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Optimization conditions for native microalgal strains grown on high ammonia-containing wastewater and their biomass utilization

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Abstract: Excessive microalgal blooms can be caused by waste disposal into natural water bodies resulting in the destruction of aquatic life. However,, microalgae are also known to efficiently remediate pollutants. After the treatment of wastewater, microalgae absorb specific nutrients and can enhance the production of bioproducts. Growing microalgae as an alternative to wastewater treatment and bioproduct production has received considerable attention due to its rapid growth rate, efficient waste removal, tolerance to stress conditions and ability to accumulate valuable products. In addition, these microorganisms have a high photosynthetic rate of CO_2 fixation, oxygen production and need no arable land for their cultivation. Nevertheless, in spite of these theoretical advantages, the issues surrounding the re-use of naturally existing microalgal strains need further exploration in respect to their isolation, identification and lab growth under stress conditions. The true potential of microalgae regarding wastewater treatment and energy has yet to be fully developed. The current cultivation system does not seem to be economically feasible as most of the strains used are commercially purchased. Indigenous microalgae could be the possible answer. Ammonia, one of the major constituents of most wastewaters, contributing to odor, taste, toxicity, and eutrophication is of utmost concern. The present review focuses on the growth of microalgae under high stress of ammonia in wastewater media. It also aims to present a clear-cut methodology for the isolation of microalgae from its indigenous habitat, its growth strategy under different trophic modes of nutrition, nutrient uptake, lipid, and fatty acid production. In addition, some solutions to the problem of how to make microalgae cost-effective and more sustainable are discussed in detail.

Key words: microalgae, wastewater treatment, bioproducts: ammonia

Introduction

Concern about the harm of wastewaters, greenhouse gasses, and energy crises has accelerated over recent years and the need for sustainable and renewable solutions becomes ever more urgent. One approach to the presence of major nutrients in wastewaters is to apply the mechanism of algal nutrient uptake. Microalgae cultivation has attracted the attention of researchers because of its high photosynthetic rate of CO_2 fixation and for its ability to grow in a variety of eutrophied water which is unfit for agriculture, animal or human use. Algae do not require any arable land and are able to remove the contaminants and nutrients from wastewaters efficiently (Duffy et al. 2009; Abinandan and Shanthakumar 2015; Hussain et al. 2017; Hussain et al. 2019). Microalgae can grow rapidly on non-arable land containing wastewaters, provide a good source of biomass and high-value bioproducts and biofuels (Cheng et al. 2008; Savage 2012; Atiku et al. 2016; Fatmei et al. 2016; Shah et al. 2019), as well as bio-fertilizers (Tripathi et al. 2008; Garcia-Gonzalez and Sommerfeld 2016; Din et al. 2017). They offer an alternative to other water treatment procedures by producing valuable biomass, uptaking organic wastes and metals and treating wastewater as a growth medium (Hwang et al. 2016). Since the modern world is greatly reliant on efficient ways to treat wastewater, algae, which can naturally adapt to wastewater may be a viable solution. The indigenous microalgal strain used for wastewater treatment is more productive than the commercially available strains (Zhou et al. 2011). One of the major constituents of most wastewaters is ammonia, which causes eutrophication in both fresh and marine water habitats (Liang et al. 2013). A number of studies

Table 1. Ammonia removal efficiency (%) of microalgal strains and biomass production (g L^{-1}) of microalgae grown in different media with different ammonia concentrations (mg L^{-1})

Microalgae	Strain	Media	Concentration	NH_3-N removal	Biomass	References
Monoraphidium sp.	NA	BG11	172.54 ±1.78	100	1.31	Lü et al. 2011
Chlorella vulgaris	ESP-6	biogas slurry	135.5–143	96.3	0.757	Aikawa et al. 2013
Chlorella vulgaris	UTEX 2714	mother liquor	429 (20 times dilution)	100	1.16	Aida et al. 2017
Chlorella sorokiniana	UMN266	synthetic wastewater	80–160	80	0.872	Kim et al. 2017
Chlorella vulgaris	UTEX 2714	TAP	254.5	98	1.43	Hussain et al. 2017
Chlamydomonas reinhardtii	NA	centrate wastewater	70	83	0.82	Kong et al. 2010

have focused on methods of removing ammonia from different wastewater bodies (Huang et al. 2014; Du et al. 2016; Ding et al. 2017). The microalgae-based ammonium removal process has so far been found to be the most effective among all the process studied (Table 1).

However, a major obstacle in microalgal use in the wastewater treatment process is strain selection. Growing microalgae for biomass, requiring light, CO_2, inorganic salts, water, and optimum temperature, is far more expensive than crop growth (Chisti 2007; Sarah et al. 2019; Ullah et al. 2019). Being already adjusted to prevailing climatic conditions and the stress environment, local strains have a distinct advantage over commercial strains for growth in wastewater media and are cost-effective. Therefore more suitable for further experimentation. Microalgae may become a model for the removal of wastes, including ammonia, from wastewaters enhancing their accumulating and/or leaching potentials. Based on the above hypothesis, our aim was to isolate the microalgae present in local leachate wastewater bodies and screen them for a high rate of ammonia content in wastewaters associated with high biomass valorization. The wastewater born microalgae tend to be facultative heterotrophs, as the wastewater has considerable loads of organic contents and low light penetration (Zhou et al. 2011). Therefore, the species could be grown in both heterotrophic and autotrophic conditions. Microalgal species showing better growth in the media should be grown in synthetic wastewaters. The best-grown species could be checked as a potential tool for tolerance and the recovery of NH_3-N in different NH_3-N concentrations. Determining the most optimal strain for the best nutrient removal efficiency and high biomass could lead to benefits for foods, feeds, fertilizers and other high-value products.

Wastewater treatment using microalgae

Along with microalgae-based technology, several other attempts have been made to treat wastewaters. Fermented food waste could enhance denitrification from domestic wastewaters when processed together (Zhang et al. 2016). Biodegradable polymer, as a solid phase denitrification agent could also improve wastewater treatment technology (Zhang et al. 2016). In addition, ultraviolet (UV) could cause the disinfection of antibiotic-resistant *Escherichia coli* present in different kinds of wastewaters (Zhang et al. 2017). Similarly, vertical and horizontal flow constructed wetlands have been combined to complement each other in order to achieve higher treatment efficiency (Vymazal 2011). A UPLC-MS/MS analytical method has been developed and validated for the simultaneous determination of enrofloxacin (ENR) and ciprofloxacin (CIP) at environmental relevant concentrations in piggery wastewater, piggery wastewater solids, agricultural soil and groundwater with good performance characteristics (Parpounas et al. 2017). Using solar energy, advanced oxidation processes (AOPs) represent an effective alternative for the removal of the pollutants (Marcelino et al. 2015). However, the above-studied procedures are both costly and time consuming. Therefore, microalgae are considered as a cost-effective agent for treating wastewater with renewable product production. The ability of microalgal could be enhanced to remove 100% of ammonia depending upon the quality of wastewaters and the nature of the selected strains and procedures adopted (De-Bashan et al. 2004; Pires et al. 2013). Microalgal strains which are highly tolerant to wastewaters and NH_3-N, with a high oxygen producing and CO_2 capturing rate, high biomass, and lipid productivity, could be used for wastewater treatment (Wang et al. 2016). Among the most commonly used strains, *Chlorella* (Du et al. 2012; AlMomani and Örmeci 2016; Ramsundar et al. 2017), *Chlamydomonas* (Kong et al. 2010; Kothari et al. 2013) and *Scenedesmus* (Zhang et al. 2015; Duangjan et al. 2016; Lutzu et al. 2016) are selected for their easy cultivation, their potential for high biomass production, ammonia, phosphorus removal from wastewater and the production of valuable products.

Microalgae as a source of biofuel

A reduction of the heavy dependence on fossil fuels and the consequent emission of the greenhouse gases that cause global warming has become an urgent necessity. Internal combustion engines and liquid hydrocarbon usage need to be phased out and an alternative solution found. Although alternatives exist, such as first and second generation biofuel there are also limitations.

First generation biofuel obtained from agricultural crops is a good source of biofuel, however, its production involves the usage of farmland, thereby reducing the amount of agricultural land available for edible crops that could be grown for consumption. This has given rise to the search for second-generation biofuel from non-edible crop plants like *Jatropha* sp. which has a long life cycle and requires extensive land and nutrients. Thus, as a result of the above-mentioned drawbacks the development of industrial-scale 3G and 4G biofuel i.e. algae-to-biofuels is of primary importance. Apart from 3G algal biofuel, algae metabolic engineering is the basis for the fourth generation biofuel which can overcome the above-mentioned issues (Lü et al. 2011). Microalgae have a very limited negative ecological impact as compared to other plants; they need no arable land, possess superior photosynthetic and carbon capturing ability, can produce higher biomass, have a short life cycle, and can be grown in all kinds of water, including highly concentrated wastewater. Moreover, they are a source of high lipid content with a bioproduct producing ability that offers a very practical alternative (Aikawa et al. 2013; Araya et al. 2015; Cavonius et al. 2015; Aida et al. 2017; Kim et al. 2017; Shuaib et al. 2017). Lipids from algae is currently a focus of great interest and are efforts are being made not only to discover higher lipid producing algal strains but also to enhance their lipid producing capability. To produce high-quality renewable biofuel, algae from sugar mill ponds have shown enhanced lipid productivity when provided with artificial light, CO_2 and a higher temperature (Bensonet et al. 2016). In another study, *Oedogonium*, a freshwater filamentous green-alga, in deficient nitrogen conditions that caused nutrient starvation, continued co-solvent hydrothermal liquefaction to produce nonpolarbiocrude, carbon dioxide hydrogenation. Hydrotreating has been optimized for high-quality biofuel and wastewater treatment (Cole et al. 2016). In addition, *Chlorella pyrenoidosa*, a rapidly growing alga, rich in protein and low in lipid contents, was employed for its high lipid yielding ability under the hydrothermal liquefaction process. The microalga produced 65.4% biocrude oil at 28°C and 120 minutes retention time thus introducing a novel treatment process which could be exploited to treat wastewater with Hydrothermal liquefaction (HTL) of algal biomass, which would recycle CO_2 from HTL gaseous products and produce nutrients form aqueous products while supporting algal production (Yu et a. 2011).

Apposite strain selection

Uni-algal culture isolation

The most important step is to collect indigenous microalgal strains. Microalgal samples from different areas of wastewater are collected using sterile bottles. The sample is then preserved using 4% formalin to ensure long term storage. Unialgal strains can be obtained using the protocol (Zhou et al. 2011). To achieve sufficient biomass of the candidate strain, samples containing algae are condensed using a centrifuge. To remove the zooplankton, the concentrated biomass is diluted with distilled water and passed through a plankton net (60 µm) and collected again using a glass filter (0.45 µm). For bacterial removal, cells present on the filter are washed with autoclaved salt water several times and inoculated onto a polystyrene sterile nonpyrogenic 24-well flat bottom culture cluster, containing BG11 media (Rippka et al. 1979; Imtiaz et al. 2017) with different dilution strengths. The strains are enriched for 10 days under the continuous light of a 6,000 Lux at 28°C and 120 rpm on a shaker incubator. Wells which have turned green, indicating the presence of algae, are transferred to sterilized plates containing solid BG11 media and placed in a Tissue culture box. Unialgal strains can be obtained by selecting individual green colonies for sequential sub-culturing. Liquid cultures are obtained by inoculating a colony of the purified strain from the agar plate to a 12 well culture cluster, containing 4 mL of autoclaved BG11 media. The transfer must be made in completely aseptic conditions in a biosafety cabinet; before each transfer the loop and the other equipment needs to be sterilized using dry heat. The culture is then incubated in a photo-incubator at 15°C for 3 days under continuous light with an intensity of 6,000 lux.

Autotrophic/heterotrophic culture condition

Selected microalgal strains are isolated from local leachate wastewaters after which they undergo the following process. Liquid culture colonies from the 12-well culture cluster are aseptically transferred to a 250 mL flask containing 100 mL of BG11 media. The BG11 media are prepared according to two modes of nutrition, i.e. autotrophic and heterotrophic. The BG11 media for the autotrophic mode is (g L^{-1}): K_2HPO_4.3H2O, 0.04; $MgSO_4.7H_2O$, 0.075; $CaCl_2.2H_2O$, 0.036; citric acid, 0.006; ferric ammonium citrate, 0.006; EDTA, 0.001; $NaNO_3$, 1.5; Na_2CO_3, 0.02; trace metal mix A5, 1.0 mL in which the trace metal mix A5 solution is composed of (g L^{-1}) H_3BO_3, 2.86; $MnCl_2.4H_2O$, 1.81; $ZnSO_4.7H_2O$, 0.222; $NaMoO_4.2H_2O$, 0.39; $CuSO_4.5H_2O$, 0.079; and $CoCl_2.6H_2O$, 0.05.

To establish the heterotrophic culture, the BG-11 medium, in which Na_2CO_3, the inorganic carbon source, is replaced with glucose in dark conditions. The artificial media are supplied with 10 g L^{-1} of glucose as a direct organic source of carbon (Zhou et al. 2012). The cultures are then grown in darkness for a period of 5 days, after which the culture is transferred to new het-

erotrophic media for two further times. Complete heterotrophic behavior is then achieved in the autotrophic microalgae. Using an incubator, the culture is kept at 25±2°C under a continuous cool-white fluorescent light illumination at 6,000 lux. After 10 days of cultivation the algal cells are separated from the culture media using a centrifuge at 2,000 rpm for 5 min, followed by washing with de-ionized water and another centrifugation – suspension route.

Strain selection and synthetic wastewater

In the attempt to find a successful algal strain for high ammonia tolerance there are currently several species under consideration around the world (Grzebyk et al. 1998; Indarti et al. 2015; Udom et al. 2013; Zhang et al. 2013; Abdelaziz et al. 2014; Drira et al. 2016; Passos et al. 2016; Ruiz-Martinez et al. 2012; Canedo-López et al. 2016; Dahmani et al. 2016; Franchino et al. 2016; Kumari et al. 2016; Montemezzani et al. 2016; Pereira et al. 2016).The selected strains are inoculated onto a polystyrene sterile nonpyrogenic 24-well flat bottom culture cluster containing synthetic wastewater. The synthetic wastewater may be a modification of BG11 media obtained by adding a different concentration of NH_4Cl. The media are enriched with 114, 228 and 456 g L^{-1} of NH_4Cl to raise the ammonia level to 300, 600 and 1,200 g L^{-1} respectively. The media aresupplied with 10 g L^{-1} glucose for heterotrophic growth. Growth is monitored daily under a microscope.Strains that show better performance in a good cluster are further investigated.

Microalgal growth and nutrient analyses

From the well-mixed culture broth in the bioreactors, seven-milliliters of samples are taken every 24 hrs for algal growth and synthetic wastewater nutrient analyses. Five milliliters of the algal samples are used for the measurement of the total suspended solids (TSS) using standard procedure (APHA 1995). While 2 mL liquid samples for nutrient analysis are collected from the media and centrifuged at 7,000 rpm for 10 min and supernatants are then collected and properly diluted for analyses of ammonia-nitrogen (NH_3-N). Measurements of TN, TP, NH_3-N and chemical oxygen demand (COD) are performed following the Hach photometric procedures (Hach 2008). The removal rates (%) for NH_3-N are calculated by dividing the variance of the first day and final day concentrations by the first-day concentration, and then multiplying them by 100:

$$R\ (\%) = (R_t - R_0/R_t) \times 100 \qquad [1]$$

where: R is nutrient removal efficiency and R_t and R_0 are the nutrient concentrations at day t and day 0.

Strain identification

The genomic DNA of microalgal cells is extracted using a QiagenDNeasy Plant Mini Kit (Qiagen, Germantown, USA) according to the available protocol. To disrupt the microalgal materials, a slight amount of algae (ca. 0.25 g) and 200 µL of Buffer AP1 are placed in a 1.5 mL micro-centrifuge tube and ground with a pestle. Next, an additional 200 µL of Buffer AP1 plus 4 µL of RNase-A stock (100 mg mL^{-1}) is added. The mixture is then ground, mixed using a vortex, and incubated for 15 min at 65°C to lyse the cells (mixed 2–3 times during incubation). Additionally, 130 µL of Buffer AP2 is added to the lysate and mixed, incubated on ice for 5 min to precipitate proteins, polysaccharides, and detergents, and then centrifuged at 14,000 g for 5 min. The lysate is transferred to a QIA shredder spin column and centrifuged at 14,000 g for 2 min. The supernatant is then transferred to a fresh 1.5 mL micro centrifuge tube, to which 1.5 volumes of Buffer AP3/E are added and mixed. This mixture is applied to the DNeasymini spin column, centrifuged at 6,000 g for 1 min, and then placed into a fresh tube with 500 µL Buffer AW. The mixture is centrifuged at 6,000 g for 1 min and the filtrate discarded. Next, 500 µL of Buffer AW is added to the column and centrifuged for 2 min at 14,000 g for 2 min to dry the membrane. The column is next placed in a fresh 1.5 mL microcentrifuge tube and 100 µL of Buffer AE at 65°C is added onto the membrane. Then the sample is incubated for 5 min at room temperature, and centrifuged at 6,000 g for 1 min to elute the DNA. The elution step is repeated once more to give a final volume of 200 µL. DNA is diluted 5, 10, 20, 50, or 100-fold for use in PCR amplification. For PCR amplification, a diluted portion of extracted DNA is used to amplify the 18S rRNA gene with two primers, 16S1N (forward, 50-TCCTGCCAGTAGTCATATGC-30) and 16S2N (reverse, 50-TGATCCTCT/CGCAGGTTCAC-30) (Grzebyk et al. 1998). Genomic DNA template, 50-100 ng, is mixed with the polymerase chain reaction (PCR) mixture containing 50 µL X PCR buffer (TaKaRa, Otsu, Japan), 0.2 mMdNTP, 0.2lM of 16S1N, and 16S2N primers, respectively, and 2.5 units of Taq polymerase, which is then denatured at 94°C for 5 min. The PCR program is run for 30 s at 94°C, 30 s at 50°C, 2 min at 72°C for 40 cycles, and a 7 min final extension at 72°C. The PCR products are purified using a QiAquick gel extraction kit, and then sent to the Genomics Center for DNA sequencing. The resulting 18S rRNA gene sequences are aligned and compared to the nucleotide sequences of some known microorganisms in the GenBank database.

Biomass utilization

Total lipid extraction

Microalgal cells are harvested using a centrifuge and then dried with a freeze dryer for lipid content analysis. The lipids are extracted using a single step extraction method (Zhou et al. 2011). Approximately 0.1 g of dried algae powder is weighed into clean, 25 mL screw-top glass tubes, in which 10 mL 2:1 chloroform-methanol (v/v) mixture is added. Extraction is carried out in a 30°C water bath for 30 min with 100 rpm rotation speed. Upon completion of the reaction, the algal solid residues are removed by passing the suspension through a Whatman 934-AH glass fiber filter. The filtrate is then transferred to another clean, 25 mL screw-top glass tube containing 2 mL 0.9% NaCl solution to wash out the water-soluble components. After centrifugation, a biphasic system without any interfacial fluff is obtained. The volume of the lower phase containing essentially all the algal lipids extracted is measured, and 3 mL of the lower phase transferred into a weighed, clean, 5 mL glass tube. Organic solvent is then dried off under an N-EVAP Analytical Nitrogen Evaporator. Duplicate lipid determination is made for every sample. The lipid content of dry weight is calculated according to the following formula:

$$LW\ (g\ g^{-1}) = (m_2 - m_0) \times V/\ (3 \times m_1) \qquad [2]$$

where LW stands for "lipid content based on dry weight", m_1 is the weight of the algae powder, m_0 is the weight of the empty 5 mL glass tube, m_2 is the weight of the 5 mL tube with the dried lipids, and Vis is the total volume of the lower phase after washing. The total lipid concentration (TC) is calculated by multiplying the lipid content of dry weight by the TVSS on the 4th day. The equation is listed below:

$$TC\ (g\ L^{-1}) = LW \times TVSS. \qquad [3]$$

Fatty acid isolation

Algae cells are harvested through centrifugation and then dried with a freeze dryer before analysis. Fatty acid content and composition analysis is performed by following two consecutive steps including preparation of fatty acid methyl ester (FAME) and GC-MS analysis. FAME is prepared using a one-step extraction-transesterification method (Indarti et al. 2005) with minor modification. 100 mg of dried algae samples are weighed into clean, 25 mL screw-top glass bottles, to which 10 mL mixture of methanol, concentrated sulfuric acid, and chloroform (4.25:0.75:5) are added. Transesterification is carried out in a 90°C water bath for 90 min. Upon completion of the reaction, the chloroform layer containing FAME is carefully collected and subjected to GC-MS analysis. The GC is equipped with a flame ionization detector and a DB-5-MS capillary column. The oven temperature is set at 80°C, and held for 5 min, then raised to 290°C at a rate of 4°C min^{-1}, and held at 290°C for 5 min, while the injector and detector temperature are set at 250 and 230°C, respectively. The carrier gas (helium) is controlled at 1.2 mL min^{-1}. Chromatographic data is recorded and integrated using Agilent data analysis software. The compounds are identified in the NIST Mass Spectral Database and quantified by comparing the peak area with that of the external standard (C18:2).

Conclusion

Indigenous microalgae can significantly recycle nutrients from waste particularly using wastewater as a medium of growth. Microalgae can reduce CO_2, produce O_2, treat wastewater and can produce biomass and bioproducts. To harness the full potential of microalgae, wide and extensive research is needed to investigate its, as yet unknown qualities. Among thousands of different kinds of microalgae, only a limited number are under consideration. Microalgal strains that inhabit wastewater are tolerant to a higher level of waste in comparison to commercially available strains. These indigenous strains could be used for wastewater treatment, biomass, and the production of bioproducts.

Conflict of interest

All the authors declare that they have no conflict of interest.

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