ratio improves biomass production (Amado & Vazquez, 2015). Pan et al. (2017) reported that increasing C/N ratio up to 76:1 enhanced astaxanthin production, albeit resulting in decreased cellular carotenoid and astaxanthin content. The authors also observed significant alteration in expression of 9 proteins involved in the synthesis of astaxanthin with varying C/N ratio. Generally, C/N ratio < 5 negatively influences the synthesis of carotenoids in *P. rhodozyma* (Vustin et al., 2004; Zhuang et al., 2020). The C/N ratio of the control media was approximately 6:1, however since molasses contain various nitrogenous substance, these in addition to the peptone supplementation increased the total nitrogen in molasses-based hence lowering the C/N ratio and subsequently the production of astaxanthin in this media. Furthermore, at higher carbon and nitrogen loading the oleaginous red yeast *Rhodotorula glutinis* is reported to produce lipids at the expense of astaxanthin. *P. rhodozyma* is known also be oleaginous yeast and can accumulate a large quantity of lipids and as such astaxanthin content is negatively correlated with fatty acid content (Xiao et al., 2015). The presence of alternative carbon sources (like organic acids and lipids) in molasses probably increased the carbon loading in molasse media and led to lipid synthesis at the detriment astaxanthin. It is thus evident from the above that for increased carotenoid production it is imperative to use a proper C/N ratio. Currently, there is a controversy in published literature as to whether accumulation of astaxanthin is growth associated or not, while some authors report that it is growth associated, there are contrary reports that suggest otherwise (Schmidt et al., 2011). In this current study it is difficult to make a conclusion in this regard, while it seems accumulation of astaxanthin is growth-associated considering both maximum astaxanthin was recorded at the same time with maximum biomass of each media, the growth medium that yielded highest biomass (SM) did not correspondingly yield the highest astaxanthin content. Nonetheless, the results observed in this study suggest astaxanthin is associated with the stationary growth phase. In addition, while some researchers like (Stoklosa et al., 2018) have reported that yeast extract supplementation improves both growth and astaxanthin production in *P. rhodozyma*, Ramirez et al. (2000) reported that yeast extract supplementation inhibited astaxanthin synthesis. In the study growth media were supplemented with yeast extract, it is however unclear at this point the effect of yeast extract on growth and astaxanthin production on the strain of *P. rhodozyma* used in this study.

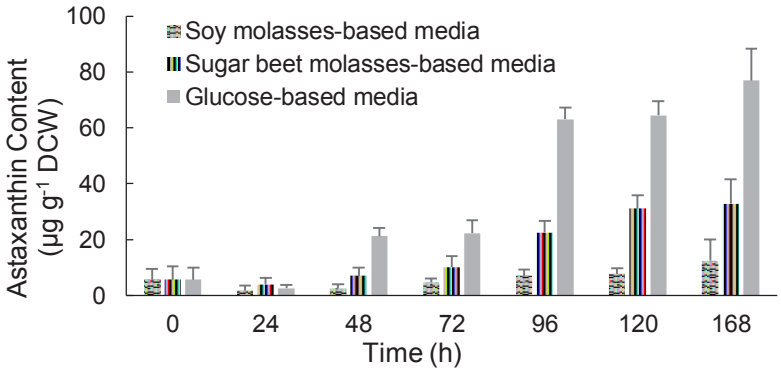

Figure 6. Astaxanthin production in different media.

Increasing the sugar concentration of SM to compensate for unassimilable sugar ultimately led to a decrease in the maximum astaxanthin accumulated by *P. rhodozyma* in SM (from 21.5 μg g^{-1} to 12.4 μg g^{-1} of DCW). Active culture growth during the first 24 h coupled with intense anabolic processes has been reported to inhibits carotenoid synthesis (Xiao et al., 2015). Increasing the sugar concentration in SM resulted in more favorable growth conditions in the initial stages, this increased growth (as evident in the biomass yield) and most likely anabolic process, hence the decrease in astaxanthin synthesis. Molasses come along alone with several compounds some of which hamper carotenoid synthesis. Phenolic for instance have been suggested to inhibit carotenoid synthesis. Results of study by Stoklosa et al. (2019) showed that significant reduction of phenolics by detoxification of sweet sorghum hydrolysate with activated carbon markedly increased biomass and astaxanthin productivity of *P. rhodozyma*. Soy molasses is reputed to be an excellent source of phenolics such as isoflavones while sugar beet molasses likewise is reported to contain substantial amount of phenolic compounds including Gallic acid (Chen et al., 2015; Zhong & Zhao, 2015). The lack of pretreatment of molasses used in the present implies phenolic compounds probably contributed to the low astaxanthin production in molasses-based media. In addition, it has been reported that carotenogenesis, especially with regard to secondary carotenoids like astaxanthin, is associated with stress response (Barredo et al., 2017). Changes in environment of culture media overtime such as depletion of nutrients, accumulation of waste, changes in pH etc., most likely are the corresponding stresses during the stationary phase that promote astaxanthin synthesis. Though changes in pH of media during the course fermentation was not monitored in this present study, it is generally known that pH of media during fermentation changes. While results of studies by Urmau et al. (2019) showed that pH of media increases over the time of fermentation, Stoklosa et al. (2018) generally recorded a lower final pH of media in contrast to pH of media before fermentation in shake flask cultures but a higher final pH in bioreactor experiments. In cultivation of *P. rhodozyma* pH is known to affects cell growth and astaxanthin production differently for each strain. Stoklosa et al. (2018) observed higher astaxanthin was obtained in medium with lower final pH at the end of cultivation. Similarly, higher concentration of astaxanthin was obtained by lowering pH of medium during fermentation (Schewe et al., 2017). These results show that pH is a significant stress that affect astaxanthin production and the lack of pH monitoring and control in the present study represents a major limitation. By and large, like biomass yield, amount of astaxanthin obtained with agric-food wastes is variable. While up to 2.07 mg g^{-1} of astaxanthin was obtained using sorghum bagasse hydrolysate (Stoklosa et al., 2019), a maximum of 293 μg g^{-1} was obtained with mesquite pods extract even after optimization (Villegas-Méndez et al., 2019). Montanti et al. (2011) also obtained astaxanthin yield of 0.36 mg g^{-1} and 0.23 mg g^{-1} with sugarcane bagasse and barley straw hydrolysate respectively. Even though these yields are several folds higher than that obtained in the present study, it is important to note that the cultivation conditions, strain of yeast and media composition (in terms of both concentration and types of compounds) in these studies differ. These parameters contribute significantly to astaxanthin yield. For instance, under the same cultivation conditions and media composition different strains of *P. rhodozyma* bioaccumulate distinct amounts of astaxanthin (Amado & Vazquez, 2015; Montanti et al., 2011). Moreover, astaxanthin yield of the same strain varies on different agro-industrial byproducts (Villegas-Méndez et al., 2019). Furthermore, pH,

temperature and light are crucial factors that affect astaxanthin bioaccumulation. The optimum level these also vary from one strain/mutant to another (Schmidt et al., 2011). Thus, even though the results of the present study show *P. rhodozyma* Y1654 can grow and accumulate astaxanthin in both soy and sugar beet molasses, optimization of the above parameters is needed to improve astaxanthin production in these media. This notwithstanding, at a less glucose concentration (10 g L^{-1}) Villegas-Méndez et al. (2019) reported that *X. dendrorhous* ATCC 24202 cultivated at 20 °C produced significantly higher astaxanthin as compared to the strain used in the present study. Thus, it is highly probably that the strain used in the present study is inferior in terms of astaxanthin production or the cultivation condition where not optimum for astaxanthin production as there is no prior data on optimum condition for increased astaxanthin by *P. rhodozyma* Y1654.

CONCLUSION

The results of this study reveal that soy molasses and sugar beet molasses are good inexpensive sources of sugar for cultivation of *P. rhodozyma* Y1654. However, a significant fraction of sugar present in soy molasses, composed mainly of raffinose and stachyose, was unassimilable by *P. rhodozyma* Y1654 suggesting it lacks pre-requisite enzymes like α-galactosidase. Hence, to improve utilization of sugar in soy molasses it is necessary to pre-hydrolyze complex sugars. For production of astaxanthin by *P. rhodozyma* Y1654, molasses-based media greatly lags behind the standard glucose media, with sugar beet molasses being superior to soy molasses. To achieve comparative astaxanthin yield, molasses-based media needs further optimization.

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