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Dual role of *Chlorella sorokiniana* and *Scenedesmus obliquus* for comprehensive wastewater treatment and biomass production for bio-fuels

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ABSTRACT

Microalgal treatment of raw sewage presents many complexities, mainly resulting from the inability of the algal species to sustain increased physiological stresses due to variable nutrient levels and high concentrations of organics. *Chlorella sorokiniana* and *Scenedesmus obliquus* have been identified to tolerate higher amounts of organic loading and physiological stresses. Nutrient removal, pathogen removal, and lipid accumulation with secondary or tertiary effluents have been demonstrated independently for these organisms. However, their potentials for accomplishing these objectives simultaneously with raw sewage have not been investigated. This study presents comprehensive investigations of applicability of *C. sorokiniana* and *S. obliquus* to wastewater treatment without the requirement for any additional treatment. *S. obliquus* showed greater potential for removing organic carbon (76.13 ± 1.59% COD removal), nutrients (98.54 ± 3.30% N-removal, 97.99 ± 3.59% P-removal) and comparable pathogens removal (99.93 ± 0.12% total coliforms removal, 100% faecal coliform removal) in comparison to *C. sorokiniana* (69.38 ± 1.81% COD removal, 86.93 ± 3.49% N-removal, 68.24 ± 11.69% P-removal, 99.78 ± 0.12% total coliforms removal, 100% faecal coliform removal) with 15 days of cultivation with filtered raw sewage, but also encountered increased levels of stress (F_v/F_m of 0.48 ± 0.03) which accounted for increased lipid accumulation in the cells (23.26 ± 3.95% w/w) but might also affect their biomass productivity and treatment potential in longer applications. In comparison, *C. sorokiniana* demonstrated better adaptability to physiological stresses (F_v/F_m of 0.53 ± 0.01) and may be suitable for achieving comprehensive treatment and sufficient lipid accumulation (22.74 ± 3.11% w/w) without compromising these potentials during prolonged applications. These results highlight the importance of selecting algal species with better stress resistance to extend their applicability for comprehensive wastewater treatment and lipid production.

1. Introduction

Wastewater generation has always been associated with the development of human societies. Effective and efficient treatment of wastewater is critical to achieve sustainable growth of algae and to reduce the demand for fresh water, which already is a scarce resource in many parts of the world. Over the years, much effort has been devoted to the development of efficient wastewater treatment processes. These can be broadly categorized into primary treatment, secondary treatment, and tertiary treatment based on the specific objectives of the processes (Arceivala and Asolekar, 2007). These processes cumulatively are very energy and cost intensive (McCarty et al., 2011), and hence have limited overall applicability in economically weaker societies. An ideal process should be able to achieve comprehensive wastewater treatment with minimum energy and cost input.

The role of microalgae in wastewater treatment has gained prominence due to their potential for nutrient uptake from wastewater for growth, and more importantly due to the value of algal biomass generated and other by-products (Christenson and Sims, 2011; Pittman et al., 2011; Rawat et al., 2011). Many algal species have demonstrated great potential for the dual applications of wastewater treatment and production of biomass for bio-fuel or other valuable products (Rawat et al., 2011). In many independent

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studies, microalgae have also demonstrated potential for removal of organic carbon and pathogens in addition to nutrients. For example, *Chlorella* sp. have been reported to remove 83% COD from domestic wastewater (Wang et al., 2010). In addition, faecal coliforms and other pathogens are sufficiently removed from wastewater with prolonged cultivation of microalgae (Ansa et al., 2012). Increase in pH, dissolved oxygen production, and excretion of algal metabolites having antibacterial properties are cited as the main reasons for pathogen removal in algal cultivations (Ansa et al., 2011, 2012). These applications have however been restricted largely to secondary or tertiary treated effluents (Ji et al., 2013a; Srirangan et al., 2011). Applications of algal cultivation to raw sewage mainly from animal livestock have been reported. These applications often encounter a number of difficulties, viz. unbalanced C:N:P ratios, chromaticity, need for pre-treatment etc (Ji et al., 2013b). Still, the applicability to raw sewage depends largely on the ability of selected algal species to tolerate the high and variable organic loading of these wastewaters.

Tolerance to the high organic loading associated with domestic wastewaters has been reported for *Chlorella* sp. and *Scenedesmus* sp. (Kümmerer, 2008; Palmer, 1969). In addition, *Chlorella* sp. have been reported to have good nutrient removal potential with sufficient lipid production for bio-fuels from a wide variety of wastewaters (Choi and Lee, 2015; Filippino et al., 2015; Ramanna et al., 2014; Ryu et al., 2014; Xu et al., 2015). Similarly, *Scenedesmus* sp. have also demonstrated good nutrient removal and lipid accumulation potentials with wastewater (Xin et al., 2010). Nutrient removal, pathogen removal, and lipid accumulation with secondary or tertiary effluents have been demonstrated in independent studies for both these organisms. However, their potentials for achieving all these objectives simultaneously, namely comprehensive wastewater treatment with sufficient lipid production using raw sewage without any additional treatment process(es), have not been investigated or are rare. The present study was designed to investigate the applicability to raw sewage and compare their respective potentials in detail for comprehensive wastewater treatment that includes nutrient removal, pathogen removal, and organic carbon removal; and lipid yield in the biomass. To the best of our knowledge, no other study has covered such detailed information i.e. removal of organic load, nutrients (N&P), coliforms as well as physiological health of algae, biomass production and its lipid yield as well. The use of raw sewage without any treatment and use of post chlorinated wastewater has not yet been reported. The success of batch experiments once translated to pilot scales would reduce the use of fresh water by several fold. In addition, it would improve the economics of the micro-algal biomass production (for bio fuel) which is a major techno-economic barrier to commercial algal bio fuel production currently.

2. Material and methods

2.1. Algae culture

Chlorella sorokiniana (genbank accession number: AB731602.1) and *Scenedesmus obliquus* (genbank accession number: FR751179.1) were isolated from the Durban region of KwaZulu Natal, South Africa and purified by subsequent sub-culturing using the streak plate method (Ramanna et al., 2014). Seed culture was prepared by inoculating BG11 medium with *C. sorokiniana* and *S. obliquus* in 1 L flasks. Cultures were maintained at room temperature (22 ± 2 °C) with 16:8 h light:dark cycle under illumination from Gro-Lux lamps (80 μmol m^{-2} s^{-1}). To maintain turbulent conditions, the culture was continuously shaken at 80 rpm on orbital shaker (OrbiShake Shaker, Labotec, South Africa). The pH adjustment of the medium to 7 was done using either 1 M H_2SO_4 or 1 M NaOH.

2.2. Experimental details

The growth, comprehensive wastewater treatment, and lipid accumulation potentials of *C. sorokiniana* and *S. obliquus* at different wastewater concentrations were observed for 15 days. A flow chart of the experimental design is presented in Fig. 1. The raw sewage obtained from eThekwini municipality of South Africa was used in this study and processed in following manner before actual growth experiments. The raw sewage was first filtered through 0.25 mm stainless steel filter then aerated for 8 h with commercial pump (flow rate ≥ 10 L/min) which resulted in some foaming due to the presence of inherent fatty components. This build-up foam was skimmed regularly, and after 8 h of aeration, foaming decreased substantially. Aeration was carried out to ensure maximum removal of oil and grease contents. This suggested that the sewage to be used in experiments was relatively free from external fatty components. Moreover, this also ensures and lipid quantification during the batch experiment would predominantly be from algal biomass. All the experiments were conducted in 2 L flasks under continuous aeration in growth chambers (at an illumination of 80 μmol m^{-2} s^{-1}with16:8 h light:dark cycle at 22 ± 2 °C). The light intensity was measured by a light meter (MT 940, Major Tech, South Africa). The filtered raw sewage was diluted with post chlorinated effluent (residual chlorine < 0.01 mg L^{-1}) from a wastewater treatment plant to achieve different nutrient concentrations (25, 50, 75, and 100% wastewater). All the experimental sets were inoculated with 5% of stock culture of *C. sorokiniana* (biomass

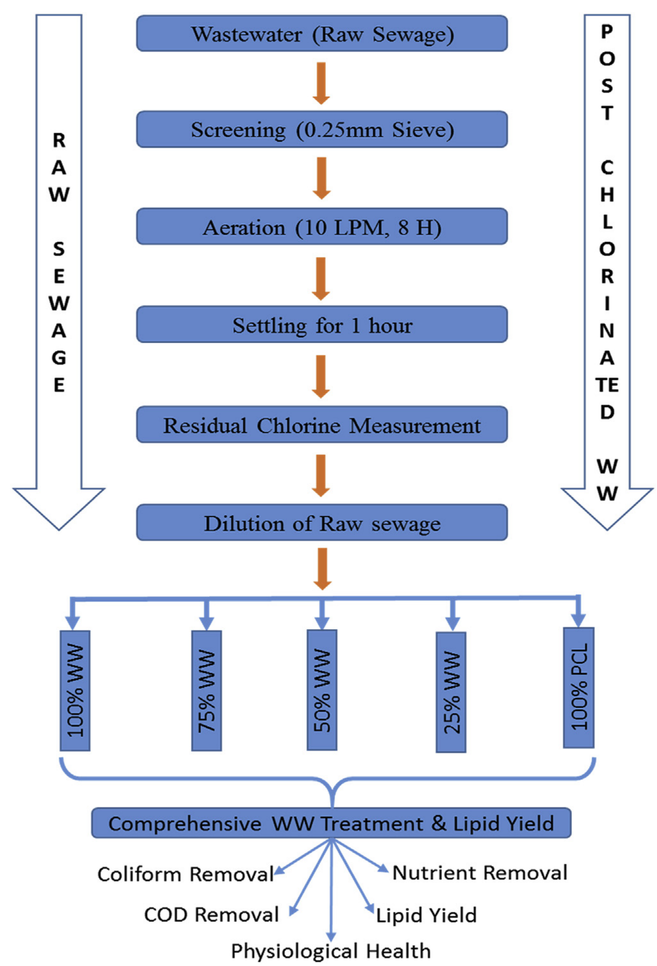

Fig. 1. Flow chart of the experimental design.

concentration was 92.46 mg L^{-1}) and *S. obliquus* (biomass concentration 96.21 mg L^{-1}). Table 1 presents the characteristics of the filtered raw sewage and the post chlorinated effluents used during experiments. Every 3rd day, 50 mL culture was withdrawn from each flask for analysis. 10 mL sample was used to determine specific growth rate (by measuring optical density at 680 nm) and physiological health by pulse amplitude modulated (PAM) fluorometry. Remaining 40 mL sample was centrifuged (20 min at 1509 g) and the supernatant was used further for physico-chemical and microbiological analysis and thick algal slurry was used for gravimetric analysis. Algal dry weight biomass of all the sets was determined (each after three days) gravimetrically according to standard method 2540-D (APHA, 1998). Consistent air supply (2 L min^{-1}) in the culture was maintained throughout the experiment by continuous aeration using portable air pumps, air valves and ceramic diffusers.

2.3. Measurement of growth and physiological health of algae

The growth of the cultures was continuously monitored by taking the optical density of the culture at 680 nm using UV visible spectrophotometer (Spectroquant Pharo 300, Merck, Germany) and gravimetric analysis of the biomass. The gravimetric analysis data is not presented. A Dual-PAM (pulse amplitude modulation) 100 chlorophyll fluorometer (Heinz Walz Gmbh, Effeltrich, Germany) was used for non-invasive fluorescence measurements. The sample was dark adapted before measurements to open all photo system-II (PS-II) reaction centres in the chlorophyll. The quantum efficiency of PS-II charge separation (F_v/F_m) was calculated as per following equation (Ramanna et al., 2014):

$$F_v/F_m = (F_m - F_o)/F_m \qquad (1)$$

where F_v is the variable fluorescence resulting due to maximum fluorescence F_m and minimum fluorescence F_o in a dark adapted sample.

2.4. Physico-chemical analysis

The pH was measured with a pH meter (Orion Dual Star, Thermo Scientific). The nitrate, nitrite and orthophosphate concentrations in the samples were measured by Gallery™ Automated Photometric Analyzer (Thermo Scientific, USA). The chemical oxygen demand (COD) was determined by closed reflux titrimetric method. All measurements were done in triplicate.

2.5. Microbiological analysis

Colilert kit was used for the coliform and *Escherichia coli* analysis. The colilert is based on defined substrate technology (IDEXX Laboratories, Inc., USA). The β-galactosidase present in the colilert medium gets metabolised in o-nitrophenyl, with a colour change from colourless to yellow; whereas, *E. coli* use β-glucuronidase to metabolize 4-methyl-umbelliferyl into fluorescent 4-methyl-umbelliferone. A 10 mL supernatant obtained from centrifugation was diluted with sterilized water. The growth media pouches provided with colilert were dissolved in the diluted supernatant and transferred into IDEXX Quanti-trays and sealed. The IDEXX Quanti-trays were incubated at 37 °C and counted after 24 h. The *E. coli* counting was done in UV chamber.

Table 1
Characteristics of raw sewage and post chlorinated effluent.

Parameter	Raw sewage	Post chlorinated effluent
pH	6.93 ± 0.28	7.65 ± 0.21
Alkalinity, mg L^{-1}	240.00 ± 2.35	10.67 ± 1.15
COD, mg L^{-1}	320.07 ± 3.78	48.12 ± 1.15
N-NH_3, mg L^{-1}	52.23 ± 1.21	1.45 ± 0.86
N-NO_2^-, mg L^{-1}	0.00 ± 0.00	0.36 ± 0.58
N-NO_3^-, mg L^{-1}	0.40 ± 0.13	3.75 ± 0.33
P-PO_4^{-3}, mg L^{-1}	8.47 ± 0.23	0.48 ± 0.15
Total coliform, cfu 100 mL^{-1}	$1.27 \times 10^7 \pm 10^4$	0.00 ± 0.00
Fecal coliform, cfu 100 mL^{-1}	$2.00 \times 10^6 \pm 10^2$	0.00 ± 0.00

2.6. Lipid extraction and quantification

At the end of the experiment, 1 L of algal culture was centrifuged (20 min at 1509 g) and lipid extraction from the biomass was carried out by microwave assisted solvent extraction. The effects of various concentrations of wastewater on the lipid yield were assessed by comparing it with control biomass grown in BG11 medium. The centrifuged biomass was kept overnight at −84 °C in a bio-freezer (Glaciar NU9668E, Nuaire, Japan), followed by freeze drying using a lyophilizer (Mini Lyotrap, LTE scientific Ltd. United Kingdom). 500 mg biomass from each set was taken and mixed with 20 mL solvent mixtures (2:1 ratio of chloroform and methanol) and subjected to microwave digestion (Milestone S.R.L., Italy) at 100 °C for 10 min at 1000 W following the method of Guldhe et al. (2014b). The solvent layer was decanted by filtration and residual biomass was again subjected to such repeated solvent extraction for maximum lipid recovery. All the extracted solvent aliquots were mixed and washed with distilled water followed by centrifugation. The supernatant was collected and dried to constant weight at 70 °C. The percentage lipid yield per gram of dried biomass was determined gravimetrically.

3. Results and discussion

3.1. Growth potential of algae with raw sewage

The applicability of microalgae for comprehensive treatment of raw sewage is of great interest as it circumvents the need for additional treatment processes. Such applications have largely been investigated with secondary or tertiary treated effluents for major algal species (Ji et al., 2013a; Srirangan et al., 2011). Since, *C. sorokiniana* and *S. obliquus* both have predominantly been found to be suitable for nutrient removal with nutrient rich wastewaters, it is imperative to investigate their potential for such comprehensive treatment of raw domestic sewage and to compare their potentials (Pittman et al., 2011). A primary objective of this work was to investigate the growth potentials of *C. sorokiniana* and *S. obliquus* with raw sewage without any detrimental effect on culture physiology. Fig. 2 presents the growth results of *C. sorokiniana* (Fig. 2a) and *S. obliquus* (Fig. 2b) at different concentrations of raw sewage. The suitability of sewage for algal growth could be readily observed, since essential nutrients are already present in it. At all sewage dilutions, reasonable growth for both species was observed, except when nutrient levels were drastically lowered after dilution (WW-25). This further establishes the algal productivity on raw sewage qualitatively. A more detailed quantitative estimate of the effects of various sewage dilutions on the biomass production of both species could also be investigated from these figures. As observed, *C. sorokiniana* achieved maximum growth with WW-75 (75% raw sewage) and maintained a higher biomass level for the duration of the experiment (Fig. 2a). The nutrient content of such wastewater (WW-75) was sufficient to sustain the growth while the level of dilution in raw sewage provided more favourable growth conditions in comparison to raw sewage with no dilution (WW-100). This is also supported by the lag phase of two days as observed for

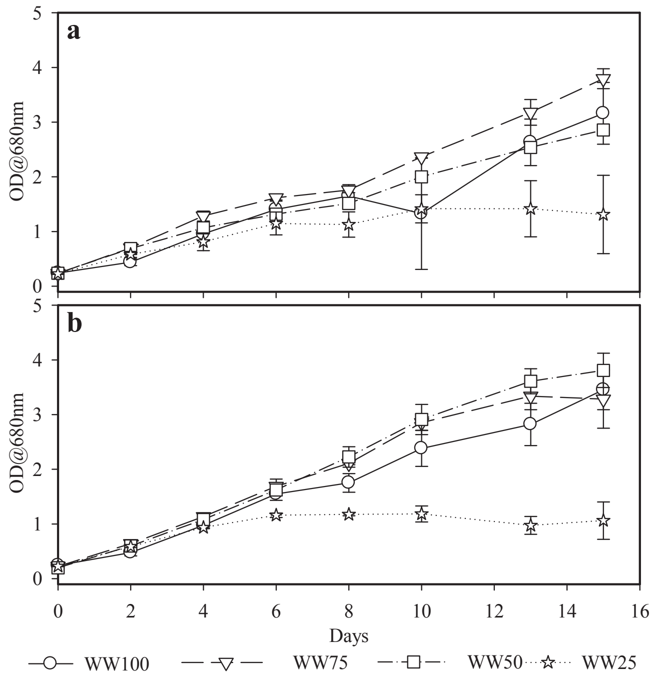

Fig. 2. Effect of different wastewater levels on the growth of (a) *C. sorokiniana*, (b) *S. obliquus*.

raw sewage, which is due to the necessary acclimatization of culture to such raw wastewater. In comparison, other dilution levels of wastewater (eg. WW-75, WW-50, and WW-25) provided more favourable growth conditions as no such lag was observed (Fig. 2a). Also, the raw sewage (WW-100) provided similar cultivation conditions for growth of *C. sorokiniana* as 50 percent diluted sewage (WW-50). The reduced nutrient content in WW-50 had the similar effects as the need for acclimatization with raw sewage, and the growth was comparable in both wastewaters. In contrast, growth of *C. sorokiniana* declined after six days with WW-25, due to exhaustion of nutrients. These results indicate the potential of raw sewage to be used for the cultivation of *C. sorokiniana* with 75% raw sewage as the optimal condition for growth. Cultivation of *C. sorokiniana* with raw effluent from palm oil mill was also found to be suitable (Nwuche et al., 2014).

In case of *S. obliquus*, comparable growth was observed for six days of cultivation with raw sewage (WW-100), and its two dilutions (WW-75 and WW-50). After six days, raw sewage performed sub-optimally in comparison to other two and this may be due to higher sustained physiological stress (refer Section 3.2) with such raw sewage (Fig. 2b). The 50% sewage was found to be optimal cultivation media for *S. obliquus* for the overall experimental duration, while WW-75 provided marginally sub-optimal conditions. In contrast, WW-25 was found to be limited in nutrients and did not support growth.

3.2. Effects of using raw sewage on the physiological conditions of algae

Since, the application of raw sewage for algal cultivation has been reported to present many complexities, it is important to investigate the stresses on culture physiology to determine their sustainable applications (Ji et al., 2013a). The effects of using raw sewage as a substrate on physiological conditions of *C. sorokiniana* and *S. obliquus* were observed by calculating the quantum efficiency (F_v/F_m) of reaction centres in PS-II of chlorophyll by non-invasive fluorescence measurements. This parameter has now gained acceptability for indicating the stress conditions of the culture and is widely been used in identifying stresses due to various environmental conditions such as nutrient starvation (White et al., 2011). $F_v/F_m < 0.5$ generally indicates that culture is under going physiological stress. Fig. 3 presents the evolution of F_v/F_m with cultivation of both species in raw sewage and its dilutions. For *C. sorokiniana* WW-25 resulted in low F_v/F_m values which indicate that the culture is stressed due to nutrient starvation with this cultivation medium (Fig. 3a). These effects were also observed on reduced growth of *C. sorokiniana* with WW-25 (see Fig. 2a). In comparison, WW-75 resulted in continuous F_v/F_m values above 0.5, which indicates a healthy culture. The optimal growth using this wastewater (WW-75) is also an effect of such healthier culture. For raw sewage (WW-100), *C. sorokiniana* remained in a stressed condition (low F_v/F_m) during the initial phase of cultivation. Stress levels can be explained by the need for acclimatization to raw sewage. A lag phase of growth was also observed due to such need for acclimatization (see Fig. 2a). For WW-50, the culture remained healthy as observed by F_v/F_m values, but started to experience the stresses after eight days as the nutrients started to become limiting.

In comparison, the stresses when using sewage were more prominent with *S. obliquus* (Fig. 3b). Growth with WW-25 resulted in a highly stressed culture due to nutrient limitations which is evident from continually declining F_v/F_m values. Similar stresses were observed with raw sewage (WW-100) during the initial phase of experiments. This might be due to the need for acclimatization of *S. obliquus* to raw wastewater and evidence of this is also observed in lower growth with the raw sewage (see Fig. 2b). Cultivation using WW-75 maintained the culture in a relatively healthy state for 10 days of operation after which F_v/F_m started to decline. Decline was also observed with WW-50 after four days of cultivation.

These results when analyzed with the corresponding growth data of *C. sorokiniana* and *S. obliquus* provide some important insights about the suitability of cultivating these species on raw sewage without any additional treatment (see Figs. 2 and 3). As observed, *C. sorokiniana* was able to acclimatize better to raw sewage and maintain relatively lower stress levels than *S. obliquus*

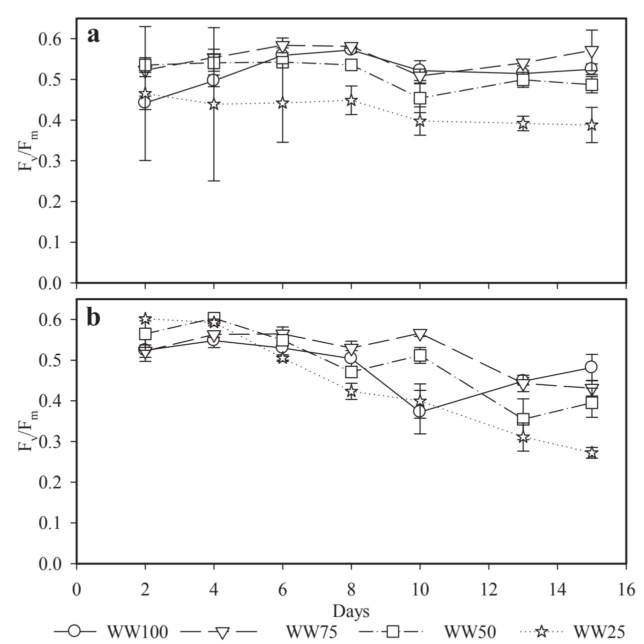

Fig. 3. Effect of different wastewater levels on the quantum efficiencies of (a) *C. sorokiniana*, (b) *S. obliquus*.

for all cultivation conditions. This better suitability is also evident by the fact that optimal growth of *C. sorokiniana* was observed with WW-75, while *S. obliquus* showed sub-optimal growth in WW-100 and WW-75. Raw sewage had to be diluted by 50% to achieve its optimal growth, which indicated the relatively unsuitable conditions using raw sewage for *S. obliquus* cultivation. Both species suffered severe nutrient stresses with WW-25 which affected their growth. Hence WW-25 is not a sustainable cultivation medium for either species. For sustainable long term cultivation, raw sewage could be diluted to 75% for *C. sorokiniana* and 50% for *S. obliquus* without the requirement for any additional treatment. These results also indicate the suitability of such sewage for the biomass production for lipid. Since *C. sorokiniana* have a well-established potential of maintaining high lipid contents (Zhang et al., 2013; Zheng et al., 2013), the application of WW-75 for their cultivation may result in a viable and economically more sustainable strategy. The need for dilution of raw sewage (WW-50) to achieve optimal growth conditions for *S. obliquus* makes such cultivation relatively more costly for this species due to additional requirements for pumping the dilution water and other involved costs, though technically feasible.

3.3. Removal of organic carbon from raw sewage

The organic carbon loading in raw sewage was 320.07 ± 3.78 mg L^{-1} COD which was lowered with subsequent dilutions by post chlorinated effluent (see Table 1). Wastewaters were treated by cultivation of *C. sorokiniana* and *S. obliquus* to investigate the organic carbon removal performance of the two species and to compare their optimal performance. Since, *C. sorokiniana* and *S. obliquus* both can undergo mixotrophic growth while consuming organic carbon (Kim et al., 2013; Mandal and Mallick, 2009), these species also removed organic carbon from sewage as presented in Fig. 3. *C. sorokiniana* removed sufficient COD with all wastewaters (see Fig. 4a), despite the final effluent COD being the highest in raw sewage (WW-100). This is due to the initial higher level of COD in WW-100 and the need for acclimatization of *C. sorokiniana* with WW-100 and the associated stresses. In contrast, the final COD levels in effluents with WW-75, WW-50, and WW-25 are all lower than with raw sewage (Table 2). *S. obliquus* also achieved COD removal with all wastewaters (Fig. 4b). Final effluent COD with WW-100 and WW-75 achieved with *S. obliquus* were comparable while better quality effluents were achieved with WW-50 and WW-25. In this study, we used raw sewage without any pretreatment except prefiltration and aeration, therefore a relatively high bacterial contamination was expected. In the initial phases, most of the COD removal could be due to the synergistic effect of bacterial and algal consortium as culture was aerated continuously. Once bacteria have degraded most of the organic matter and mineralized the media, algae could have picked up exponential growth. Therefore at initial stage, even though both the algal species were stressed, 70–90% of the total COD removal was achieved. The best effluent quality in terms of COD removal was achieved for WW-25 with both species, which is due to the reduced level of organic carbon in the influent. Other researchers have also observed comparable COD removal with algae on different wastewaters (Wang et al., 2010). It has been observed that at low nutrient concentrations, both the algae and bacteria compete which suppresses the growth of both species (Rhee, 1972). But in a nutrient rich medium, algae and bacteria co-exist and support the growth of each other (Ma et al., 2014). Zhang et al. (2012) reported that in an algae bacterial consortia, primary degradation of complex organic compounds to its metabolites (N and P species) is done by bacterial communities and such metabolites are further utilized by algal communities. Gonzalez and Bashan (2000) also reported higher

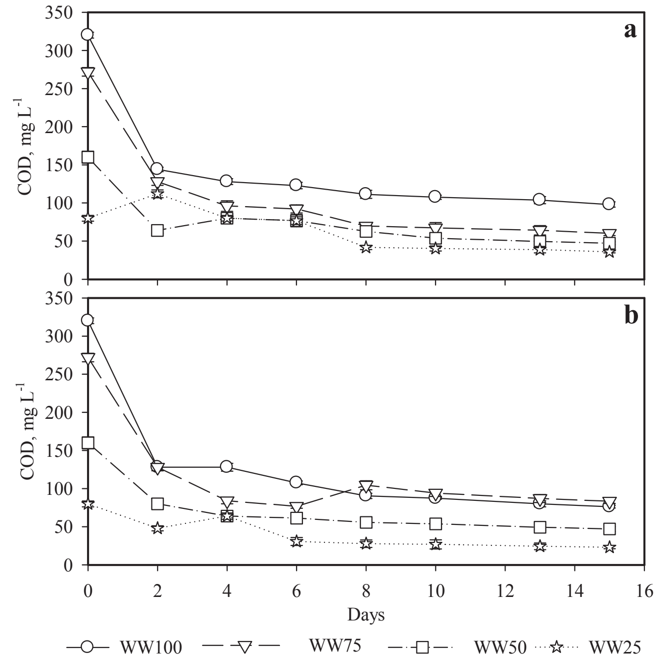

Fig. 4. Effect of different wastewater levels on the COD removal by (a) *C. sorokiniana*, (b) *S. obliquus*.

autotrophic growth of *Chlorella vulgaris* supported by some of the bacteria. Our findings are in accordance with the findings of Ma et al. (2014) who also reported that symbiotic relationships between wastewater born bacteria and algae play a significant role in the nutrient removal. Our findings are also in accordance with his studies that at initial stages, bacteria promotes the nutrient removal.

The performances of both species for COD removal are also compared to gain some insight into their potentials (Fig. 5). Lower COD in effluents after treatment with *S. obliquus* in all cases except WW-75 (Fig. 5a). Similarly, it was able to tolerate the variable COD input and the stresses associated with such variations to maintain a fairly uniform COD removal in all experiments (Fig. 5b). In comparison, COD removal potential varied with the influent COD and the growth conditions for the *C. sorokiniana*, which achieved best COD removal with WW-75 and that declined with WW-50 and WW-25. Similarly it also achieved lower COD removal for raw sewage. These results indicate the superior suitability of *S. obliquus* for COD removal with variable loading despite stressing culture conditions, possibly due to their higher mixotrophic growth potential.

3.4. Removal of nutrients from raw sewage

3.4.1. Removal of nitrogen

Nitrogen present in the raw sewage was predominantly in a reduced form as ammonia, while the post chlorinated effluents had nitrate as the dominating nitrogen form (Table 1). These compositions resulted in the changing levels of different nitrogen species with different wastewaters as per corresponding dilution. Ammonia concentrations in the influent wastewaters (WW-100, WW-75, WW-50, and WW-25) subsequently reduced with dilution (Fig. 6a), while those of nitrite and nitrate increased (Fig. 6b and c respectively). Both *C. sorokiniana* and *S. obliquus* were able to sufficiently remove ammonia from wastewaters due to uptake, with *S. obliquus* showing marginally better potential for such removal (Fig. 6a). In contrast, the treatment with both algal species resulted in an increase of nitrite levels in the final effluent after treatment

Table 2
Comparison of performance between *C. sorokiniana* and *S. obliquus*.

Parameter	*C. sorokiniana*				*S. obliquus*			
	WW-100	WW-75	WW-50	WW-25	WW-100	WW-75	WW-50	WW-25
Final Biomass as OD@680 nm	3.16 ± 0.57	3.79 ± 0.18	2.86 ± 0.07	1.31 ± 0.72	3.45 ± 0.36	3.28 ± 0.53	3.81 ± 0.31	1.06 ± 0.34
Culture condition, as F_v/F_m	0.53 ± 0.01	0.57 ± 0.05	0.49 ± 0.02	0.39 ± 0.04	0.48 ± 0.03	0.43 ± 0.02	0.40 ± 0.04	0.27 ± 0.01
% COD removal	69.38 ± 1.81	77.89 ± 2.81	70.49 ± 6.13	55.15 ± 5.63	76.13 ± 1.59	69.31 ± 2.72	070.56 ± 6.35	71.10 ± 6.28
% N-removal	86.93 ± 3.49	89.01 ± 4.28	91.85 ± 5.56	88.92 ± 9.08	98.54 ± 3.30	98.02 ± 4.23	97.29 ± 5.58	95.44 ± 8.77
% P-removal	68.24 ± 11.69	86.25 ± 5.09	96.20 ± 6.72	76.97 ± 12.60	97.99 ± 3.95	92.12 ± 5.28	89.72 ± 6.93	84.65 ± 10.49
% Total coliform removal	99.78 ± 0.12	99.88 ± 0.12	99.88 ± 0.12	99.93 ± 0.12	99.93 ± 0.12	99.98 ± 0.12	99.97 ± 0.12	99.97 ± 0.12
% Fecal coliform removal	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
Final pH	11.31 ± 0.14	11.22 ± 0.24	11.21 ± 0.12	11.12 ± 0.28	10.63 ± 0.21	10.58 ± 0.32	10.02 ± 0.41	8.65 ± 0.23
% Lipid yield	22.74 ± 3.11	25.87 ± 2.62	26.11 ± 1.86	27.68 ± 3.12	23.26 ± 3.95	25.34 ± 1.55	26.59 ± 3.12	28.36 ± 2.02

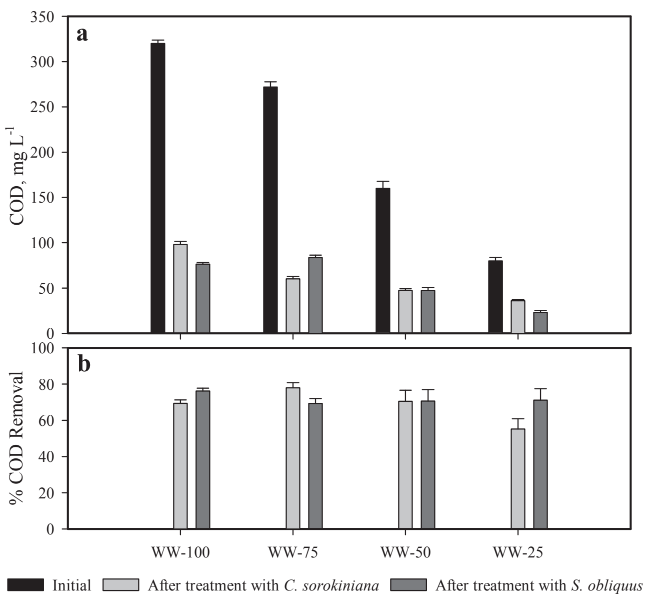

Fig. 5. Comparison of COD removal potentials of *C. sorokiniana* and *S. obliquus* with different wastewater levels (a) absolute COD removal; (b) percent COD removal.

(Fig. 6b). Such increases were significantly higher with *C. sorokiniana* and may be a consequence of more favourable conditions for oxidation of ammonia to nitrite with this species. This speculation is supported by the fact that nitrate levels also increased for WW-100 and WW-75 with *C. sorokiniana* (Fig. 6c) which could also be attributed to nitrification of ammonia. Lorenzen et al. (1998) also observed similar nitrification in the presence of microalgae and attributed it to photosynthetic oxygen. Nitrate was taken up by both species as suggested by their subsequent removals from WW-50 and WW-25. Overall, *S. obliquus* demonstrated higher nitrogen removal potential in comparison to *C. sorokiniana* for all wastewaters (Fig. 6d). In addition, both species were able to maintain their nitrogen removal potential above 85 percent removal with all variations in nutrient levels and growth conditions, with *S. obliquus* showing better removal potentials (Fig. 6e). Similarly high nitrogen removal potential has also been observed by other researchers for both *Chlorella* sp. (Wang et al., 2010) and *Scenedesmus* sp. (Zhang et al., 2008).

The distribution of various nitrogen species was also analyzed after treatment with both species and compared with initial composition in the corresponding wastewater to investigate the postulate of *C. sorokiniana* providing relatively better conditions for ammonia nitrification to nitrite and nitrate (see Fig. 7). Such conditions represent better availability of photosynthetic oxygen, and indicate the corresponding photosynthetic efficiencies of the

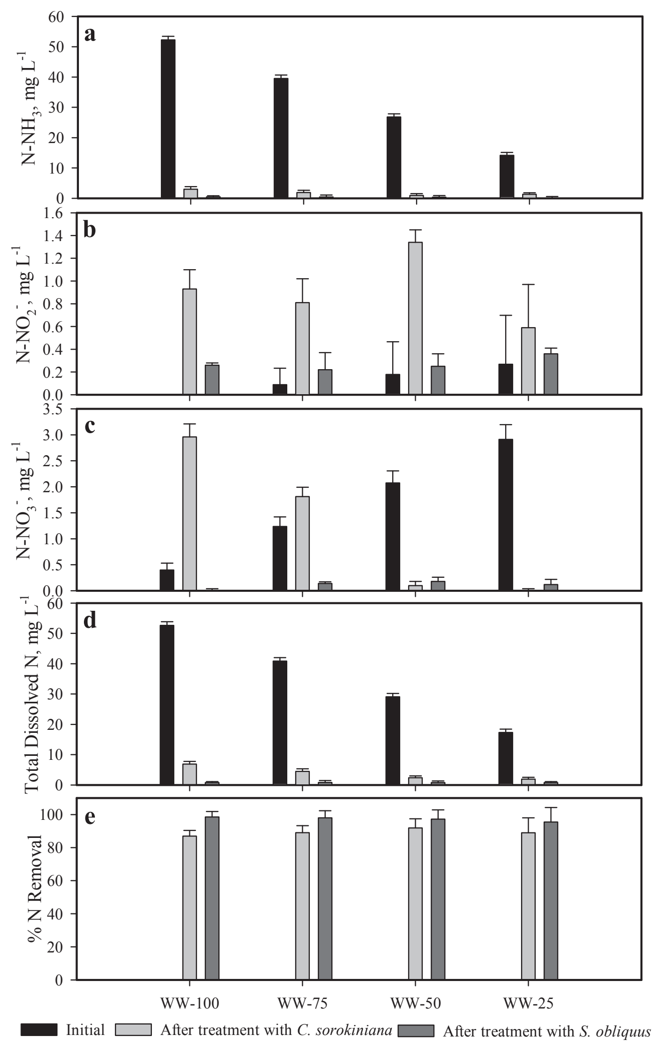

Fig. 6. Comparison of N-removal potentials of *C. sorokiniana* and *S. obliquus* with different wastewater levels (a) $N\text{-}NH_3$ removal; (b) $N\text{-}NO_2^-$ removal; (c) $N\text{-}NO_3^-$ removal; (d) total dissolved N-removal; (e) percent N-removal.

concerned algal species (Lorenzen et al., 1998). The initial composition of wastewaters had ammonia as the dominant nitrogen species whose contribution declined from 99.24% for WW-100 to 81.65% for WW-25; and the nitrate levels increased with the dilution of raw sewage from 0.76% for WW-100 to 16.81% for WW-25. For a particular wastewater, the distribution of various nitrogen species in the effluent after treatment with both *C. sorokiniana* and *S. obliquus* are also quantified. In general,

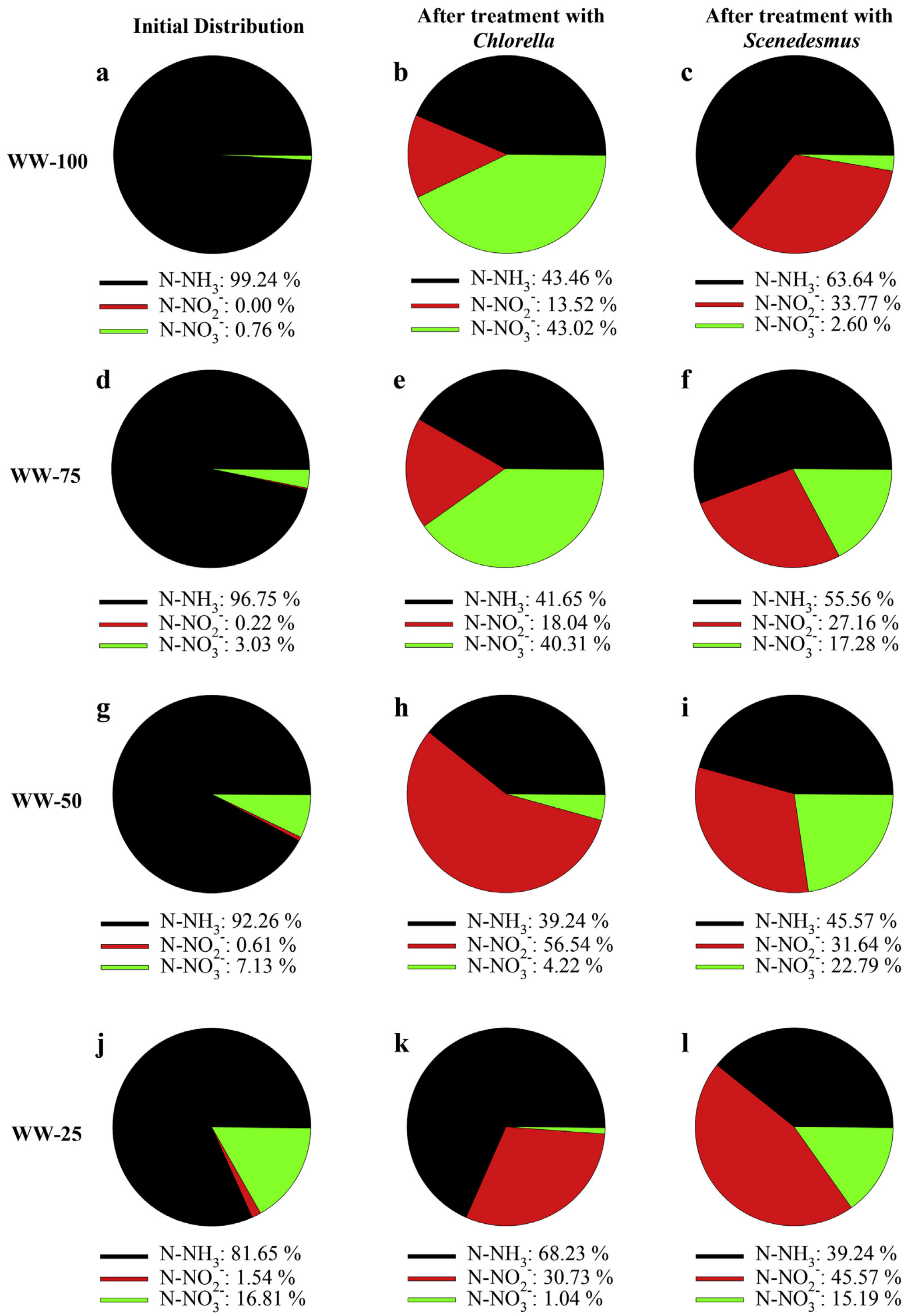

Fig. 7. Effects of treatment with *C. sorokiniana* and *S. obliquus* on the distributions of various N-species in the wastewater.

treatment with *C. sorokiniana* resulted in a more nitrified effluent with lesser ammonia and higher nitrate contributions in comparison to *S. obliquus*. The anomaly in this is observed for WW-25, where *S. obliquus* resulted in an effluent with lesser ammonia and higher nitrate and nitrite. Since, cultivation with WW-25 resulted in a stressed culture of both species after 10 days of treatment, and the absolute levels of these nitrogen species in the treated effluents are very low, and the anomaly could be a consequence of either stressed culture behaviour or experimental errors in measuring such low nitrogen levels and their distributions. These observations qualitatively suggest a higher photosynthetic efficiency and better oxidative conditions during treatment with *C. sorokiniana*, whilst better nitrogen removal is achieved with *S. obliquus*.

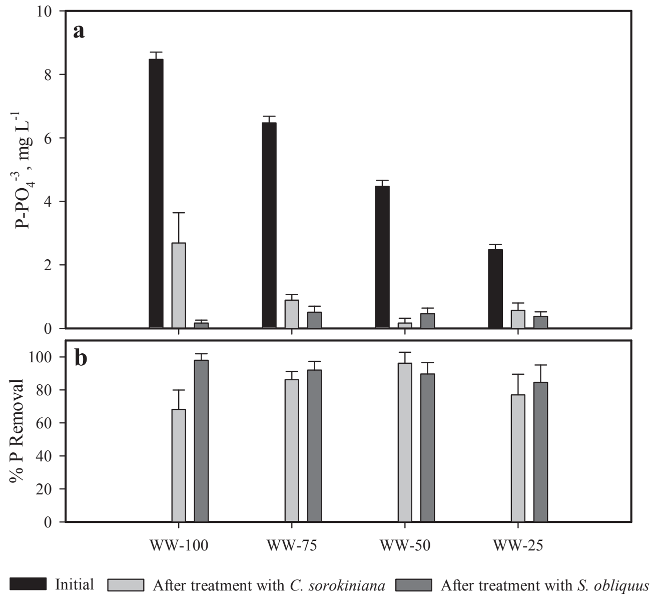

Fig. 8. Comparison of P-removal potentials of *C. sorokiniana* and *S. obliquus* with different wastewater levels (a) absolute P-removal; (b) percent P-removal.

3.4.2. Removal of phosphorus

Phosphorus was present in raw sewage as orthophosphate. Similar to nitrogen, phosphorus uptake has also been widely reported for these algae species (Wang et al., 2010; Zhang et al., 2008). Phosphate removal is compared for all wastewaters to gain insights into the potential of *C. sorokiniana* and *S. obliquus* (Fig. 8). Treatment with *C. sorokiniana* resulted in lowest phosphorus removal efficiencies from raw sewage (68.24 ± 11.69% P-removal). The stressed growth of *C. sorokiniana* and the need for acclimatization to raw sewage may have contributed to the lower removal efficiency. Phosphorus removal potentials increased with more favourable growth conditions and reduced stress levels of the culture with increasing dilutions and highest removal efficiency (96.20 ± 6.72%) was achieved for WW-50. Removal efficiencies again declined for WW-25 which could be due to stressed growth of *C. sorokiniana* with low nutrient content of this wastewater as evident in reduced growth and increased stresses. In contrast, *S. obliquus* achieved similar P-removal efficiencies with all wastewaters. In addition, *S. obliquus* demonstrated better phosphorus removal efficiencies when compared to *C. sorokiniana*.

3.5. Removal of coliforms from raw sewage

The application of both algal species during treatment resulted in the removal of coliforms from the wastewater (Fig. 9). Pathogen removal has been observed with prolonged algal cultivation such as in maturation ponds (Oswald, 1990). Treatment with *C. sorokiniana* resulted in removal efficiencies of total coliforms from 99.78 ± 0.12% for WW-100 to 99.93 ± 0.12% for WW-25 (Table 2). In comparison, application of *S. obliquus* achieved total coliforms removals from 99.93 ± 0.12% for WW-100 to 99.97 ± 0.12% for WW-25 (Fig. 9a). Complete removal of faecal coliforms was achieved for all wastewaters with both species (Fig. 9b). These results indicate the applicability of both species to achieve a high level of pathogen removal during wastewater treatment without any additional process such as chlorination, with *S. obliquus* showing marginally better potential for pathogen removal. Cultivation of both species in wastewater resulted in the increase in pH to values above 10 (Fig. 10). The removal of coliforms can be postulated to be due to such high levels of pH which are reported to have antibacterial effects on pathogens. In addition, various metabolites excreted from these algae are also reported to have bactericidal effects on pathogens (Kümmerer, 2008). To compare the dominating reason for such coliforms removal, the effects of increase in pH without any algae on coliforms were also studied. Such increased pH in

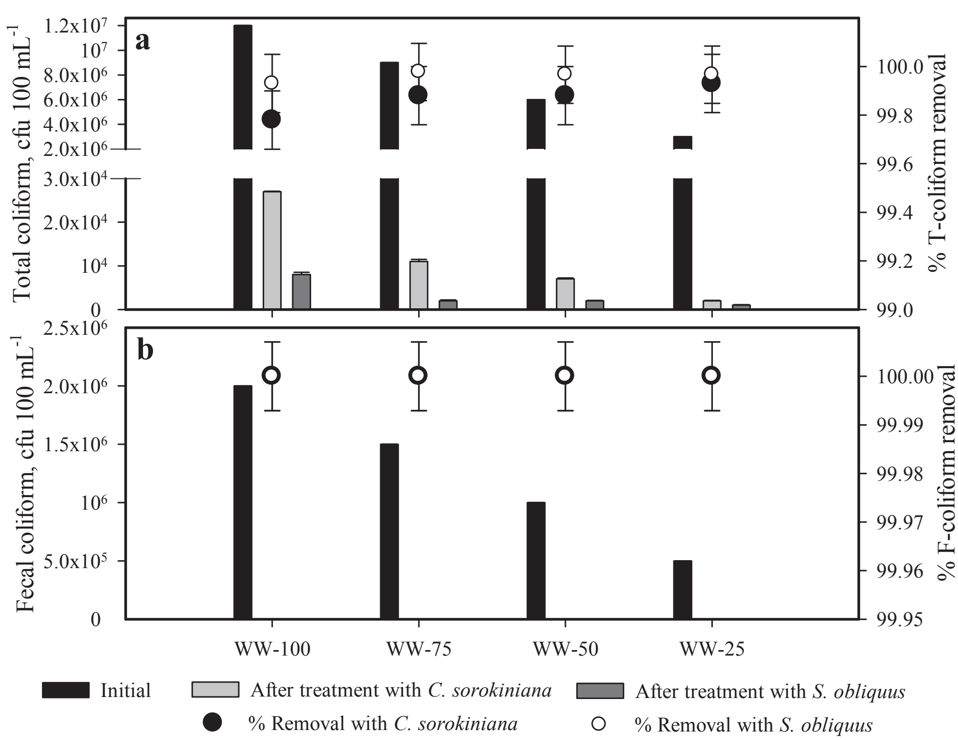

Fig. 9. Comparison of pathogen removal potentials of *C. sorokiniana* and *S. obliquus* with different wastewater levels (a) total coliforms removal; (b) fecal coliforms removal.

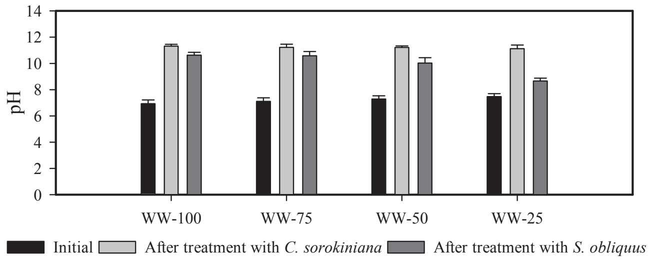

Fig. 10. Effects of treatment with *C. sorokiniana* and *S. obliquus* on the pH.

wastewaters did not result in substantial reduction of coliforms (data not shown). This suggests for the bactericidal effects of algal metabolites as the dominating factor for pathogen removal in comparison to rise in pH in the present study.

3.6. Lipid production

Algal biomass after experimental durations was used for lipid extraction. The final yields of lipids for both *C. sorokiniana* and *S. obliquus* with all wastewaters are presented in Fig. 11. In addition, the lipid production with BG11 media is also compared as a control for nutrient rich cultivation conditions. Both *C. sorokiniana* and *S. obliquus* accumulated lipids (14.17 ± 1.72% and 15.82 ± 2.23% respectively) in BG11 media. The cultivation of these species with different wastewaters resulted in a significant (ANOVA, 95% confidence level) increase in lipid accumulation with maximum yield being achieved with WW-25 for both *C. sorokiniana* (27.68 ± 3.12%) and *S. obliquus* (28.36 ± 2.02%). Since, the nutrient levels in BG11 are higher than in actual wastewaters, the cultivation of algae with real wastewater provided relatively nutrient deprived conditions and resulted in nutrient stress on algal cell. Such stresses are extensively reported to increase the lipid accumulation in the microalgae cell (Singh et al., 2014; Xin et al., 2010; Zhang et al., 2013). Other researchers have also observed higher lipid accumulation by algae with real wastewater in comparison to BG11 medium (Ramanna et al., 2014). The successive dilution of wastewater resulted in nutrient stressed conditions and hence the highest lipid accumulation was observed with WW-25 with maximum such stress, although the effect of dilution was not significant. *C. sorokiniana* and *S. obliquus* both have been reported to accumulate such high levels of lipid under nutrient stress (Guldhe et al., 2014a; Ramanna et al., 2014). The lipid accumulation potential was comparatively higher for *S. obliquus* and may be a consequence of

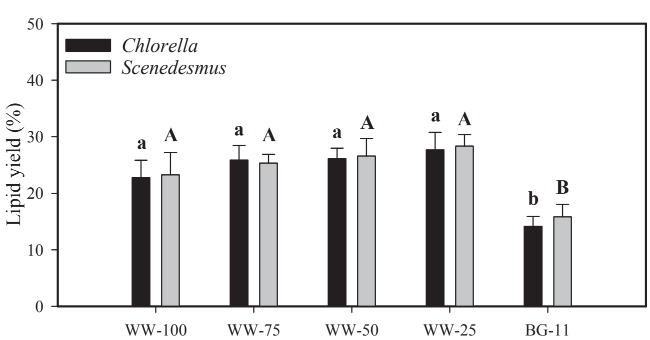

Fig. 11. Effects of different wastewater levels on the lipid yields of *C. sorokiniana* and *S. obliquus*. Letters over individual bars represent results of 1-way ANOVA at 95% confidence level. Small letters indicate ANOVA results for *C. sorokiniana*, while capital letters indicate those for *S. obliquus*. Different letters represent significant difference.

increased stress levels in the cell at similar cultivation conditions than *C. sorokiniana*.

4. Conclusion

This study investigated the role of *C. sorokiniana* and *S. obliquus* in achieving a comprehensive treatment of filtered raw domestic sewage and lipid accumulation in biomass. *S. obliquus* demonstrated better overall nutrients and pathogen removal potentials, as well as lipid accumulation in comparison to *C. sorokiniana*. However, prolonged growth on raw sewage resulted in stressed culture and which might affect the overall productivity in long term applications and hence the treatment potentials. *C. sorokiniana* demonstrated better stress resistance under similar cultivation conditions, and hence might be a suitable candidate for achieving such objectives in the long term without appreciable effects on the culture physiology. Furthermore, the additional requirement of water for dilution to provide acceptable growth conditions with *S. obliquus* might be a cost intensive alternative in comparison to *C. sorokiniana*.

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