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Evaluation of physicochemical procedures for pigment extraction from mixed microalgal consortium

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ABSTRACT

Microalgae have gained attention as feedstock for value-added chemicals production. The extraction potential of four different organic solvents such as methanol, chloroform, dimethyl sulphoxide (DMSO) and acetone was assessed for obtaining chlorophyll (*Chl a* and *Chl b*), and total carotenoids over 48 h at every 4 h intervals utilizing mixed microalgal consortium. Maximum of 4.62 μg/ml of *Chl a*, 4.78 μg/ml of *Chl b* and 1.76 μg/ml of total carotenoids were obtained with DMSO, methanol and 80% acetone after 16 h, 40 h and 36 h respectively. Maximum astaxanthin yield of 0.82 μg/ml was obtained with DMSO after 20 min, which was found to decline on further increase in extraction time. Supplementing the solvent extraction process with microwave and ultrasound increased the pigment yield. The study would facilitate utilization of natural microalgal pigments as an eco-friendly and economical alternative for synthetic colorants used in the feed and agricultural industries.

1. Introduction

Microalgae are photosynthetic organisms, which have the hallmark feature of utilizing sunlight to convert the inorganic atmospheric or industrial carbon dioxide into organic biomass, that can be potentially processed into biofuels as well as multitude of bioactive compounds (Behera et al., 2019a). The simplified unicellular nature of microalgae combined with the ability to remediate wastes, ease of cultivation has drawn the interests of researchers towards these phototrophs (Rangabhashiyam et al., 2017; Behera et al., 2019b). Although the immense potential for high value metabolite production in open raceway ponds make them a promising biofuel feedstock (Tan et al., 2020), often high upstream and downstream processing costs with low product yield hinders large-scale application (Liu et al., 2019). To improve the feasibility, it is essential to reduce the cultivation costs as it accounts for 10–20% of total process economics (Hu et al., 2018). Recent study by Gour et al. (2018) proposed synthetic medium with lower amount of nitrate as a cost-effective source to culture microalgae. As a part of integrated waste management and algal cultivation, the use of municipal and domestic wastewater as the source of nutrients has gained attention over years. Since, 1% of urine contributes to 80% nitrogen and 60% phosphorous load of domestic wastewater (Chang et al., 2013), thus to better manage the different categories of wastes, the use of source separated urine is now regarded an essential substrate for culturing microalgae. Researchers have successfully grown *Spirulina platensis* (Chang et al., 2013), *C. vulgaris* (Jaatinen et al., 2016), *Scenedesmus* sp. (Chatterjee et al., 2019) and mixed algal consortium (Behera et al., 2020a) in urine with significant nutrient removal and biomass productivity. Nevertheless, the fuel only option from algae is not considered economically feasible and often decreases the rate of returns obtained during process scale-up. Thus, there is a need to adopt an altogether different approach and study the different bioactive compounds which can be obtained by growing algal biomass using cheaper substrate. This process is further expected to boost up the global algal market by 2024, with a worth of 1.1 billion USD and compounded annual growth rate (CAGR) of 7% (Algae Market, 2019–2027).

Pigments are an essential class of bioactive compounds with prominent nutraceutical properties that has been increasingly utilized in food or feed supplements (Wang et al., 2018). Due to the toxic issues associated with the use of synthetic pigments and associated safety concerns, focus has shifted to natural pigments from microorganisms. Microalgae are considered as an essential substrate for obtaining pigments devoid of any toxic content. Chlorophyll constitutes a primary component of algal biomass, possesses antioxidant properties, and is mostly utilized as coloring agents. Carotenoids are natural liposoluble pigments found in algae, consisting of hydrocarbons and their oxygenated derivatives

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(Wichuk et al., 2014). The hydrocarbon carotenoids are named as carotenes, whereas the oxygenated derivatives are known as xanthophylls (Hu et al., 2018). These molecules are associated with photosynthetic complex and act as accessory pigments protecting the plants against photoperiodic damage. Due to their anti-oxidant, hepato-protective and cardio-protective properties, these compounds have nutraceutical applications as food/feed supplements in different industries. One of the major carotenoid of commercial importance is astaxanthin, which is known for their anti-oxidant, anti-cancerous, anti-diabetic, cardio and neuro protective properties (Wang et al., 2018).

As an irreplaceable substitute for fossil fuel, the global market for these high value pigments derived from algae (cosmetics, nutraceuticals and pharmaceuticals) worth's around $2 billion (Oilgae, 2016). The global chlorophyll extract is expected to touch $463.7 million by 2025, with a CAGR of 7.5% growing from $279.5 million (by revenue) in 2018 (Chlorophyll Extract Market Report, 2019). The global carotenoids market was valued at $1577 million in 2017 and is projected to reach $2098 million by 2025, registering a CAGR of 3.6% from 2018 to 2025 (Hu et al., 2018).

One of the key factors that determine the economic viability of algae based products is the technological development of efficient upstream and downstream processes. However, the studies related to the utilization of waste resources for high value compounds like pigments are very limited. Arashiro et al. (2020) demonstrated cultivation of *Nostoc* sp., *Arthrospira platensis* and *Porphyridium purpureum* in industrial wastewater and reported 103, 57 and 30 mg/g dry weight (dw) of phycocyanin, allophycocyanin and phycoerythrin respectively, through solvent extraction using phosphate buffer. *Scenedesmus* sp., grown in domestic wastewater having a culture density of 27.4×10^6 cells/ml, produced 49.11 μg/ml chlorophyll *a* (*Chl a*) and 24.93 μg/ml carotene following sonication based pigment extraction with 90% acetone (Durvasula et al., 2015). Most studies reported above have utilized a single operational extraction conditions. Very few researchers have reported the extracted amount of astaxanthin, chlorophyll and total carotenoids from microalgae using different solvents. Singh and Rather (2018) reported an astaxanthin yield of 55% from *Haematoccoccus pluvialis* with acetone, while only 10% yield was obtained by using methanol. Among the set of four different solvents *(i.e.)* acetone, soy oil, medium-chain triglycerides (MCT) oil and limonene, the chlorophyll extraction efficiency from *Haematoccoccus pluvialis* was found to be maximum (17.55 mg/g dry weight) with acetone (Stramarkou et al., 2016). Martinez et al. (2019) reported that the algal carotenoids yield varies with the solvent type (methanol > acetone > ethanol) and extraction time, as the pigment concentration increased from 60 min to 360 min which later declined after 720 min. Over the last decade, there has been a huge interest in the use of microwave and ultrasonication to facilitate the process of pigment extraction (Amin et al., 2018). Microwave and ultrasonic waves provide a more homogenous and stable thermal environment for recovering pigments from microalgae. Amin et al. (2018) and Pasquet et al. (2011) have reported an increase in the pigment concentration when the conventional solvent extraction process is being supplemented with physical pretreatment. Fabrowska et al. (2018) reported *Chl a*, *Chl b* and total carotenoid yield of 16.9, 9.9 and 3.0 μg/ml respectively with microwaves and 10.8, 5.1 and 0.5 μg/ml respectively during ultrasound-assisted solvent extraction process for *C. glomerata*.

Based on the Web of Science data until 2017, the number of research on pigment extraction process with the use of freshwater microalgae is much less compared to macroalgae or marine microalgae (Fabrowska et al., 2018). Due to the growing interest in freshwater algae under the biorefinery concept, the number of studies has increased exponentially in the years 2018–2021, with most of the studies related to pigment extraction from *Chlorella* sp. Also, studies done so far have mostly concentrated over a single algal strain mostly cultured in synthetic medium. Still several algal strains and mixed consortium remains unexplored. It is therefore essential to explore as well as understand the influence of different operational conditions over the pigment extraction process from native freshwater algal consortia. Also, it is noteworthy to mention that very limited studies have been done reporting the pigment concentration in algae grown in urine. Feng and Wu (2006) reported 14.29 mg/g of *Chl a*, 2.27 mg/g of total carotenoids from *Spirulina maxima* grown with 180 times diluted human urine. 0.47 mg/ml of total chlorophyll was obtained from *Spirulina* sp. grown with 10^{-6} diluted cow urine (Joshi et al., 2014). A recent study by Kumar et al. (2018) reported the variation in microalgal pigment concentration with respect to the changes in urine composition from different sources, owing to the nutrient availability. None of the studies done so far have reported the influence of different physical and solvent based extraction methods over the pigment yield of microalgae grown with urine.

The present study thus analyzed the potential of native algal consortium enriched with 6.5% (v/v) diluted human urine for obtaining different class of pigments. The effect of different solvents, treatment time and the effect of physical methods (microwave, ultrasonication) on pigment extraction processes were investigated. As per the author's knowledge, the present study is among the first of its kind to analyze the concentration of pigments from a natively isolated consortium enriched with human urine through different physiochemical extraction process. Practical implications of the research have also been presented. Such studies are essential in realizing the untapped potential of utilization of waste resources for growing algal consortium to obtain high value bioactive pigments, thus making the algal technology sustainable in future.

2. Materials and methods

2.1. Microalgae culturing

Mixed microalgal consortium from the National Institute of Technology (NIT) Rourkela open ponds were collected. To reduce the cost factor involved during cultivation, the mixed algal consortium was grown with diluted human urine (6.5% v/v) at 205 μmol photons m^{-2} s^{-1} with a photoperiod of 8:16 h light-dark cycle, at ambient temperature (30 ± 5 °C). The above concentration of urine has been selected based on the previous optimization studies done by authors (Behera et al., 2020a) and the concentration of urine as reported in (Patil et al., 2021) . Often a higher urine concentration beyond a threshold value delimits the microalgal growth and metabolism due to ammonium toxicity. The influence of inhibition on microalgal growth rate due to batch variation in salts is expected to be avoided by repeated subcultures over a period of two years.

2.2. Extraction of Chl a, b and total carotenoids

Algal cultures were harvested at the end of the exponential phase (after 10 days). Five ml of algal culture was centrifuged at 7500 rpm for 10 min, followed by repeated washing (thrice) with distilled water under the same conditions to remove any residual salts or contaminants. The pellet obtained for each was suspended with 5 ml of solvent (dimethyl sulphoxide (DMSO), acetone, chloroform and methanol), vigorously vortexed for 2 min and incubated in dark conditions at 4 °C. Pigments obtained were estimated spectrophotometrically after every 4 h (over a period of 48 h) from the supernatant obtained after centrifugation (7500 rpm, 10 min). Concentration of different extracted pigments was estimated based on the equations shown in Table 1 provided by Lichtenthaler (1987) and Wellburn (1994).

2.3. Extraction of astaxanthin

Five ml of microalgal culture was centrifuged at 7500 rpm for 10 min. The supernatant was discarded and the pellet was washed twice with distilled water. The obtained pellets were saponified using 5% KOH (w/v) and 30% methanol (v/v) followed by vortex for 2 min and incubation at 70 °C for 5 min to remove the chlorophyll, and avoid any

Table 1
Equations for estimating *Chl* a (C_a), *Chl b* (C_b), total carotenoids (C_{x+c}) and astaxanthin ($C_{astaxanthin}$).

Solvent	Equations
Acetone (80%)	$C_a = (12.25A_{663.2} - 2.79A_{646.8}) * (v / V)$
	$C_b = (21.5A_{646.8} - 5.1A_{663.2}) * (v / V)$
	$C_{x+c} = ((1000A_{470} - 1.82C_a - 85.02C_b) / 198) * (v/V)$
DMSO	$C_a = (12.47A_{665.1} - 3.62A_{649.1}) * (v / V)$
	$C_b = (25.06A_{649.1} - 6.5A_{665.1}) * (v / V)$
	$C_{x+c} = ((1000A_{480} - 1.29C_a - 53.78C_b) / 220) * (v / V)$
Methanol	$C_a = (16.72A_{665.2} - 9.16A_{652.4}) * (v / V)$
	$C_b = (34.09A_{652.4} - 15.28A_{665.2}) * (v / V)$
	$C_{x+c} = ((1000A_{470} - 1.63C_a - 103.96C_b) / 221) * (v / V)$
Chloroform	$C_a = (11.47A_{665.6} - 2A_{647.6}) * (v / V)$
	$C_b = (21.85A_{647.6} - 4.53A_{665.6}) * (v / V)$
	$C_{x+c} = ((1000A_{480} - 1.33C_a - 23.93C_b) / 202) * (v / V)$
DMSO	$C_{astxanthin} = (4.5 * A_{490} * v) / V$

All pigments were expressed in μg/ml. A is the absorbance at particular wavelength, v represent volume of extracts (in ml); V is the volume of the culture medium (in ml).

interference during spectrophotometric estimation. Pellet remaining after removal of supernatant was washed thrice with deionized water. The remaining pellet was suspended with 5 ml DMSO strongly vortexed for 2 min, then incubated at 70 °C over a period of 10–40 min for the recovery of astaxanthin. Above mentioned procedure was followed until the pellet becomes colorless and the astaxanthin obtained were quantified spectrophotometrically from the supernatant obtained *via* centrifugation (7500 rpm for 10 min) at 490 nm as per the equation mentioned in the study by Liu (2018) given in Table 1. The extraction study for astaxanthin was limited with only DMSO as solvent, due to the unavailability of equations to determine the pigment concentration spectrophotometrically using acetone, methanol and chloroform. The absorbance maxima for specific pigment, depends on the pigment itself and the solvent utilized for extraction and not on the microalgal species present (Casella et al., 2020). The absorbance peak obtained in spectrophotometer at 490 nm [even-though reported for *Haematococcus pluvialis*] can be used to measure astaxanthin (other ketocarotenoids absorbance are negligible and have small spectral peaks) in all microalgae and also in case of the mixed microalgal consortium.

2.4. Extraction of pigments with physical methods

In order to study the influence of physical pretreatment methods like microwave and ultrasonication over the yield of pigments, the above-mentioned procedures before incubation with the extractant/solvent was subjected to either microwave at 180 W for 1 min or ultrasonicated at 40 kHz for 10 min, similar to the procedure of Fabrowska et al. (2018). The concentration of pigments was estimated after the optimal time period (showing maximum yield) through the spectrophotometer by using the equations given in Table 1.

2.5. Statistical analysis

All the experiments were done in triplicates and the mean and standard error were calculated using XLSTAT integrated with Microsoft Excel 2016. Statistical analysis was done using OriginPro 2016 (64-bit) through Tukey test for one-way analysis of variance (ANOVA). The means and the differences were considered significant with alpha < 0.05 at 95% confidence interval.

3. Results and discussion

3.1. Microalgal consortium composition and growth characteristics

The algal culture in diluted urine (6.5% v/v) consisted of several microalgal species and was predominantly populated by multiple microalgal species. The microalgal consortium composition was identified *via* cellular morphology observed under light microscope and consisted of *Chlorella* sp. (prominent spherical small cells); *Scenedesmus* sp. (flat, cylindrical, slightly curved cells); *Spirulina* sp. (spirally coiled filamentous structure); *Synechocystis* sp. (small, circular cells mostly occurring in colonies). The microscopic image of the consortium and the different species of microalgae identified based on the morphological differences has been shown in the previous study by Behera et al. (2020b). The stability of the microalgal culture composition and the ecology/community structure was maintained and established by repeated sub-culturing and examination under light microscope at the end of exponential phase. The detailed growth and nutrient removal profile and microscopic analysis of microalgal consortium have been already reported by (Behera et al., 2020a, 2020b). After 10 days, the microalgal biomass content of 2520 mg/l was obtained with a specific growth rate of 0.26/day. Microalgal productivity of 211 mg/l/day was obtained after a period of 10 days. Biomass content of 800 mg/l and 730 mg/l with *Spirulina platensis* and *Chlorella vulgaris* respectively have been reported with diluted human urine by Chang et al. (2013) and Jaatinen et al. (2016). Recent study by Chatterjee et al. (2019) reported 460 mg/l and 424 mg/ l biomass content for *Scenedesmus* sp., in 15 times and 20 times diluted human urine respectively. The variation in biomass content obtained is due to the differences in the culture and other operational conditions. Further, the nature of microalgae, their ability to acclimatize and the availability of nutrients in usable form due to compositional variation in urine also influences the algal growth rate and biomass content.

3.2. Yield of Chl a, Chl b and total carotenoids

As shown in Figs. 1–3, the nature of solvents and the extraction time have a considerable effect over the algal biomass and its pigment extraction efficiency. *Chl a* was best extracted with DMSO after 16 h, compared to other solvents. 4.62 μg/ml of *Chl a* was obtained within 16 h, which was found to be statistically significant with p value < 0.05, at $n = 3$, compared to other treatments interval. Beyond 16 h, the pigment concentration was found to be almost constant. The corresponding values for *Chl b* and total carotenoids under the same extraction conditions were found to be 3.09 μg/ml and 0.92 μg/ml respectively (Fig. 1).

Maximum of 4.78 μg/ml of *Chl b* was obtained with methanol after an incubation period of 40 h which was statistically significant with p value < 0.05, at $n = 3$ compared to other incubation time period (Fig. 2). Under the same experimental conditions, methanol showed a yield of 3.96 μg/ml *Chl a* and 0.5 μg/ml of total carotenoids. Acetone, on the other hand, proved to be most efficient in the extraction of total carotenoids. Treatment with 80% acetone solution resulted in a yield of 1.76 μg/ml after a period of 36 h. A considerable amount of *Chl a* (3.97 μg/ml) and *Chl b* (2.21 μg/ml) were also detected with the same operational parameters. An increase in extraction time resulted in no significant improvement in the total carotenoid yield. It is noteworthy to mention that chloroform proved to be the least effective and didn't show any detectable pigments yield. Instability of pigments and subsequent degradation during the extraction process often results in the absence of detectable amount of pigments in the extractant solution. Dominguez-Bocanegra et al. (2004) reported that with Hexane: Acetone: Ethyl alcohol as solvent mixture in ratio of 100:70:70, chlorophyll extraction from *H. pluvialis* was highest, amounting to 3.3 μg/ml at 200 h, which gradually decreased to 2.0 μg/ml after 250 h. Caesar et al. (2018) reported *Chl* $(a + b)$ productivity of 547 mg/m^2 by DMSO using green microalgae after 90 min. Extraction with acetone and methanol from *Haematococcus pluvialis* resulted in 7.03 mg/g and 7.81 mg/g of carotenoids respectively after 360 min, which declined further after 720 min (Martinez et al., 2019). The difference in the pigment yield might be due to the difference in the microalgal species and the experimental conditions including the nature of solvent and the extraction time. The

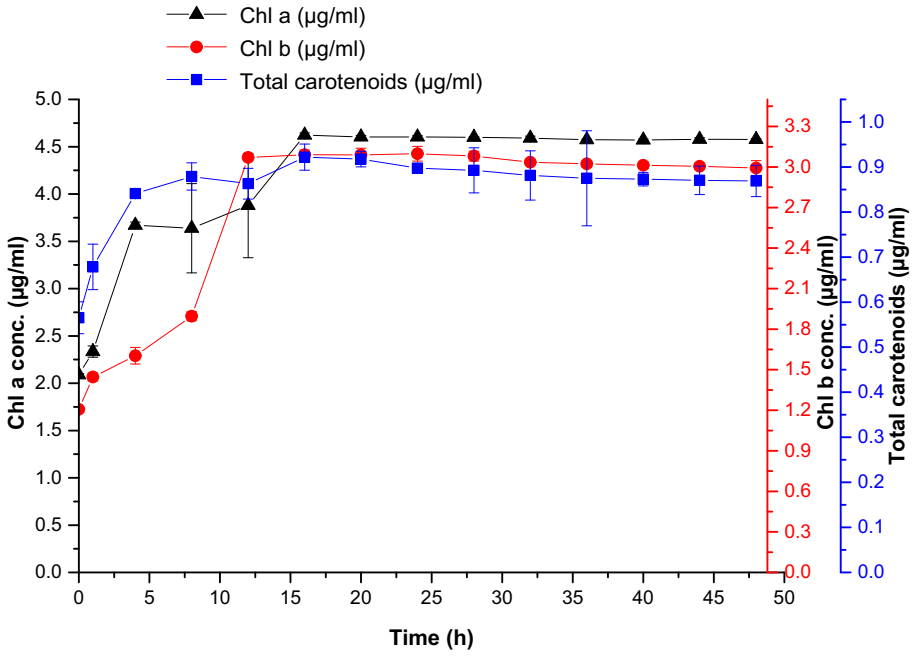

Fig. 1. Variation in concentration of *Chl a*, *Chl b* and total carotenoids with time during extraction with DMSO. Data represented are mean ± standard error at n = 3.

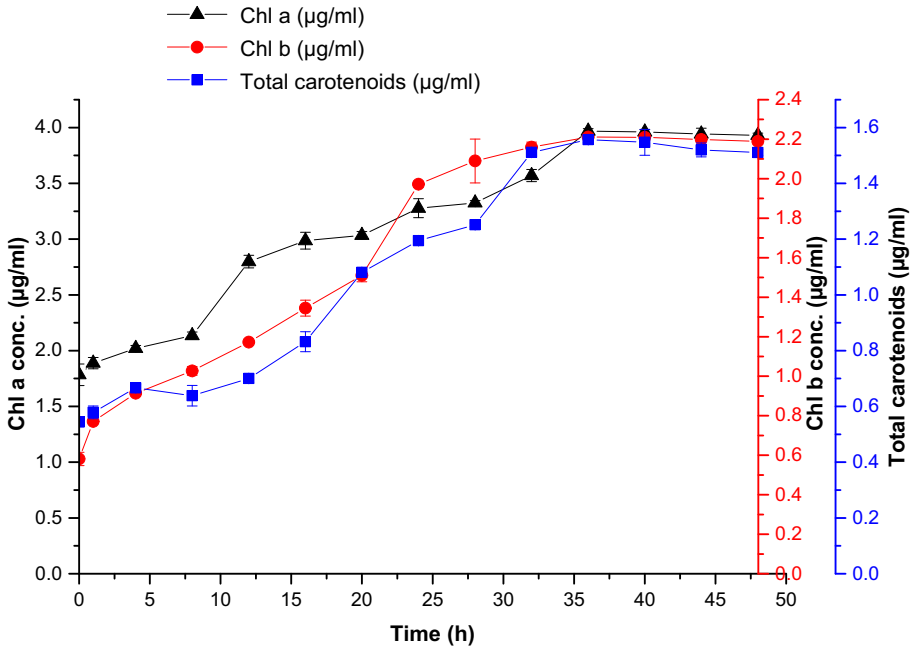

Fig. 2. Variation in concentration of *Chl a*, *Chl b* and total carotenoids with time during extraction with acetone. Data represented are mean ± standard error at n = 3.

extractability of pigments depends on the strength of solvent. Strong solvent could disrupt the cells faster, thus increase in extraction time might not have any effect on the pigment yield. On the other hand, less strong solvents might not be able to disrupt the cells sufficiently, thus an increase in extraction time will increase the yield to a certain point, beyond which it gets saturated and become constant. Nevertheless, the strength of solvent and its disruption capacity depends on the cell wall rigidity of algal cells.

3.3. Yield of astaxanthin

Maximum astaxanthin content of 0.82 µg/ml was obtained with DMSO after 20 min which later slightly declined as shown in Fig. 4. Astaxanthin yield of 4 µg/ml was obtained for freshwater microalgae *Chlorella sorokiniana* and 2 µg/ml for marine microalgae *Tetraselmis* using acetone with 10 min extraction time (Raman and Mohamad, 2012). 3.97 µg/ml and 2.91 µg/ml of astaxanthin from *C. sorokiniana* grown in Bold's Basal Media (BBM) using acetone and methanol respectively after 35 min respectively was reported by Rasid et al. (2014). Authors also reported that 0.73 µg/ml and 0.66 µg/ml

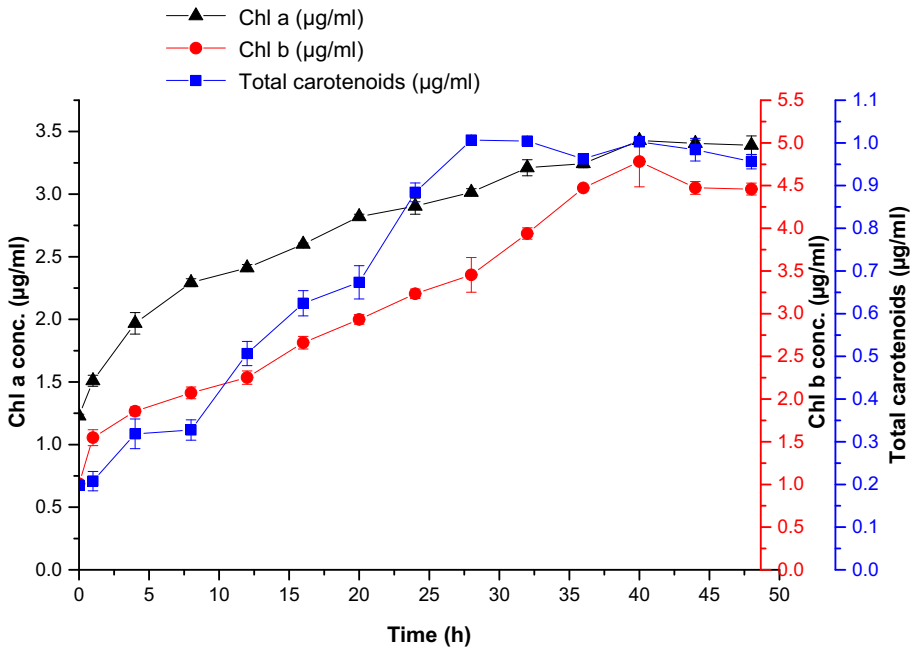

Fig. 3. Variation in concentration of *Chl a*, *Chl b* and total carotenoids with time during extraction with methanol. Data represented are mean ± standard error at n = 3.

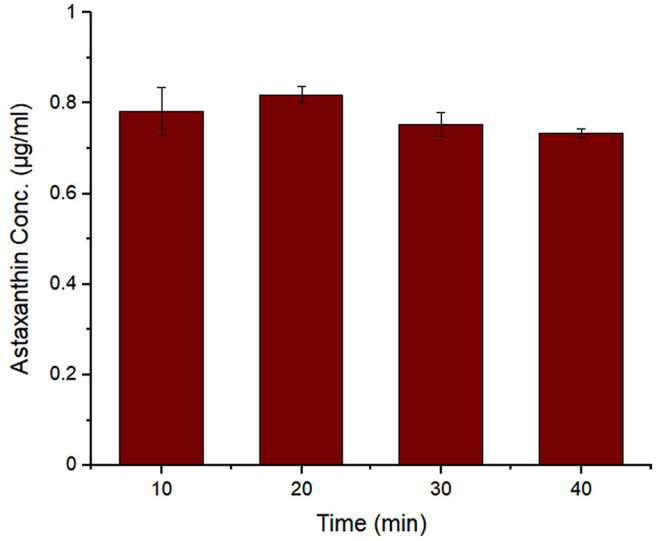

Fig. 4. Variation in concentration of astaxanthin with time during extraction with DMSO. Data represented are mean ± standard error at n = 3.

astaxanthin from *Tetraselmis* sp. F/2 media supplanted with seawater using acetone and methanol after 10 min respectively. Aburai et al. (2015) reported a yield of 4.1 mg/g of astaxanthin from *Scenedesmus* species. Kawasaki et al. (2020) reported a unique orthologs of protein corresponding to astaxanthin binding carotenoprotein in *Scenedesmus* sp., Oki-4N. Since, the algal consortium utilized in the present study through microscopic examination showed the presence of green microalgae belonging to mostly *Chlorella* sp., and *Scenedesmus* sp., these strains might act as the prominent source of astaxanthin content in the consortium. Variation in astaxanthin yield was due to the differences in metabolic efficiency of microalgae to accumulate astaxanthin. Also the strength of solvents utilized determines the overall yield. Apart from microalgae and solvent type, the extraction time also has a profound influence over the astaxanthin yield. Zou et al. (2013) reported an increase in astaxanthin yield from 10.83 µg/g after 30 min to 17. 85 µg/g after 120 min following extraction with ethanol in ethyl acetate (50:50). As evident in the present study and also reported by Dong et al. (2009), the yield of pigment increases until the equilibrium concentration is reached beyond which the extraction time has no or almost negligible effect.

3.4. Effect of physical methods on pigments yield

Microwave-assisted extraction (MAE) and ultrasonication assisted extraction (UAE) resulted in an almost 50% increase in each of the cases as represented in Table 2. Maximum yield of *Chl a* of 8.87 ± 0.99 µg/ml was obtained with MAE by DMSO after 16 h. Similarly, the highest yield of *Chl b* (7.63 ± 0.28) µg/ml and total carotenoids (4.02 ± 0.15) µg/ml were obtained with methanol and acetone-based extraction supplemented with microwave irradiation after 40 and 36 h respectively. A maximum astaxanthin yield of 3.19 ± 0.07 mg/l was obtained with MAE in the presence of DMSO after 20 min extraction time. The maximal values in each of the cases was found to be statistically significant with p

Table 2
Concentration of pigments from microalgae by MAE and UAE methods. Data represented are mean ± standard error at n = 3.

Solvent	*Chl a* (µg/ml)	*Chl b* (µg/ml)	Total carotenoids (µg/ml)	Astaxanthin (µg/ml)
Microwave-assisted extraction (MAE)				
DMSO	8.87 ± 0.99	5.21 ± 0.19	3.54 ± 0.23	3.19 ± 0.07
Acetone	6.89 ± 0.42	4.63 ± 0.14	4.02 ± 0.15	–
Methanol	6.21 ± 0.19	7.63 ± 0.28	2.30 ± 0.17	–
Ultrasound-assisted extraction (UAE)				
DMSO	6.24 ± 0.25	4.12 ± 0.10	2.50 ± 0.18	2.18 ± 0.04
Acetone	5.02 ± 0.38	4.01 ± 0.12	3.33 ± 0.15	–
Methanol	5.83 ± 0.06	6.52 ± 0.21	2.00 ± 0.17	–

value < 0.05, at $n = 3$ compared to other treatments. The concentration of the pigments was also found to increase when the solvent extraction process was supplemented with ultrasonic waves (Table 2). However, the yield was found to be comparatively lower than MAE. This also suggests that the algal species in the consortium has robust cell wall characteristic which was found to be disrupted effectively on supplementing the chemical extraction process with physical pretreatments thereby, resulting in higher yield of pigments. Similar kind of results has also been obtained by Pasquet et al. (2011) and Fabrowska et al. (2018). Amin et al. (2018) reported a yield of 17.19 μg/ml of chlorophyll from *Chlorella* sp. with ultrasound-assisted solvent extraction (45 kHz) using methanol after 120 min. As per Liyan et al. (2009), MAE is considered an efficient and economic method for recovery of astaxanthin from *H. pluvialis*. Treatment with 141 W microwave power for 83 s resulted in recovery of 594 μg of astaxanthin per 100 mg of *H. pluvialis* biomass, comparatively higher than the control without pretreatment. Pasquet et al. (2011), with the utilization of MAE at 50 W, 56 °C, after 3–5 min for treating *D. tertiolecta* reported 0.12% (dw) β-carotene, 0.45% (dw) *Chl a* and 0.13% (dw) *Chl b*, comparatively more than UAE and control without physical treatment. *A. plantensis* on treatment with 10 mM ethanol ammonium acetate combined with microwave power at 400 W, 60 °C after 15 min, resulted in extraction of 0.014% (dw) of β-carotene (Esquivel-Hernández et al., 2017). Compared to UAE, MAE in addition to cell wall disruption, also increases the solvent temperature thereby decreasing its viscosity, thus facilitating the permeability of the desired bioactive compounds into the solvent (Pasquet et al., 2011; Fabrowska et al., 2018). MAE being a fast, reproducible, homogenous and effective method could be easily and profitably utilized at industrial level to extract pigments from algal biomass without the use of additional pressure (Kapoore et al., 2018).

It is noteworthy to mention that supercritical fluid extraction (SFE) with carbon dioxide (CO_2) is one of the most common and popular pigment extraction technique that provide high efficiency, good yield in less extraction time and have zero impacts on the chemical or biological properties of products (Kapoore et al., 2018). However, the extraction system is complicated and involves very high capital costs compared to other physical methods. Further, another serious drawback that the SFE with CO_2 faces is the low solubility of polar compounds, thus might require co-solvents which might sometimes reduce the selectivity and extraction efficiency for pigments. As per the recent review by Kapoore et al. (2018), cell disruption techniques like microwave and ultrasonic extraction have been commonly used for lipid extraction, and have recently gain popularity for obtaining pigments and high value products, though the studies are very few. Microwave heat causes evaporation, thus increasing the pressure inside the cell causing it to burst and release the intracellular content. Microwave irradiation increases cell porosity, disrupts hydrogen bonds causing dissolved ions to migrate, thus facilitating easier extraction. Similarly, ultrasonication exposes the cells to high intensity sound pressure waves in alternating compression and refraction cycles, resulting in cell disruption thereby, releasing out the intracellular contents. These physical techniques could act as a suitable cost-effective and efficient alternative to obtain high value pigments.

3.5. Practical implications and applicability of research

It could be hypothesized in comparison with the studies by Raman and Mohamad (2012) and Rasid et al. (2014) for *Chlorella* sp., and Aburai et al. (2015) and Kawasaki et al. (2020) for *Scenedesmus* sp., that the above-mentioned species present in the consortium when grown under limited nutrient content present in urine might have prominently contributed to the accumulation of carotenoids. Further, to certain extent, *Spirulina* sp., might also be responsible for carotenoid production in the algal consortium, similar to that of the study by Esquivel-Hernández et al. (2017). *Synechocystis* sp., under normal physiological growth conditions do not accumulate ketocarotenoids. Thus, *Synechocystis* sp., present in the consortium might not have any role over the carotenoid production.

The yield of pigments is not only dependent on the solvent used, the operational conditions like the incubation time and physical cell disruption technique, but also on the growth medium utilized for culturing microalgae as shown in Table 3. Total pigment content (*Chl a* + *Chl b* + carotenoids) for *C. debaryana* grown in wastewater was found to be lower compared to that of the algae grown in BBM (Arora et al., 2016). The content of *Chl a* was much more reduced compared to *Chl b* and carotenoids as the presence of organic carbon in wastewater and resulting mixotrophic conditions reduces dependency over photosynthesis, relieving photoinhibition. This redirects the carbon in cells to synthesize more energy related compounds, rather than pigments. Kumar et al. (2018) reported 1.62 μg/ml, 0.76 μg/ml and 0.93 μg/ml of *Chl a*, *Chl b* and total carotenoids from *C. sorokiniana* using 10% cow urine. The study also reported a variation in yield of pigments with the use of 10% human urine. Thus, the nutrient composition of the wastewater based media (*i.e.*) the nitrogen and phosphorous load also affects the algal growth, thereby the pigment yield.

As evident from Table 3, the results obtained in the present study was comparatively lower than those reported in literature. The reason might be ascribed to the nutrient composition of the medium utilized, apart from the extractability of the solvents and other operational conditions as highlighted in the previous sections. Often, under nutrient limited conditions, the photosynthetic carbon flux is directed towards lipid synthesis rather than promoting pigment accumulation or biomass growth. Lai et al. (2019) reported that under nutrient limited conditions, the nitrogenous compounds in biomass declines, thereby reducing the concentration of pigments, though this condition can trigger starch/lipid accumulation. Apart from nutrient, salinity also impacts the growth and pigment concentration. Similar conclusions were also obtained from the study by Moura et al. (2020) which reported 35% higher chlorophyll and total carotenoid yield for *D. tertiolecta* grown in BBM compared to alternative medium with the same salts as BBM dissolved in seawater. Thus, it could also be reasoned that the presence of residual amount of salts in the urine based medium might also have hindered the accumulation of pigments in microalgae during the growth. Further, the Table 3 also provides conclusive evidence that mixed consortium are not only robust enough to grow in waste resources without much cross-contamination effects, but also shows better yield compared to single axenic cultures. For instance, the study by Beigbeder et al. (2019) have reported that mixed consortium consisting of *Chlorella* sp., *Scenedesmus* sp., and *Acutodesmus* sp. produced 25.80 μg/ml total chlorophyll and 5.90 μg/ml total carotenoids grown in Bold Basal Medium (BBM) following extraction with methanol. The values reported by authors were found to be higher than the pigment yield obtained in case of single axenic culture as utilized in the study by Arora et al. (2016) and Amin et al. (2018) having single axenic *Chlorella* sp., Nevertheless, more studies on pigment extraction from mixed microalgal consortium are necessary to derive much more authentic comparison. Also, it is noteworthy to mention that the variation in yield obtained in case of pigments present in mixed consortium might be attributed to the differences in the individual microalgal species and also the operational conditions utilized during extraction.

The present study has taken into consideration of a native mixed microalgal consortium, since it was acclimatized with the local environmental conditions and was growing in wastewater which possibly contained sewage from the urinals of the nearby hostels. The mixed consortium was selected instead of a single axenic culture as it is more robust to changes in growth conditions and resistant to cross-contamination by other microbes. The diluted human urine could act as a low cost nutrient medium for growing microalgae, which could be utilized in a biorefinery concept to extract pigments followed by biofuel production. The ethical and socio-economic concerns linked with the use of urine grown algal biomass as a source of products for food and pharmaceutical industries are limited. However, the pigments extracted

Table 3
Comparison of the pigments yield obtained in the present study with literature.

Species	Medium	Solvent	Physical treatment	Extraction time	Pigment	Yield (μg/ml)	References
Chlorella sp.	Synthetic fertilizers	Methanol	UAE	2 h	Total chlorophyll	17.19	Amin et al. (2018)
C. sorokiniana	BBM	Acetone	No	35 min	Astaxanthin	3.97	Rasid et al. (2014)
Scenedesmus sp.	Domestic wastewater + TAP medium	Acetone	UAE	24 h	*Chl a*	49.11	Durvasula et al. (2015)
C. debaryana	Domestic wastewater	Methanol	No	24 h	(*Chl a* + *Chl b* + carotenoids)	12.38	Arora et al. (2016)
C. debaryana	Sewage wastewater	Methanol	No	24 h	(*Chl a* + *Chl b* + carotenoids)	11.88	Arora et al. (2016)
C. debaryana	Paper mill effluent	Methanol	No	24 h	(*Chl a* + *Chl b* + carotenoids)	10.49	Arora et al. (2016)
C. debaryana	Dairy wastewater	Methanol	No	24 h	(*Chl a* + *Chl b* + carotenoids)	12.13	Arora et al. (2016)
C. debaryana	BBM	Methanol	No	24 h	(*Chl a* + *Chl b* + carotenoids)	14.73	Arora et al. (2016)
Mixed algal consortium (*Chlorella* sp., *Scenedesmus* sp., *Acutodesmus* sp.)	BBM	Methanol	No	24 h	Total chlorophyll	25.80	Beigbeder et al. (2019)
Mixed algal consortium (*Chlorella* sp., *Scenedesmus* sp., *Acutodesmus* sp.)	BBM	Methanol	No	24 h	Carotenoids	5.90	Beigbeder et al. (2019)
C. vulgaris	Jaworski medium	Methanol	No	15 min	Total carotenoids	3.8	Gupta et al. (2016)
Mixed algal consortium	Diluted human urine	DMSO	No	16 h	*Chl a*	4.62	Present study
Mixed algal consortium	Diluted human urine	DMSO	MAE	16 h	*Chl a*	8.87	Present study
Mixed algal consortium	Diluted human urine	DMSO	UAE	16 h	*Chl a*	6.24	Present study
Mixed algal consortium	Diluted human urine	Methanol	No	40 h	*Chl b*	4.78	Present study
Mixed algal consortium	Diluted human urine	Methanol	MAE	40 h	*Chl b*	7.63	Present study
Mixed algal consortium	Diluted human urine	Methanol	UAE	40 h	*Chl b*	6.52	Present study
Mixed algal consortium	Diluted human urine	Acetone	No	36 h	Total carotenoids	1.96	Present study
Mixed algal consortium	Diluted human urine	Acetone	MAE	36 h	Total carotenoids	4.02	Present study
Mixed algal consortium	Diluted human urine	Acetone	UAE	36 h	Total carotenoids	3.33	Present study
Mixed algal consortium	Diluted human urine	DMSO	No	20 min	Astaxanthin	0.82	Present study
Mixed algal consortium	Diluted human urine	DMSO	MAE	20 min	Astaxanthin	3.19	Present study
Mixed algal consortium	Diluted human urine	DMSO	UAE	20 min	Astaxanthin	2.18	Present study

via the growth of microalgae in urine after sterilization could be utilized as biostimulants due to their antioxidant potential for promoting the resistance of plants to environmental stress conditions. These pigments could also be utilized as colorants for feed products.

4. Conclusions

The present study analyzed the potential of a mixed microalgal consortium grown in 6.5% (v/v) diluted urine for producing different pigments. Pigment concentration was found to be influenced by the selectivity of solvent, incubation time and physical treatment. Supplementing the conventional solvent based pigment extraction process with ultrasonic and microwaves resulted in an increase in pigment concentration. Astaxanthin yield was increased from 0.82 μg/ml to 3.19 μg/ml after 20 min during microwave-assisted extraction with DMSO. Such studies would help in exploring the potential of pigments from algae grown in urine, thus facilitating large-scale commercialization of algal technology.

CRediT authorship contribution statement

AJ: Conceptualization, Data curation, Investigation, Writing - Original draft preparation
BB: Conceptualization, Data curation, Writing - Original draft preparation
BP: Conceptualization, Funding acquisition, Writing - Reviewing and editing, Supervision, Final approval.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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