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Mixotrophic cultivation of *Scenedesmus dimorphus* in sugarcane bagasse hydrolysate

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Running head:

Microalgae conversion of bagasse into lipids

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Authors Contribution:

MM performed all experiments, manuscript drafting.

QAH & AA worked on collecting and arranging data.

FJ helped in statistical analysis.

AR helped in revising the manuscript.

PMS supervised and facilitated the project, results interpretation of experiments.

JIQ contributed in critical discussions.

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Abstract:

Overuse of the fossil fuels to fulfill existing energy requirements has generated various environmental problems like global warming. Emergence of environmental issues due to burning of the fossil fuel resources has provoked researchers to explore alternative sources of fuel. In this scenario, microalgal biofuels could present a promising alternative fuel if produced cost-effectively without competing for freshwater resources and arable land. Aim of the present study was to grow microalgae by employing lignocellulosic waste for production of lipids. *Scenedesmus dimorphus* NT8c was chosen based on its ability to tolerate heat, rapid growth and ease of harvesting by overnight settling. Biochemical composition and growth parameters of microalgae were analyzed when cultivated mixotrophically on sugarcane bagasse hydrolysate, a low-value agricultural by-product that is currently underutilized. Despite a slight increase in turbidity in the medium, *S. dimorphus* NT8c cultures raised mixotrophically in 5 gL^{-1} sugarcane bagasse hydrolysate displayed significantly higher growth rates compared to photoautotrophic cultivation with an overall biomass productivity of 119.5 mg $L^{-1}d^{-1}$, protein contents of 34.82% and fatty acid contents of 15.41%. Thus, microalgae cultivated mixotrophically are capable of photosynthesizing while metabolizing and assimilating organic carbon, significant increases of biomass and lipid productivity can be achieved. However, high supplementation with organic

carbon can result in unfavorable levels of turbidity and bacterial growth, reducing microalgal biomass productivity.

Keywords: autotrophic; mixotrophic; *S. dimorphus* NT8c; FAME analysis; photosynthetic pigments

Introduction

A steady increase of both human population and their energy demand has resulted in dangerous outcomes from deforestation and fossil fuel exploitation [1,2]. Consequently, interest in different fuels such as biodiesel, bioethanol, biohydrogen and biomethane from non-food biomass and industrial effluents which otherwise represent a major portion of solid waste has been increased [3,4,5]. Biomass wastes such as lignocellulosic, food-industrial and post-harvesting agriwastes are reservoirs of polymeric carbon sources from photosynthates and organic nutrients that are currently underutilized [6].

Feedstocks for biodiesel are characterized as first, second and third generation. Edible oils and bioethanol from food crops are included in first generation feedstocks. Second (lignocellulosics) and third (microalgae) generation biofuels are considered advanced biofuels as they may not need to compete for agricultural land, biodiverse landscapes or freshwater resources [7]. Microalgae are advantageous over other biofuel crops as they have the ability to

grow throughout the year [8], need less water [9], can be cultivated on non-arable land as well as in brackish or sea water [10] and accumulate 20-50% lipids of their dry weight (DW) [11, 12]. However, the costs of microalgal biomass production need to be reduced. It has been reported that many microalgal species show potential to grow on various agro-industrial wastes [13, 14, 15, 16]. Moreover, it has also been reported that photomixotrophic cultivation leads to high cellular fatty acid content [17, 18, 19]. The capability of microalgae to grow on organic carbon substrates increases the likelihood of implication of lignocellulosic wastes/feedstocks which in turn increases net productivity and decreases cultivation costs. Sugarcane bagasse is a low-cost and abundantly available lignocellulosic substrate. Bagasse is mainly composed of cellulose (40-50%), hemicelluloses (30-35%) and lignin (20-30%) [20]. Sugarcane bagasse, the main by-product of the sugarcane industry, is being converted into sugars via chemical, thermal and enzymatic hydrolysis [21]. Two main carbohydrates found in lignocellulosic hydrolysate are D-glucose which is obtained mainly from cellulose and D-xylose from hemicelluloses [22]. Now a days, the most valuable biofuels such as bioetahnol, biogas, biohydrogen and biodiesel which are produced commercially from extortionate feedstocks like sugarcane, corn and oil crops, may also be produced from low-cost materials like sugarcane bagasse, corn stover and microalgae.

Present study elucidates the low-cost cultivation of fast-growing, heat-tolerant and easy-to-harvest microalgae (*S. dimorphus* NT8c) [23] employing sugarcane bagasse as carbon source. *Scenedesmus dimorphus* was cultivated previously in cattle digestate [24], in palm oil effluents [25], brewery waste water [26] and sugarcane bagasse [27].Another alga, *Chlorella* spp. has been

demonstrated to grow mixotrophically and heterotrophically on many organic carbon sources, but in our experience, this alga is difficult to harvest cost-effectively as it does not settle easily. In contrast, there are insufficient data and no recent studies about lipid and biomass productivities of *Scenedesmus dimorphus* cultivation on sugarcane bagasse as organic carbon source. The present study is the first to focus on low-cost bioconversion of lignocellulosic waste to biodiesel by exploiting highly efficient oleaginous microalgal isolate *Scenedesmus dimorphus* NT8c.

Materials and Methods

Pretreatment of lignocellulosic substrate:

Sugarcane bagasse was obtained from harvested sugarcane fields in Queensland, Australia (Fig.1). Bagasse was pretreated/hydrolyzed with 0.5% sulphuric acid and then autoclaved at 121^{o}C for 15 min [28]. After autoclaving, the mixture was filtered by using 0.45 µm Millipore filter paper and filtrate was used for further carbohydrate analysis and inoculation. Three different growth cultivation conditions were analyzed in the present study (Table1). All cultivation conditions were carried out in triplicates using separately grown cultures.

Culture conditions and growth parameters

Microalgae *S. dimorphus* NT8c originates from the Northern Territory, Australia where they had evolved to withstand high temperatures and salinity fluctuations [23]. Initially, microalgae stock culture was maintained at 25±2^{o}C in 250 mL Erlenmeyer flasks containing working

volumes of 200 mL in Bolds Basal Medium (BBM) [29], supplemented with 5 and 10g/L sugarcane bagasse hydrolysate under mixotrophic conditions with constant aeration through bubbling with an aeration rate of 11 L min^{-1} and a photoperiod of 16:8 hours (light:dark period) with fluorescent lights at 120 µmol photons $m^{-2}s^{-1}$mounted at the top of flasks. The inoculum concentration for photoautotrophic microalgal cultivation was 0.1 g DWL^{-1} in BBM. All experiments were carried out under batch cultivation and in triplicates using three separately grown cultures per treatment. To determine the growth rates, optical density (OD) was measured at 440nm on daily basis. pH was measured daily with a pH meter. Colorimetric API Test Kits were used along with a UV/VIS spectrophotometer (Model U-2800, Hitachi, Tokyo, Japan) to measure nitrate and phosphate contents at 545 nm and 690 nm, respectively, unless and until the nutrients became depleted. All microalgal cultures were routinely examined under the compound microscope (Olympus CX21LED) for other microalgal contamination and bacterial growth (Fig.2) by using a fluorescence microscope (Zeiss Axio Fluorescence Microscope). In the case of mixotrophic conditions, sugarcane bagasse hydrolysate was separately added in BBM medium as substrate with an initial bagasse concentration of 5 and 10 g L^{-1}. When cells attained the stationery growth period, they were centrifuged at 4800 rpm for 10 min and pellets were freeze-dried and stored at -20^{o}C for further biochemical analyses. Supernatants were also stored at 4^{o}C.

Microalgae growth

Microalgal dry weight (DW) was measured by maintaining the suspensions in non-pyrogenic 50mL pre-weighted sterile tubes before centrifugation at 4800 rpm for 5 min.

Supernatants were then removed and tubes with algal pellets were kept in a drying oven at 60^{o}C until constant weight was reached [30]. The biomass productivity was obtained by using the following formula:

$$P = \frac{\Delta X}{\Delta t}$$

Where P is biomass productivity ($mgL^{-1}d^{-1}$), ΔX is the variation of biomass concentration ($mg\ L^{-1}$) in specific cultivation times Δt (day) [31].

Quantification of pigments

a) Carotenoids

A total of 10 mg of lyophilized dry biomass was added to 10 ml acetone, and was crushed in a mortar and pestle. The mixture was then vortexed. Then, 10 ml of hexane and 5 ml of 10% NaCl were added. The mixture was centrifuged at 3000g at 4°C for about 5 minutes. The process was repeated until the mixture became colorless. Supernatants obtained were dried in a centrifugal evaporator. 2.5ml of methanol: dichloromethane (50:50) was then added into supernatant for the HPLC analysis. A total of 20µL extracts were then injected to YMC C30 carotenoids column (Waters, Milford, MA, USA). HPLC-Photodiode Array (HPLC-PDA; Shimadzu, Kyoto, Japan) analysis was undertaken as previously described [32, 33].

b) Chlorophyll

Chlorophyll a and b were measured spectrophotometrically. For that purpose, microalgae were concentrated by centrifuging a known volume of culture to pellet. A few ml of ice-cold 90% acetone was added, mixed thoroughly and grounded with a glass rod. Then 10ml of 90% acetone was added and the mixture was placed in a refrigerator for 24 h, before the supernatant was poured off into a graduated glass centrifuge tube and if necessary, re-extracted with fresh acetone. Again the supernatant was poured off and combined with the supernatants extracted earlier. The solvent (90% acetone) was used as blank against all readings. For chlorophyll (a and b), the absorbance was read at 664nm and 647nm [34] and calculated by using the following formula:

$$\text{Chlorophyll a} = 11.93\ E_{664} - 1.93\ E_{647}$$

$$\text{Chlorophyll b} = 20.36\ E_{647} - 5.50\ E_{664}$$

Total protein estimation:

Total soluble protein contents were measured by using the Lowry method [35] with slight modifications. A total of 10 mg of lyophilized microalgal biomass was crushed and then suspended in 10mL of lysis buffer (5 mL L^{-1} of Triton X-100, Chem-Supply; 0.3722 gL^{-1}ethylenediaminetetracetic acid disodium salt, Chem-Supply; 0.0348 g L^{-1} of phenyl methyl sulfonyl fluoride, Sigma Aldrich) for approximately 30 min. Then, 0.1 mL of 10% (w/v) sodium dodecyl sulphate (SDS) solution was mixed with 0.1 mL of the lysis buffer mixture. Proteins

were extracted with the help of a CB-X Protein Assay Kit. Absorbance was read at a wavelength of 595nm. The total protein content (%) of the biomass was calculated as:

$$Protein\ (\%) = \frac{\mathrm{CVD}}{\mathrm{m}} \mathrm{x}100$$

Where C is protein concentration (mg L^{-1}), V is the volume of lysis buffer, D is dilution factor, M is the quantity of biomass (mg).

Fatty Acid Methyl Ester (FAME) profiling

In a vial, 10 mg of lyophilized microalgal dry biomass was mixed together with 100µL of heneicosanoic acid dissolved in heptane and 2mL 5% acetyl chloride in methanol. The vial was placed in a water-bath heated at 90°C for 1h. To the mixture, 2mL of hexane and 2mL of 0.9% (w/v) sodium chloride solution were added. The mixture was then vortexed and centrifuged at 2000 rpm for 5min. After a clear separation of layers, 1mL of the hexane layer was transferred to a 2-mL vial and 100 µL of heptadecanoic acid dissolved in heptane was added before fatty acid methyl ester (FAME) analysis. A Shimadzu GCMSQP2010ULTRA system was used to analyze and quantify the fatty acids of each microalgal sample. A Stabilwax® (Crossbond® Carbowax® polyethylene glycol) capillary column (30mm x 0.25mm ID x 0.5 µm) was used with helium as carrier gas at a constant linear velocity of 42.7 cm/s. The temperature of the injection port was set at 250°C and 1 µL of sample was injected in split mode with a split ratio of 10 [36]. The mass spectrometer was operated with the ion source and interface temperatures at 200°C and 250°C, respectively. The mass range was from 40 to 500 m/z. Fatty acids were identified using a

Supelco® 37-component FAME mix standard purchased from Sigma-Aldrich and verified using the NIST14 library. Heptadecanoic acid was used as the internal standard, while heneicosanoic acid was used as the recovery standard for the analysis [37]. Total fatty acid (TFA) content was determined as the sum of all of the fatty acids.

Sugar analyses

Samples of cultures were filtered using 0.25 µm filters in order to remove any suspended cells before final analyses. Filtered samples were stored in HPLC vials in a -20°C freezer until analysis. For each culture, three samples were taken. For the profiling of sugars, an Agilent HPLC Alltech carbohydrate column was used to speciate glucose, fructose, xylose, maltose, sucrose and maltotriose. The method was established to work between 0.25% and 0.5% levels of sugars i.e (25 – 500 mg 100^{-1} mL). All samples were sonicated before running and diluted as per requirement so that they can be read by the ELSD detector. The mobile phase was 75% acetonitrile in water, with a 1mL/min flow rate. An Alltech Carbohydrate column, 4.6mm x 250mm was employed. ELSD was set to the sugars method with a gas flow rate of 2mL min^{-1} and a temperature of 87^{o}C [38].

Statistical analyses

All data are expressed as mean ± standard deviation. Results were analyzed statistically by using the software Minitab 16.

Results and discussion

Microalgae are capable of growing both autotrophically as well as heterotrophically. Many microalgae have ability to grow mixotrophically in the presence of an organic carbon source (acetate, glycerol, glucose, etc.), and this leads to high productivities as compared to autotrophic growth. The capability of microalgae to grow on organic carbon substrates was tested by using lignocellulosic wastes/feedstocks from sugarcane bagasse.

Effect of substrate loading on the algal growth

Biomass productivity of *S. dimorphus* NT8c was analyzed for both photoautotrophic (BBM) and two mixotrophic cultivation modes (SCBH-I and SCBH-II). Biomass productivity for the algae raised through photoautotrophic (BBM) and mixotrophic (SCBH-I and SCBH-II) cultivation (96.44, 119.25 and 105.92, mg $L^{-1}d^{-1}$, respectively; Fig. 3). Growth was highest in the medium containing 5g L^{-1} of bagasse but a decrease was measured when grown mixotrophically in medium containing 10g L^{-1} of bagasse. This may be due to the darker color of the medium because of high amounts of sugarcane bagasse and thus reduced light penetration. High concentrations of lignocellulosic waste were found not to be suitable for microalgal growth [28]. Growth and lipid productivities were also previously found to be higher in mixotrophically grown microalgae than in autotrophic or heterotrophic cultivation modes [39]. Abreu *et al*. [40] reported greater growth rates, high lipid and protein productivities of *Chlorella vulgaris* while grown mixotrophically in cheese whey (carbon and N source) as compared to growth under photoautotrophic conditions. In another study, *C. vulgaris* showed maximum biomass accumulation when grown mixotrophically on glucose than under photoautotrophic growth [41].

It has been reported that glucose enhanced high growth rates and high respiration rates in many microalgae as compared to many other carbon sources utilized like sugars alcohols, monohydric alcohols, organic acids, sugar phosphates and many other sugars [42]. Glucose alone can drive only one metabolic pathway, but sugars from sugarcane bagasse (such as xylose, glucose and many other carbohydrate components) can help in driving many metabolic pathways leading to increased biomass and oil productions [43, 44].

Effect on photosynthetic pigments

The carotenoid pigments analyzed for *S. dimorphus* NT8c in the present study were cis-β carotene, trans-β carotene, violaxanthin and lutein. Under photoautotrophic cultivation, lutein, cis-β carotene, trans-β carotene and violaxanthin were 1595.8±120.2, 451.2±209.4, 1971.2±209.4 and 400.8±166.2 μg g^{-1}DW, respectively. For biomass raised mixotrophically in medium SCBH-I, lutein, cis-β carotene, trans-β carotene and violaxanthin were 1314.8±253.0, 192.2±28.8, 935.6±173.3 and 217.01±46.7 μg g^{-1}DW, respectively, and these values were found 17.6%, 52.5%, 57.4% and 45.75% lower than the respective figures obtained for photoautotrophic cultivation. For biomass raised mixotrophically in medium SCBH-II, lutein, cis-β carotene, trans-β carotene and violaxanthin were 772.9±183.8, 105.9±18.9, 475.0± 114.9 and 112.5±34.2 μg g^{-1}DW, respectively, and these values were found 51.5%, 75.9%, 76.5% and 71.8% lower than the respective figures obtained for photoautotrophic cultivation (Fig. 4). Thus, substrate loadings had a negative correlation with the accumulation of carotenoids by microalgal cells. In another study, *Scenedesmus obliquus* showed a decrease in pigment concentration under

mixotrophic conditions compared to autotrophic cultures [45]. Chu *et al*. [46] also reported that carotenoids and chlorophyll contents decreased with an increase of glucose concentrations.

Following photoautotrophic cultivation, chlorophyll a and b were 4.36±0.68 and 4.52±0.15 µg mg^{-1}DW, respectively. For biomass raised mixotrophically in medium SCBH-I, chlorophyll a and b were 2.88±0.08 and 1.82±0.06 µg mg^{-1} DW, respectively and which are 59.9% and 79% lower than the respective figures obtained for photoautotrophic cultivation. For biomass raised mixotrophically in medium SCBH-I, chlorophyll a and b were 2.94±0.12 and 1.19±0.28 µg mg^{-1}DW, respectively and which are 91% and 89% lower than the respective figures obtained for photoautotrophic cultivation (Fig. 5). According to the previous report, utilization of external carbon source under mixotrophic conditions affect negatively on production of photosynthetic pigments [47]. In another study, glucose inhibited carbon dioxide fixation by ten folds and resulted in decreased photosynthetic pigment contents under mixotrophic conditions [48]. Chlorophyll decrement could be due to decreasing nitrogen levels as nitrogen deprivation forced cells to utilize the intracellular nitrogen pool for further cell growth. Photosynthetic pigments like chlorophyll present the most easily consumable intracellular nitrogen pools [49]. Based on the low chlorophyll production, it can be concluded that microalgae have used preferentially the available carbon sources as energy in a heterotrophic metabolism rather than CO_2 in autotrophic metabolism [40].

Effect on protein contents

Total protein content (% DW) of *S. dimorphus* NT8c was determined for both photoautotrophic (BBM) and two mixotrophic cultivation modes (SCBH-I and SCBH-II). Following photoautotrophic cultivation protein content was 29.62±0.10%of DW. For biomass raised mixotrophically in medium SCBH-I, protein content was 34.09±0.13%of DW and the value was found 15.09% higher than the respective figure obtained for photoautotrophic cultivation. For biomass raised mixotrophically in SCBH-II, protein content was 34.82±0.24% DW and the value was 17.55% higher than the respective figure obtained for photoautotrophic cultivation (fig. 6). Hu and Gao [17] also observed an increase in protein content in *Nannochloropsis* sp. when grown mixotrophically. Thus, increased production of protein in microalgae depends on the utilization of light and other organic carbon sources when grown mixotrophically [45].

Effect of cultivation methods on fatty acid profiles

The degree of unsaturation and quantification of fatty acids are two significant key factors in determining the suitability of oil as biodiesel or other purposes (e.g. omega-3 rich oil). FAMEs of *S. dimorphus* NT8c were analyzed for both photoautotrophic (BBM) and the two mixotrophic cultivation modes (SCBH-I and SCBH-II). It was found that for the biomass raised through photoautotrophic cultivation, monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA) and saturated fatty acids (SFA) were 36.55, 37.65 and 30.98 mg g^{-1} DW, respectively (Fig. 8). For the biomass raised through mixotrophic cultivation (SCBH-I), MUFA, PUFA and SFA were found to be 52.04, 48.60 and 40.02 mg g^{-1} DW, respectively. For the

biomass raised through mixotrophic condition (SCBH-II), MUFA, PUFA and SFA were found to be 37.09, 32.39 and 32.32mg g^{-1} DW, respectively. It was evident from the results that SFA, MUFA and PUFA and also total fatty acid (TFA) contents increased in the mixotrophic (SCBH-I) cultivation mode as compared to the photoautotrophic (BBM) cultivation mode. There was no significant increase in SFA, MUFA and PUFA of the biomass raised mixotrophically in 10 gL^{-1} of initial SCBH. It was found that C14:0, C16:0, C18:0 and C22:0 increased in algal biomass raised through mixotrophic (SCBH-I) cultivation as compared to photoautotrophic (BBM) cultivation. C14:0, C16:0 and C18:0 decreased for the biomass raised mixotrophically in the medium SCBH-II as compared to BBM and SCBH-I cultivation modes. C20:0 was detected in biomass raised mixotrophically (SCBH-I) but not under photoautotrophic (BBM) and mixotrophic (SCBH-II) cultivation. C22:0 was also not detected in the mixotrophic (SCBH-II) cultivation mode (Fig. 7). Microalgae that produce high amounts of SFA are ideally preferred for biodiesel feedstock as high saturation leads to good ignition quality and maximum oxidative stability and thus a high quality biodiesel production [50, 51]. SFA, PUFA and MUFA profiles are the key parameters that help in determining the quality of biodiesel according to the defined European Standards of biodiesel [52]. FAME profiling is a major factor that determines the suitability of a feedstock for biodiesel production [53]. Fatty acids synthesized in the present study ranged mainly from C16 to C18 and the same was reported in a study on four microalgal strains conducted by Abou-Shanab *et al*. [54]. The same was also observed in *Scenedesmus acutus* while grown mixotrophically in sugarcane bagasse [28].

Sugar consumption

Mixotrophic cultivation of *S. dimorphus* NT8c at two initial sugar concentrations was investigated (Fig. 8). Glucose was completely consumed when 5 and 10 g L^{-1}were provided as initial sugarcane bagasse concentrations. Furthermore, for the biomass cultivated mixotrophically in SCBH-I medium, glucose concentrations at day 0 and day 9 were 204.4±5.93 and 0.00±0.00 µg mL^{-1}, respectively and the consumption rates of glucose for the respective days were 40% and 100%. For biomass raised mixotrophically in SCBH-II medium, glucose concentrations at day 0 were 476.6±1.25 µg mL^{-1} and became non-detectable on day 9 (glucose consumption of 34% and 100%, respectively). Xylose could not be consumed completely for the cultures with 5 and 10 g L^{-1} initial sugar concentration. There are various intensive studies on culturing microalgae on D-glucose but very limited reports on D-xylose utilization. Hawkins [55] observed the capability of a *Chlorella* strain to grow on D-xylose after irradiating with UV. However, there are no reports available yet to determine the ability of wild microalgae to utilize xylose as a sole carbon source mixotrophically [21]. This may be due to the lack of effective metabolic pathways and/or uptake systems. Harsall [56] reported that D-xylose was not utilized by microalgae for chemosynthesis, rather it acted as an inhibitor for the photosynthetic process. In another study, *Neochloris oleabundans* displayed a high growth rate when cultivated in 10g L^{-1} of glucose but showed no growth when xylose and arabinose were used as a carbon sources [57].

Conclusion

This study showed that sugarcane bagasse hydrolysate served as a potential and economical candidate to cultivate *Scenedesmus dimorphus* NT8c for carotenoids and fatty acids production. Fatty acids content and productivity were increased during mixotrophic cultures as compared to the photoautotrophic conditions. Main fatty acid profiles obtained were between C16-C18 indicating a good quality biodiesel production.

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Conflict of interest

The authors declare no conflicts of interest.

References

1. Jha, S. & Bawa, K.S. (2006). Population growth, human development, and deforestation in biodiversity hotspots. *Conserv. Biol*., 20, 906-912.
2. Kouroussis, D. & Karimi, S. (2006). Alternative fuels in transportation. *Bull. Sci. Technol. Soc*., 26, 346–355.
3. Manzoor, M., Ma, R., Shakir, H.A., Tabssum, F. & Qazi, J.I. (2016). Microalgal-bacterial consortium: a cost effective approach of wastewater treatment in Pakistan. *Punjab Uni J. Zool*., 31, 307-320.

4. Javaid, H., Manzoor, M., Qazi, J.I., Xiaochao, X. & Tabssum, F. (2017). Potential of oleaginous yeast as economic feedstock for biodiesel production. Biologia, 63, 217-234.
5. Ahmad, Q.A., Yang, S-T., Manzoor, M. & Qazi, J.I. (2017). Moderate alkali-thermophilic ethanologenesis by locally isolated *Bacillus licheniformes* from Pakistan employing sugarcane bagasse: a comparative aspect of aseptic and non-aseptic fermentations. *Biotechnol Biofuels*, 10, 105.
6. Hannon, M., Gimpel, J., Tran, M., Rasala, B. & Mayfield, S. (2010). Biofules from algae: Challenges and potential. Biofuels, 1(5), 763-784.
7. Demirbas, A. (2009). Progress and recent trends in biodiesel fuels. Energy Conversion and Management, 50, 14-34.
8. Schenk, P.M., Thomas-Hall, S.R., Stephens, E., Marx, U.C., Mussnug, J.H., Posten, C., Kruse, O. & Hankamer, B., (2008). Second generation biofuels: high-efficiency microalgae for biodiesel production. Bioenergy Research, 1**,** 20-43.
9. Dismukes, G. C., Carrieri, D., Bennette, N., Ananyev, G. M. & Posewitz, M. C. (2008). Aquatic phototrophs: efficient alternatives to land-based crops for biofuels. Curr. Opin. Biotechnol. 19**,** 235–240.
10. Searchinger, T., Heimlich, R., Houghton, R. A., Dong, F., Elobeid, A., Fabiosa, J., Tokgoz, S. & Hayes, D.Y. (2008). Use of US croplands for biofuels increases greenhouse gases through emissions from land-use change. *Science.*319, 1238–1240.

11. Metting, F. B. (1996). “Biodiversity and application of microalgae”. Journal of Industrial Microbiology and Biotechnology17, 477 -489.
12. Spolaore, P., Joannis-Cassan, C., Duran, E. & Isambert, A. (2006). Commercial application of microalgae. Journal of Bioscience and Bioengineering, 101, 87–96.
13. Mulbry, W., Kondrad, S., Pizarro, C. & Kebede-Westhead, E. (2008). Treatment of dairy manure effluent using freshwater algae: algal productivity and recovery of manure nutrients using pilot-scale algal turf scrubbers. Bioresour.Technology, 99, 8137-8142.
14. Chinnasamy, S., Bhatnagar, A., Hunt, R.W. & Das, K.C. (2010). Microalgae cultivation in a wastewater dominated by carpet mill effluents for biofuel applications. Bioresour. Technol.**,** 101, 3097–3105.
15. Sturn, BS., Peltier, E., Smith, V. & deNoyelles, F. (2012). Control of microalgal biomass and lipid production in municipal wastewater-fed bioreactors, Environmental Progress & Sustainable Energy, 31(1), 10-16.
16. Manzoor, M., Tabssum, F., Javaid, H. &Qazi, J.I. (2015). Lucrative future of microalgal biofuels in Pakistan: a review. Int. J. Energy Environ. Eng. 6, 393-403.
17. Hu, H. & Gao, K. (2003). Optimization of growth and fatty acid composition of unicellular marine picoplankton, *Nannochloropsis* sp., with enriched carbon sources. Biotechnol. Lett.25, 421–425.

18. Feng, FY, Yang, W, Jiang, GZ, Xu, YN. & Kuang, TY. (2005). Enhancement offatty acid production of *Chlorella* sp. (Chlorophyceae) by addition ofglucose and sodium thiosulphate to culture medium. Process Biochem, 40, 1315–131.
19. Oh SH, Kwon MC, Choi WY, Seo YC, Kim GB, Kang DH, Lee SY. & Lee HY. (2010). Long-term outdoor cultivation by perfusing spent medium for biodiesel production from *Chlorella minutissima*. J Biosci Bioeng, 110, 194–200.
20. Inyang, M., Gao, B., Pullammanappallil, P., Ding, W.C. & Zimmerman, A.R. (2010). Biochar from anaerobically digested sugarcane bagasse. Bioresour. Technol., 101, 8868–8872.
21. Gamez, S., Cabriales, J.G., Ramırez, J.A., Garrote, G. & Vazquez, M. (2006). Study of the hydrolysis of sugar cane bagasse using phosphoric acid. J. Food Eng., 74, 78-88.
22. Zheng, Y., Yu, X., Li, T., Xiong, X & Chen, S. (2014). Induction of D-xylose uptake and expression of NAD(P) H-linked xylose reductase and $NADP^+$ -linked xylitol dehydogenase in the oleaginous microalga *Chlorella sorokiniana*. Biotechnology for Biofuels, 7**,** 125.
23. Duong, VT, Skye TH & Schenk, PM. (2015). Growth and lipid accumulation of microalgae from fluctuating brackish and sea water locations in South East Queensland-Australia. Frontier in Plant Science. 6, 359.
24. Cici, A. & Bravi, M. (2014). Production of the freshwater microalgae *Scenedesmus dimorphus* and *Arthrospira platensis* by using cattle digestate. Chemical Engineering Transactions. 38, 85-90.

25. Fakir, KL, Zahira, Y., Renganthan, R., Sobri, TM. & Masrinda, TS. (2013). Bioremediation of palm oil mill effluents (POME) using *Scenedesmus dimorphus* and *Chlorella vulgaris*. Advanced Science Letters. 19(10), 2914-2918.

26. Lutzu, GA, Zhang, W. & Liu, T.(2015). Feasibility of using brewery wastewater for biodiesel production and nutrient removal of *Scenedesmus dimorphus.* Environmental Technology, 37(12), 1568-1581.

27. Manzoor, M., Skye, TH & Schenk, PM. (2018). *Scenedesmus dimorphus* NT8c conversion of sugarcane bagasse hydrolysate into biodiesel. New Biotechnology, 44, S124.

28. Rattanapoltee, P. & Kaewkannetra, P. (2014). Utilization of agricultural residues of pineapple peels and sugarcane bagasse as cost-saving raw materials in *Scenedesmus acutus* for lipid accumulation and biodiesel production. Appl. Biochem. Biotechnol. 10, 949-964.

29. Nichols, H.W.(1973). Growth media – freshwater. In: Stein, J. (Ed.), Handbook of Phycological Methods, Culture Methods and Growth Measurements. Camb. Univ. Press, pp. 7–24.

30. Aslam, A., Skye, TH, Mughal, TA & Schenk, PM.(2017). Selection and adaptation of microalgae to growth in 100% unfiltered coal-fired flue gas. Bioresource Technology, 233, 271-283.

31. Ho, S.-H., Chen, C.-Y. & Chang, J.-S. (2012). Effect of light intensity and nitrogen starvation on CO_2 fixation and lipid/carbohydrate production of an indigenous microalga *Scenedesmus obliquus* CNW-N. Bioresour. Technol., 113, 244–252.

32. Ahmed F., Fanning K., Netzel M., Turner W., Li Y. & Schenk P. M. (2014). Profiling of carotenoids and antioxidant capacity of microalgae from subtropical coastal and brackish waters. Food Chem., 165, 300–306.

33. Alsenani, F., Wass, TJ, Ma, R., Eltanahy, E., Netzel, ME & Schenk, PM. (2019). Transcriptome wide analysis of Chlorella reveals auxin-induced carotenogenesis pathway in microalgae. Algal Research, 37, 320-335.

34. Jeffrey, S.W. & Humphrey, G.F. (1975). New spectrophotometric equations for determining chlorophyll a, b c1 and c2 in higher plants, algae and natural phytoplankton. Biochem. Physiol. Pflanz., 167, 191-194.

35. González López CV, García MCC., Fernández FGA., Bustos CS., Chisti Y. & Sevilla JMF. (2010) Protein measurements of microalgal and cyanobacterial biomass. Bioresour. Technol. 101, 7587–7591.

36. Lim, DK, Garg, S., Timmins, M., Zhang, ES., Thomas-Hall, SR., Schuhmann, H., Li, Y. & Schenk, PM. (2012). Isolation and evaluation of oil-producing microalgae from subtropical coastal and brackish waters. PLoSONE**,** 7, 40751.

37. Ma, R., Skye, TH., Chua, ET., Eltanahy, E., Netzel, ME., Netzel, G., Lu, Y. & Schenk, PM. (2018). LED power efficiency of biomass, fatty acid and carotenoid production in *Nannochloropsis* microalgae. Bioresour. Technol., 252, 118-126.

38. Manzoor, M., Jabeen, F., Skye, TH., Altaf, J., Younis, T., Schenk, PM. & Qazi, JI.(2019). Sugarcane bagasse as a novel low/no cost organic carbon source for growth of *Chlorella* sp. BR2. Biofuels, https://doi.org/10.1080/17597269.2019.1580970.

39. Cheirisilp, B. & Torpee, S. (2012). Enhanced growth and lipid production of microalgae under mixotrophic culture condition: effect of light intensity, glucose concentration and fed-batch cultivation. Bioresour. Technol., 110, 510–516.

40. Abreu AP., Fernandes, B., Vicente, AA., Teixeira, J. & Dragone, G. (2012). Mixotrophic cultivation of *Chlorella vulgaris* using industrial dairy waste as organic carbon source. Bioresour. Technol., 118, 61–66.

41. Kong, W., Song, H., Cao, Y., Yang, H., Hua, S. & Xia, C. (2011). The characteristics of biomass production, lipid accumulation and chlorophyll biosynthesis of *Chlorella vulgaris* under mixotrophic cultivation. African J. Biotech., 10, 11620-11630.

42. Gabov, K., Hemming, J. & Fardim, P. (2017). Sugarcane bagasse valorization by fractionation using a water-based hydrotropic process. Industrial crops and products.

43. Yang, C., Hua, Q. & Shimizu, K., 2000. Energetics and carbon metabolism during growth of microalgal cells under photoautotrophic, mixotrophic and cyclic light-autotrophic/dark-heterotrophic conditions. Biochem. Eng. J., 6, 87–102.

44. Zhao, G., Yu, J., Jiang, F., Zhang, X. & Tan, T. (2012). The effect of different trophic modes on lipid accumulation of *Scenedesmus quadricauda*. Bioresour. Technol. 114, 466–471.

45. El-Sheekh, M. Mostafa., Bedaiwy, Y. Mohamed., Osman, E. Mohamed & Ismail, M. Mona. (2012). Mixotrophic and heterotrophic growth of some microalgae using extract of fungal-treated wheat bran. International Journal of Recycling of Organic Waste in Agriculture. 1, 12.

46. Chu, WL., Phang, SM. & Hock, GS. (1995). Influence of carbon source on growth biochemical composition and pigmentation of *Ankistrodesmus convolutus*. J. Appl. Phycol., 7, 59–64.

47. Deng, X-Y., Xue, C-Y., Chen, B., Amoah, PK., Li, D., Hu, X-L. & Gao, K. (2018). Glucose addition induced changes in the growth and chemical compositions of a freshwater microalga *Chlorella kessleri*. J. Chemical Techn. Biotech. 94(4), 1202-1209.

48. Kong, W-B., Yang, H., Cao, Y-T., Song, H., Hua, S-F. & Xia, C-G.(2013). Effect of glycerol and glucose on the enhancement of biomass, lipid and soluble carbohydrates production by *Chlorella vulgaris* in mixotrophic culture. Food Technol. Biotechnol.51, 62-69.

49. Yang, J., Rasa, E. Tantayotai, P., Scow, KM., Yuan, H.& Hristova, K.R.(2011). Mathematic al model of *Chlorella minutissima* UTEX2341 growth and lipid production under photoheterotrophic fermentation conditions. Bioresour. Technol., 102, 3077–3082.

50. Hu, Q., Sommerfeld, M., Jarvis, E., Ghirardi, M., Posewitz, M., Seibert, M. & Darzins, A. (2008). Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. Plant J., 54, 621-639.

51. Knothe, G. (2008). “Designer” biodiesel: optimizing fatty ester composition to improve fuel properties. Energy Fuels., 22, 1358-1364.

52. Ramos, MJ., Fernández, CM., Casas, A., Rodríguez, L. & Pérez, A. (2009). Influence of fatty acid composition of raw materials on biodiesel properties. Bioresour. Technol.100, 261-268.

53. Knothe, G. (2009). Improving biodiesel fuel properties by modifying fatty esters composition. J. Energy Environ Sci. 10, 1039-1054.

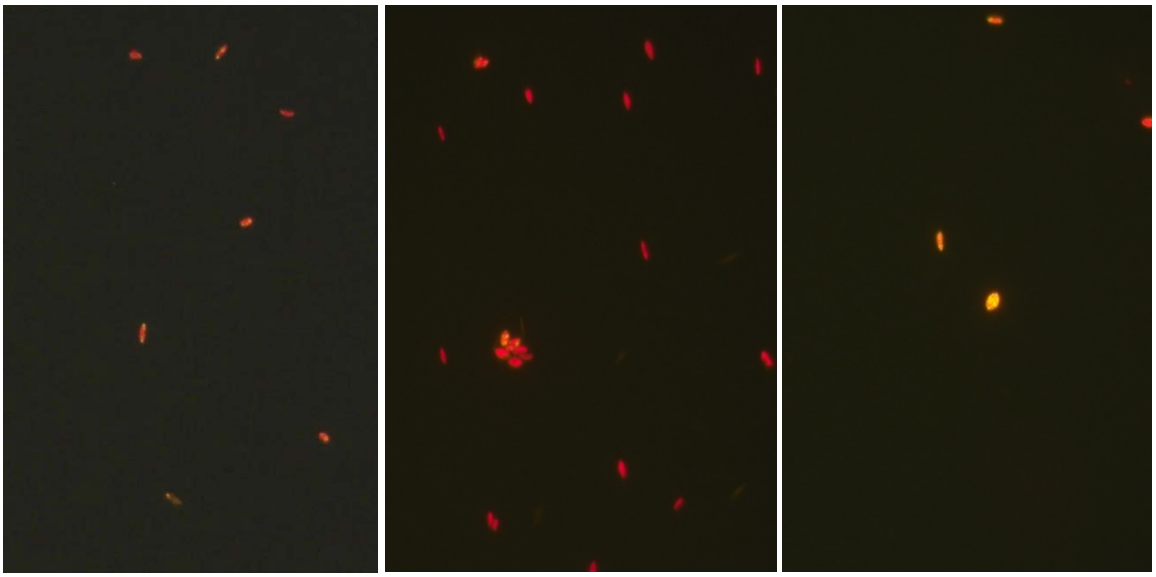
54. Abou-Shanab, RAI., Matter, IA., Kim, S-N., Oh, Y-K., Choi, J., & Jeon, B-H. (2011). Characterization and identification of lipid-producing microalgae species isolated from a freshwater lake. Biomass Bioenerg., 35**,** 3079-3085.

55. Hawkins, RL. (1999). Utilization of xylose for growth by the eukaryotic alga, *Chlorella*. Curr. Microbiol., 38**,** 360–363.

56. Hassall, KA. (1958). Xylose as a specific inhibitor of photosynthesis. Nature. 181, 1273–1274.

57. Morales-Sánchez, D., Martinez-Rodriguez, OA., Kyndt, J. & Martinez, A. (2015). Heterotrophic growth of microalgae: Metabolic aspects. World J. Microbiol. Biotechnol. 31**,** 1–9.

Fig. 1: a) Sugarcane bagasse before pretreatment b) Sugarcane bagasse after pretreatment with 0.5% H_2SO_4



a **b** **c**

Fig. 2: *Scenedesmus dimorphus* NT8c after cultivation in various media. a) cells (BBM) Nile red stained cells grown in BBM; b) Nile red stained cells grown in SCBH-I; c) Nile red stained cells grown in SCBH-II. All images are shown at 40X magnification.

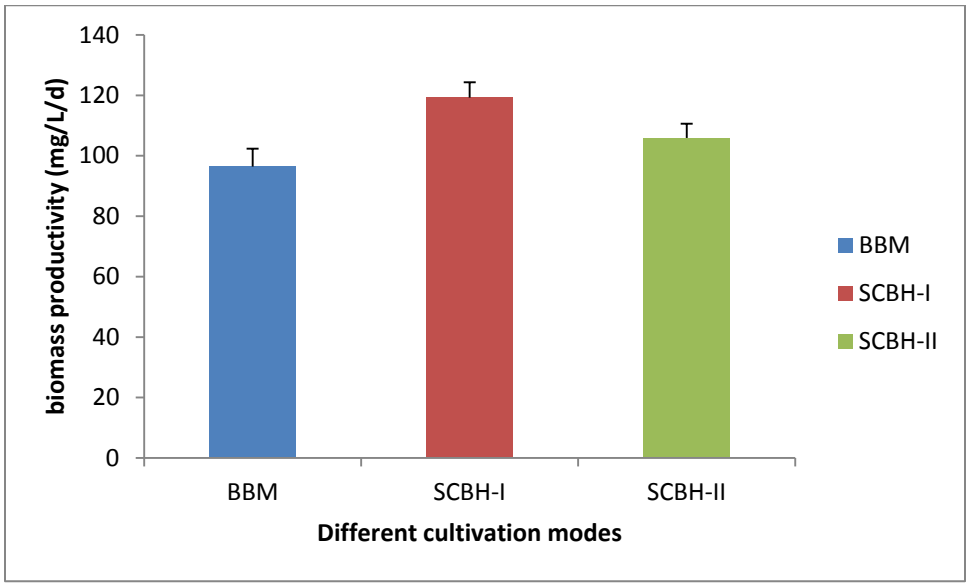


Fig. 3: Growth of *S. dimorphus* NT8c cultures in photoautotrophic (BBM) and mixotrophic medium (SCBH-I/SCBH-II) until the medium was deplete of detectable nitrate. BBM= Bolds Basal Medium; SCBH-I = Sugarcane bagasse hydolysate (5g/L); SCBH-II = Sugarcane bagasse hydolysate (10g/L). Shown are mean values ±SE of three separately grown cultures. BBM and SCBH-1 data are significantly (P<0.05) different.

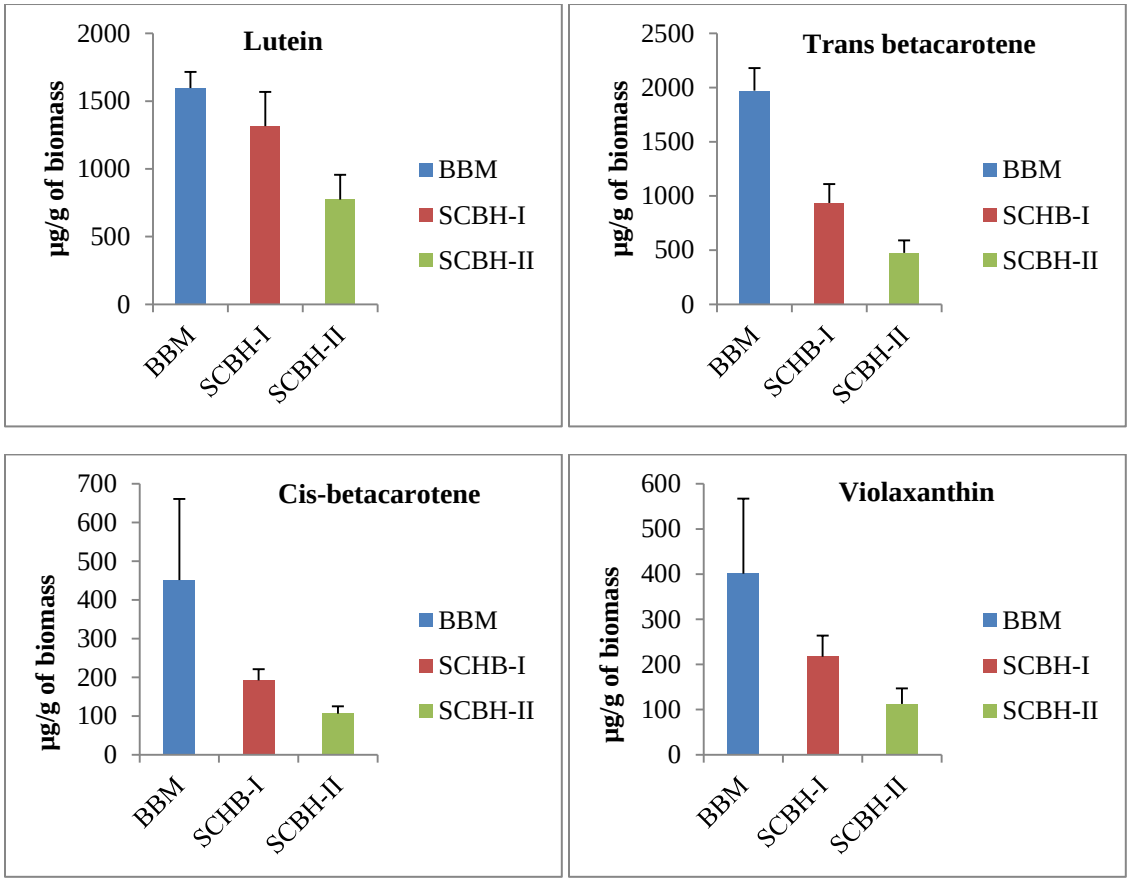


Fig. 4: Comparison of different carotenoid pigments (µg/g dry weight) in photoautotrophic (BBM) and mixotrophic (BBM+SCBH-I/SCBH-II) *S. dimorphus* NT8c cultures. BBM= Bolds Basal Medium; SCBH-I = Sugarcane bagasse hydolysate (5g/L); SCBH-II = Sugarcane bagasse hydolysate (10g/L). Shown are mean values ±SE of three separately grown cultures.

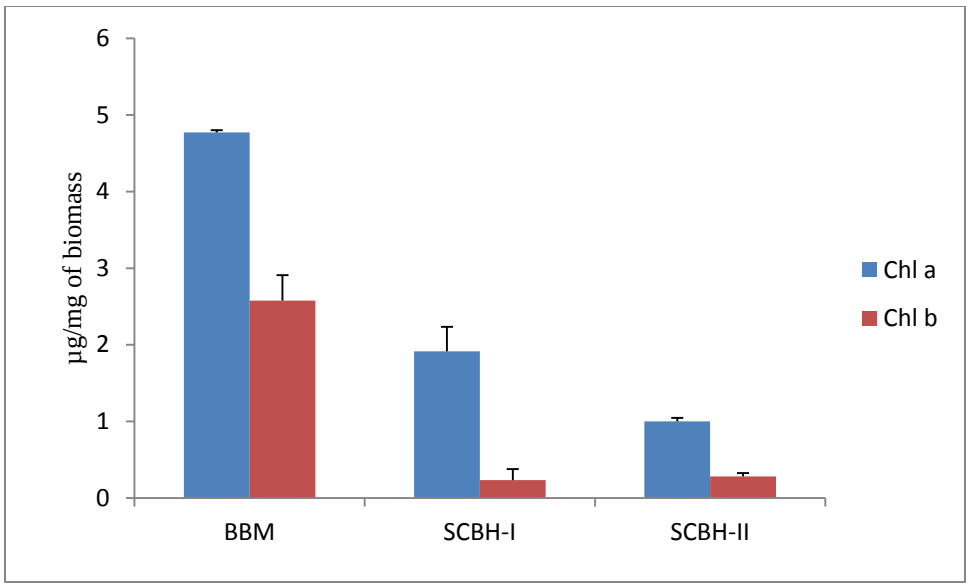


Fig. 5: Total chlorophyll of *S. dimorphus* NT8c cultures (µg/mg dry weight) in photoautotrophic (BBM) and mixotrophic (SCBH-I/SCBH-II) medium. BBM= Bolds Basal Medium; SCBH-I = Sugarcane bagasse hydolysate (5g/L); SCBH-II = Sugarcane bagasse hydolysate (10g/L).Shown are mean values ±SE of three separately grown cultures.

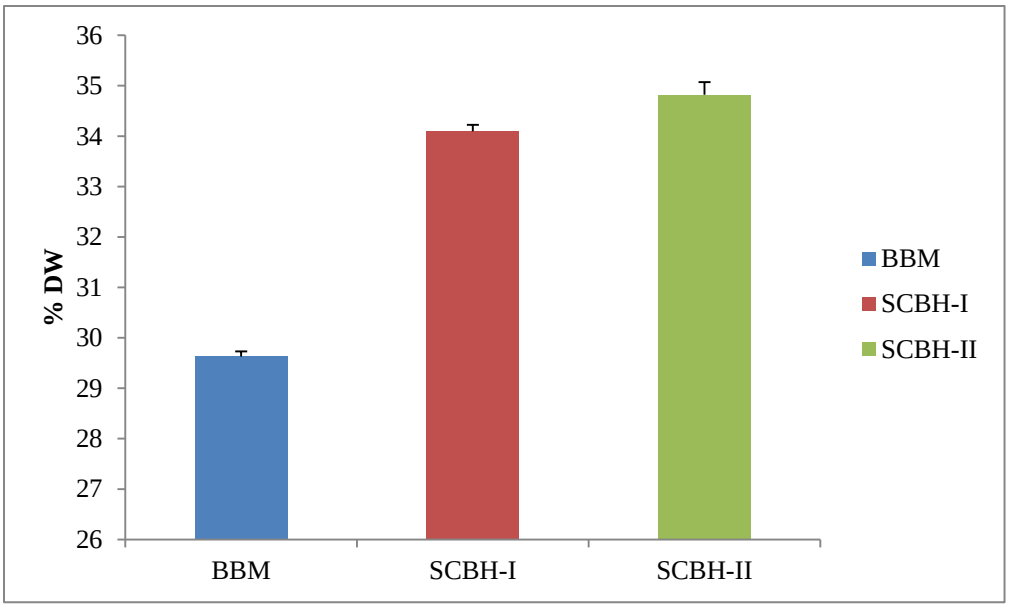
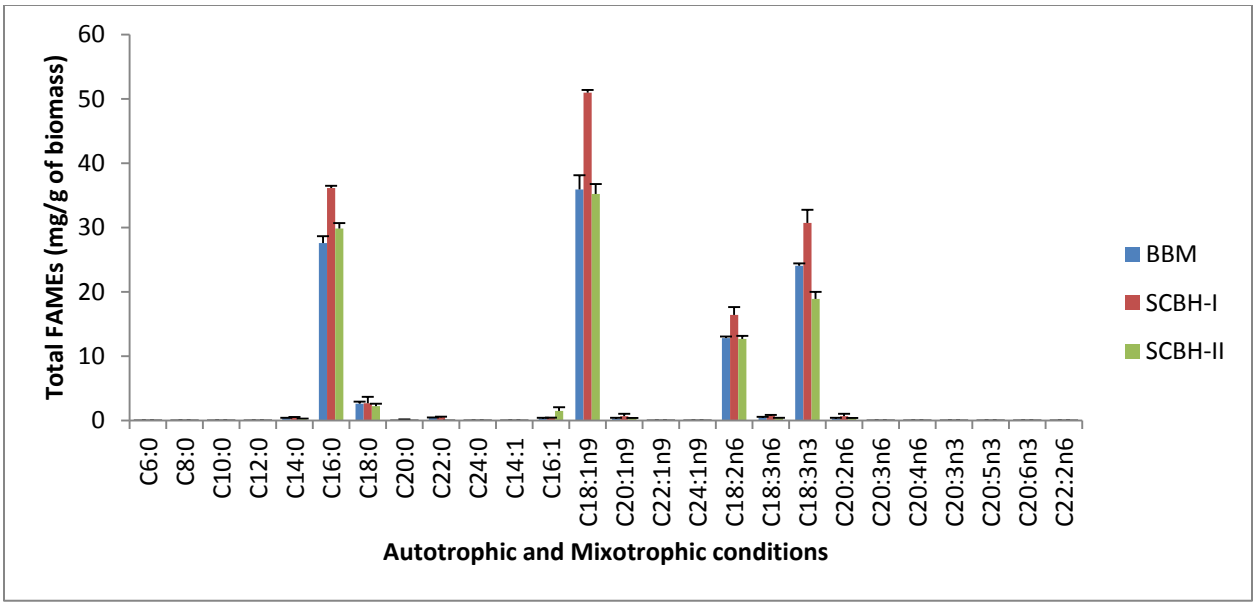


Fig. 6: Total protein contents of *S. dimorphus* NT8c cultures (%dry weight) under photoautotrophic (BBM) and mixotrophic (SCBH-I/SCBH-II) cultivation. BBM= Bolds Basal Medium; SCBH-I = Sugarcane bagasse hydolysate (5g/L); SCBH-II = Sugarcane bagasse hydolysate (10g/L). Shown are mean values ±SE of three separately grown cultures.



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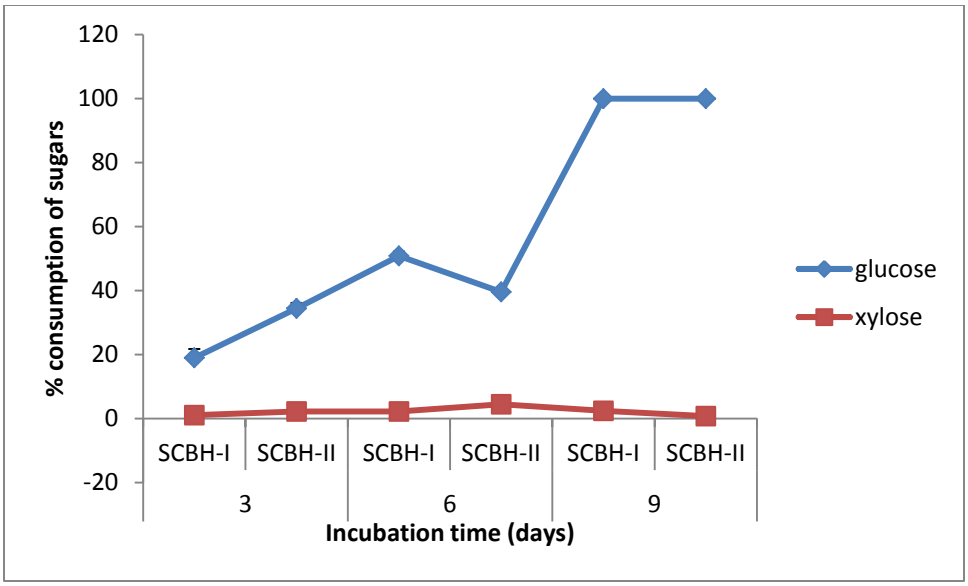

Fig. 8: Percentage consumption of sugars by *S. dimorphus* NT8c cultivated in BBM, SCBH-I and SCBH-II media.Shown are mean values ±SE of three separately grown cultures.

Table 1: Different growth conditions/media for cultivating *S. dimorphus* NT8c

Growth conditions	Carbon source
Photoautotrophic (BBM)	Air
Mixotrophic (SCBH-I)	Air and 5g L^{-1} hydrolyzed SCB
Mixotrophic (SCBH-II)	Air and 10g L^{-1} hydrolyzed SCB

(Where, BBM: Bolds Basal Medium; SCB: Sugarcane bagasse)

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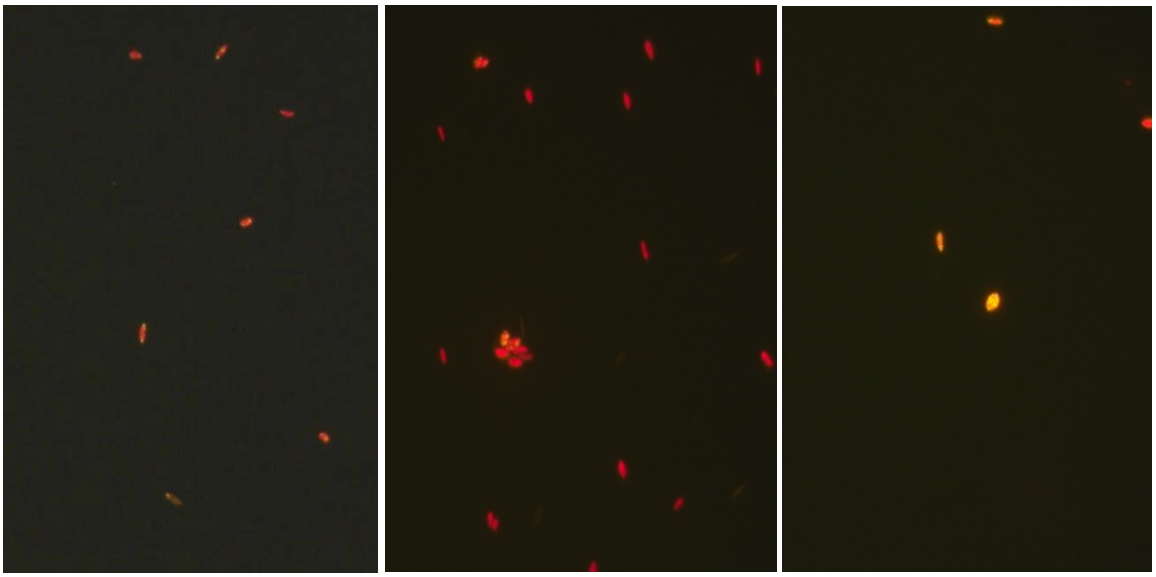

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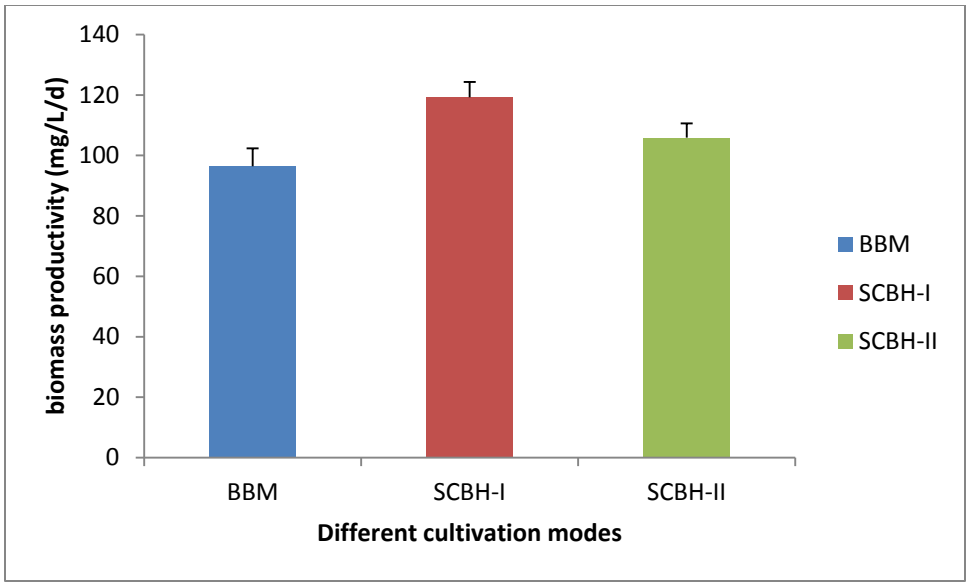

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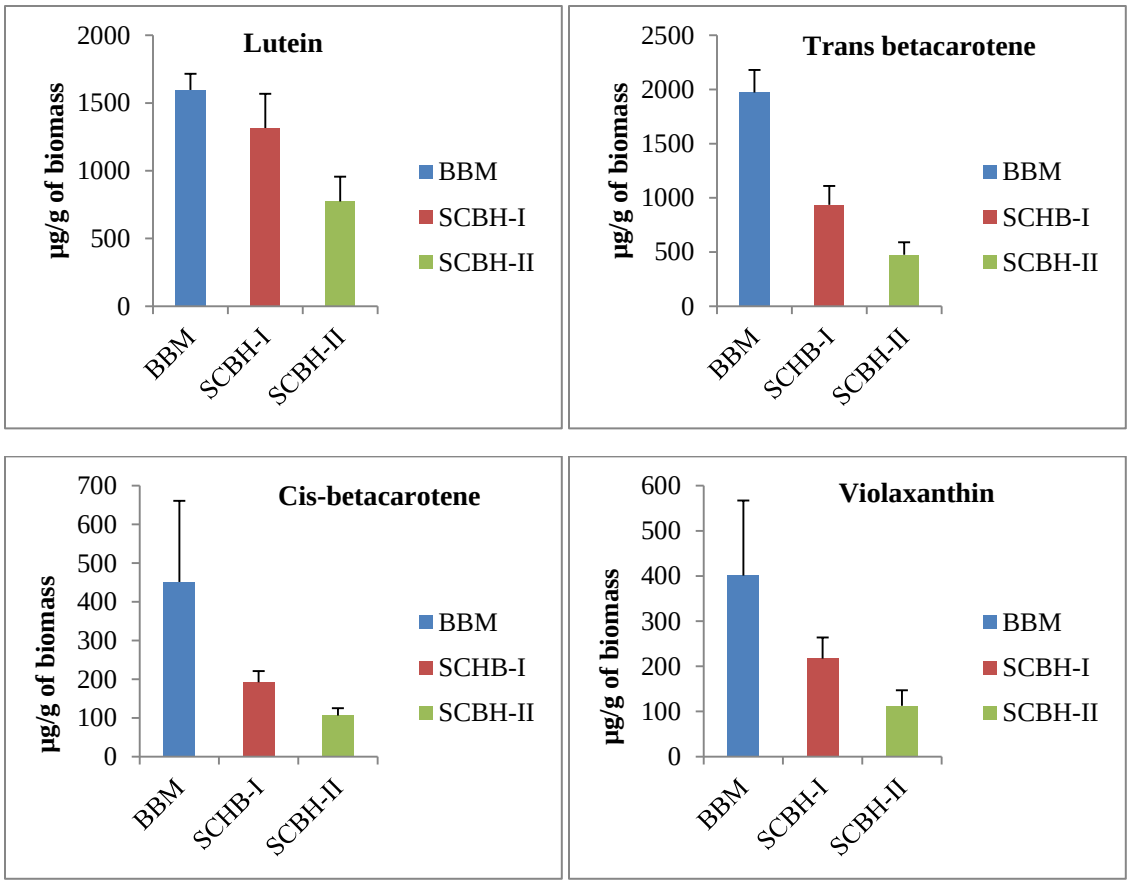

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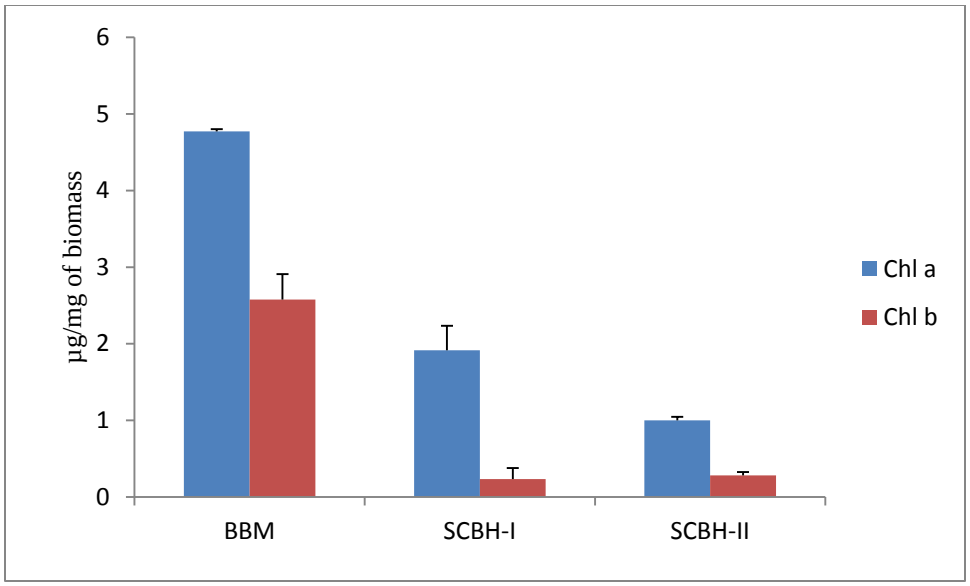

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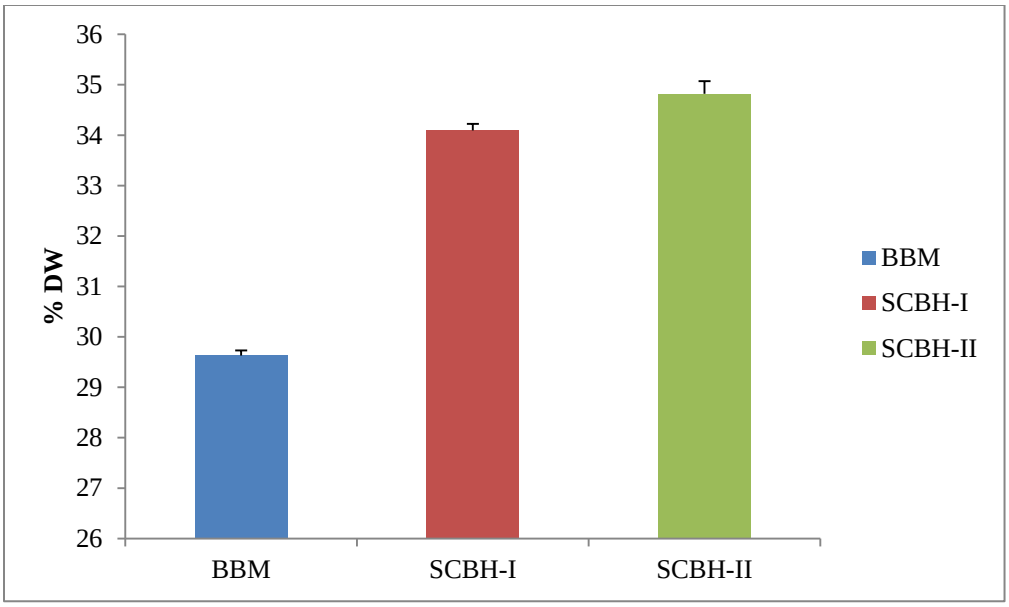

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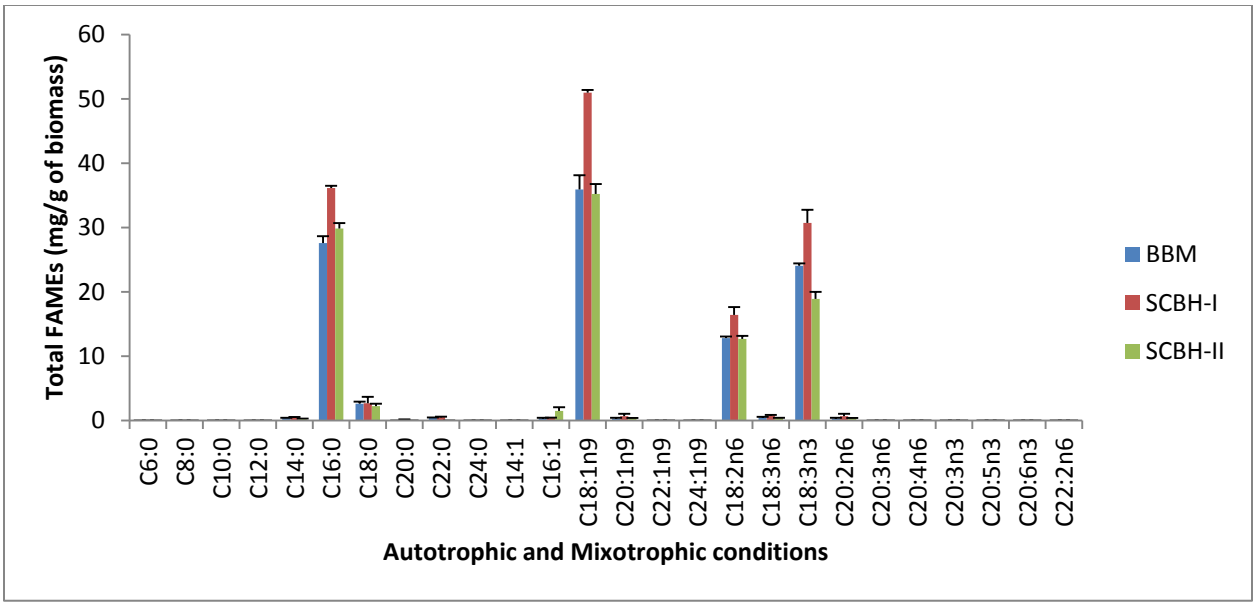

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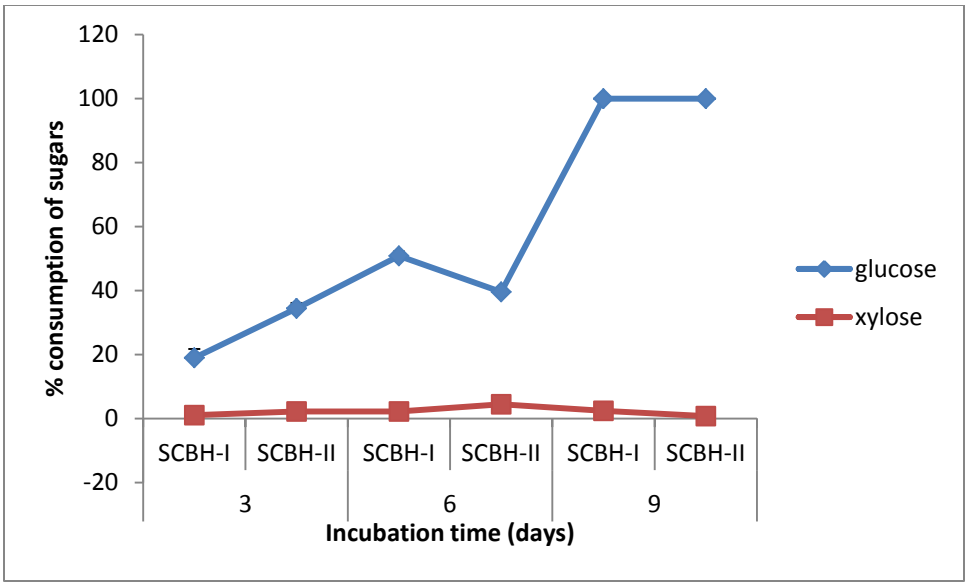

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