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Application of ozonated piggery wastewater for cultivation of oil-rich *Chlorella pyrenoidosa*

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HIGHLIGHTS

- Ozonated and autoclaved piggery wastewaters were compared for microalgae cultivation.
- The impressive nutrient removal was not affected by the sterilisation methods.
- Residual bacteria in ozonated water reduced microalgae growth by 10.4%.
- Bacteria comprised 30% of the total microorganisms in ozonated water at the end.
- Lipid production and fatty acid profile were similar in both sterilisation methods.

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ABSTRACT

Ozonated and autoclaved piggery wastewaters were compared for cultivation of oil-rich *Chlorella pyrenoidosa* by measuring nutrient removal from the medium and growth rate and lipid production of the microalgae. The removal rates of chemical oxygen demand, NH_4^+-N, total nitrogen and total phosphorus by *C. pyrenoidosa* were not influenced by both sterilisation methods. The specific growth rate and biomass of *C. pyrenoidosa* were determined by analysing the chlorophyll concentration for eliminating the disturbance of bacteria growth in culture system. Bacteria raised from the residue in the ozonated medium achieved 30% of the total microorganisms at the end of cultivation. They reduced the growth of *C. pyrenoidosa* by 10.4%, but contributed to a faster decline of the nutrient content on the first day. Lipid production and fatty acid profile did not change markedly in both sterilisation methods. The results suggest that ozonation is acceptable for piggery wastewater treatment for *C. pyrenoidosa* cultivation.

1. Introduction

Nutrient pollution arising from animal effluents can result in increasingly severe environmental problems, including water eutrophication, air pollution by ammonia volatilisation and soil degradation (Godos et al., 2010). In China, nutrient pollution by animal wastewater is posing a threat to the environment owing to inappropriate disposal.

The ability of microalgae to deplete inorganic nutrients effectively makes them an efficient bioremediation tool for wastewater treatment. The most important feature of piggery wastewater is its high concentration and good balance of organic nutrients that are suitable for the cultivation of microalgae (Travieso et al., 2006). At present, microalgae are a potential alternative for the production of biodiesel owing to their faster growth than other energy crops (Chisti, 2007). Consequently, the use of microalgae for the combined goals of biodiesel production and wastewater nutrient removal has been considered to be practical (Olguín, 2012; Rawat et al., 2011).

In many studies, microalgae have been cultivated in secondary effluent following anaerobic digestion in piggery wastewater treatment plants or diluted primary piggery wastewater (Travieso et al., 2006; Wang et al., 2010). To avoid the detrimental effects of microorganisms, wastewater should be pretreated by autoclaving; however, this is a costly and energy-intensive process, which leads to the bottlenecks of amplification of cultivating microalgae in wastewaters, indicating that this promising approach is still in its original phase (Zhu et al., 2013).

Ozone, a powerful and fastest-acting oxidising agent, not only kills both viral and bacterial pathogens rapidly and extensively but also renders some toxic chemicals and recalcitrant organic compounds in wastewaters more readily biodegradable (Silva et al., 2010; Ratpukdi et al., 2010). In addition, it has been reported

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that pretreatment of wastewater by ozonation removes undesirable odours and colours and enhances the light transmittance of the medium, which improves algal biomass and subsequent removal of inorganic nutrients from piggery effluent (Silva et al., 2010; Kim et al., 2014). Therefore, ozonation would be an effective and reliable alternative disinfection method for cultivating microalgae with piggery wastewater.

Some surviving microorganisms are still present in piggery wastewater after ozonation, which is not observed in case of autoclaving pretreatment. The characteristics of the microalgae–bacteria-based system are very important for the healthy and stable development of a microalgal culture (Subashchandrabose et al., 2011). However, seldom study has investigated the consortia of microalgae and bacteria in piggery wastewater subjected to disinfection using ozonation.

Chlorella pyrenoidosa was used in this study because it can utilise organic nutrients present in primary piggery wastewater in the mixotrophic mode to produce lipids (Wang et al., 2012). In the present study, *C. pyrenoidosa* was cultivated directly using diluted primary piggery wastewater as the medium. Ozonation modes and the effect of ozone on growth of the microalgae were compared with autoclaving. The consortia of microalgae and bacteria were analysed to evaluate the feasibility of disinfection by ozone to accelerate process scale-up.

2. Methods

2.1. Piggery wastewater used as culture media

The experiments were performed using piggery wastewater from a local pig farm as a substrate. The piggery wastewater was flocculated with chitosan as a flocculant and algin as a supplemental agent; it was then allowed to settle for several hours to precipitate any visible particulate solids. The supernatant was disinfected by autoclaving and ozonation for culturing microalgae. The concentrations of ammonium (NH_4^+-N), total nitrogen, total phosphorus and chemical oxygen demand (COD) in the piggery wastewater were determined following the protocols described previously (Clesceri et al., 1998).

2.2. Ozone generation

Ozone was generated by an air-supplied corona discharge-type ozone generator. This generator provides 300–500 mg ozone/h with air. The ozone gas was maintained at a constant flow rate of 300 mL/min and bubbled through a porous diffuser placed at the base of a vessel that transferred ozone gas into the diluted primary piggery wastewater. Ozonated samples were collected at 1, 5 and 10 min for evaluating residual ozone concentrations and disinfection efficiency. Prior to inoculation with *C. pyrenoidosa*, the ozonated effluent was exposed to air for 24 h to dissipate the dissolved ozone.

2.3. Microalgae and culture conditions

The strain of *C. pyrenoidosa* was isolated from local fresh water and maintained in modified Bristol's medium containing the following: solid ingredients: $NaNO_3$ (0.25 g/L), $K_2HPO_4{\cdot}3H_2O$ (0.075 g/L), $MgSO_4$ (0.075 g/L), $CaCl_2{\cdot}2H_2O$ (0.025 g/L), KH_2PO_4 (0.175 g/L), NaCl (0.025 g/L) and $FeCl_3{\cdot}6H_2O$ (0.005 g/L) and chemicals: 1 mL Fe-EDTA solution, 40 mL/L soil extract and 1 mL/L A_5 solution, which consisted of $ZnSO_4{\cdot}7H_2O$ (0.22 g/L), H_3BO_3 (2.86 g/L), $MnCl_2{\cdot}4H_2O$ (1.81 g/L), $CuSO_4{\cdot}5H_2O$ (0.079 g/L) and $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (0.039 g/L).

After adjusting the pH to 8.0, 500 mL of the diluted piggery water disinfected with ozone or autoclaving was placed in 3000-mL conical flasks. The culture of *C. pyrenoidosa* grown in a synthetic medium until the linear growth phase was used as the inoculum.

The culture broth was stirred by bubbling through air that had been sterilised with a 0.2-μm membrane filter at a flow rate of 0.3 L/min and incubated at 25 °C ± 2 °C. Light was continuously supplied (Philips TLD 36W/54 fluorescent lamp) at an intensity of 63 μmol/m^2/s measured at the surface of the flasks with a luminance metre (ZDS-10; Shanghai Cany Precision Instrument Ltd., China). Algae were centrifuged and harvested after culturing for 12 days.

2.4. Analytical method

2.4.1. Growth of C. pyrenoidosa

The concentration of *C. pyrenoidosa* was determined by measuring the total chlorophyll concentration ($\sum C$) using a spectrophotometric method (Becker, 1994). The concentration of microorganisms was determined by measuring the optical density at 625 nm with a UV–vis spectrophotometer (UV-6100PC, MAPADA, Shanghai, China).

Biomass concentration [dry weight of cell powder (DCW) in culture medium, g/L] in the piggery wastewater was estimated by an equation that employed total chlorophyll ($\sum C$) determinations:

$$\mathrm{DCW}\ (\mathrm{g/L}) = 0.1084 \sum C,\ R^2 = 0.9562$$

The specific growth rate was calculated by fitting the total chlorophyll for the first 8 days of cultivation to an exponential function:

$$\mu\ (\text{per day}) = \left(\ln \sum C - \ln \sum C_0\right) \Big/ t,$$

where t (day) is the time between 2 measurements and $\sum C$ and $\sum C_0$ (mg/L) are the total chlorophyll concentrations ($\sum C$) for the eighth day and beginning of a batch run, respectively.

2.4.2. Measurement of residual ozone concentrations and disinfection efficiency

Residual ozone concentrations were determined by the potassium iodide titration method (Lenore et al., 1999) and disinfection efficiency was evaluated by colony count on an LB agar plate. Sterilisation efficiency was calculated by the following equation:

$$S\ (\%) = (V_0 - V)/V_0,$$

where V and V_0 are the number of microorganisms (CFU) before and after ozonation of effluent, respectively.

2.4.3. Measurement of lipid production

The total lipid content was determined using the method of Bligh and Dyer (Iverson et al., 2001). Cell suspensions were collected and centrifuged at 6000×g for 10 min, and the pellets were then freeze-dried and stored at −20 °C until analysis. The content of lipids was calculated by the following equation:

$$C_l\ (\mathrm{g/g}) = W_\mathrm{L}/W_\mathrm{A},$$

where W_L (g) and W_A (g) are the weights of the extracted lipids and dry algal biomass, respectively.

2.4.4. Measurement and analysis of fatty acids

Fatty acids in algal cells were analysed by gas chromatography using the procedure of Slover and Lanza (1979) with some modifications. The extracted oil was saponified with saturated KOH–CH_3OH solution and then methyl esterified with boron trifluoride. Samples were analysed using a gas chromatograph (Agilent-6890, Aligent Technologies, USA) equipped with a flame