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Large Scale Cultivation and Pretreatment Optimization of Freshwater Microalgae Biomass for Bioethanol Production by Yeast Fermentation

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ABSTRACT

The rapid depletion of the world's fossil fuel reserves and global warming issues have promoted the search for sustainable alternative energy resources. In the present investigation, large-scale cultivation of naturally isolated freshwater microalgae *Asterarcys quadricellulare* strain was carried out using tertiary treated municipal wastewater as a growth medium in an open HRP pond for bioethanol production. A total of 12.091 kg of dry biomass was obtained at the end of the study. The lipid extracted carbohydrate rich spent microalgae biomass was converted to bioethanol by ethanolic fermentation. The biomass was first pre-treated with different concentrations of H_2SO_4 and HCL hydrolysis with different temperatures and reaction times. The biomass treated with a 2.0% concentration of H_2SO_4, showed maximum yields of glucose 308.38 $mg.g^{-1}$ at 100°C with 180 min reaction time. The hydrolysates derived from the hydrolysis of microalgae biomass were used as a substrate for fermentation by using *S. cerevisiae*. The obtained bioethanol was analyzed using HPLC and the purity of ethanol was 90%.

INTRODUCTION

The rapid depletion of the world's fossil fuel reserves increasing the energy demand. Fossil fuel is not a renewable energy source and is going to be inadequate due to the rapid growth in population, industrial development, and excessive energy consumption. Energy consumption could increase between 17% and 50% by 2040 relative to 2012 (IEA 2014). The available fossil fuel resources may get exhausted in less than the coming 50 years at the current rate of utilization (Abou-Shanab et al. 2011). The current levels of usage of coal, oil, and gas as a primary energy consumption account for 82% in 2012 and is to be expected to cause an increase in global atmospheric temperature above 3.6°C by 2100 (IEA 2014). On the other hand, fossil fuels are the largest contributor of greenhouse gases emitting more than 6 billion tons of CO_2 annually to the biosphere. For a sustainable energy future, the search for an alternative energy source is an important prerequisite that is renewable, economical, and environmentally friendly.

Biofuels are classified depending on the type of biomass used for their production. First-generation biofuels are primarily produced from edible food biomass resources corn and soybeans (Naik et al. 2010, Mohr & Raman 2013), and race with land and water usage for the production of food (Ho et al. 2014). The second generation of biofuels can be produced from a wide collection of different feedstocks, ranging from lignocellulosic plant biomass to municipal solid wastes, which do not battle with food resources. The major problem with this generation is developing cost-effective biomass conversion technology. Biofuels produced from algae and cyanobacteria are classified as third-generation biofuels. Biofuel based on microalgae systems is an attractive source and is a high-potential fossil fuel replacement.

Microalgal biofuel systems have the potential advantages of having higher photosynthetic efficiency, the ability to synthesize and accumulate large quantities of neutral lipids, and higher biomass productivity and multiplication rate. The microalgae are also able to grow in saline/brackish water/coastal seawater, utilize nutrients from a variety of wastewaters with the added benefit of wastewater bioremediation, produce valuable by-products (proteins, polysaccharides, pigments, hydrogen and biopolymers), and grow in culture vessels successfully throughout the year as compared to other energy crops (Sangapillai & Marimuthu 2019). Selection of the suitable microalgae strain is a crucial factor in successful biofuel production from microalgae strain. The selected strain should have rapid biomass productivity, high lipid productivity, able to survive in stressful conditions, have high CO_2 sinking

capacity, have limited nutritional requirements, and provide valuable co-products.

Microalgae can grow well in domestic, municipal, and agricultural wastewater. Growing microalgae in wastewater offers several economic and environmental merits, providing the most promising approach for sustainable ways of biofuel production. Wastewater can serve as a natural growth media rich in organic nutrients such as N, P, K, and other minor nutrients for algal cultivation which would ensure sustainability in terms of nutrient removal and wastewater remediation (Mahapatra & Ramachandra 2013). Wastewater usage for microalgae cultivation eliminates competition for freshwater, saves the cost of nutrients supplement, and reduces CO_2 emissions coupled with simultaneous nutrient removal and lipid production with wastewater treatment (Chinnasamy et al. 2010). The major drawback of growing microalgae in treated wastewater is the low amount of biomass due to the depletion of essential nutrients. This can be adjusted by the supplementation of essential nutrients to the wastewater in order to enhance microalgae growth (Abou-Shanab et al. 2013). In the present study *A. quadricellulare* (KT280061) lipid-extracted biomass was used as a substrate for bioethanol production.

MATERIALS AND METHODS

Microalgae Cultivation in HRP Pond

The green microalgae, *Asterarcys quadricellulare* strain (KT280061) isolated from freshwater were used in this study. The species was characterized morphologically and phylogenetically in a previous study (Karthikeyan & Thirumarimurugan 2017). Large-scale cultivation of *A. quadricellulare* was carried out in two high-rate production ponds (HRP) of the same size in this study. The HRP pond was 8.5 m in length, 3.0 m in width, and 0.30 m in height. The ponds were built with tarpaulin sheets also for the flexibility for pond shifting after completion of work. Five liters of optimally grown *A. quadricellulare* culture was inoculated in 45 L of modified bold basal medium (BBM) in a 100 L capacity transparent plastic tank. The tank was kept under controlled laboratory conditions for 5 days to avoid initial contamination. Later, the culture was exposed to direct sunlight for 5 days. The culture grown in transparent plastic tanks was inoculated in 450 L of modified BBM medium in a small open pond having a capacity of 750 L. The culture was allowed to grow under direct sunlight for 7 days and it was used as inoculum for the HRP pond cultivation. The total inoculum was transferred to an HRP pond containing 4500 L of 60% MTTSW-containing medium. The loss of evaporation was compensated by adding tap water every day and the culture volume was maintained at a depth of 20 cm throughout the experiment. The paddle wheels were used for mixing at a speed of 20 rpm and turned off at night. The pH of the culture medium was monitored using an automatic pH meter. The microalgae grown in a modified BBM medium served as a control. This study was conducted for 30 days.

Harvesting of Microalgae

For harvesting microalgae biomass, 500 L of grown culture pumped from the experimental pond into the storage tank was subjected to an electro-clarification process. The volume of the chamber was 100 L with one MS electrode placed at the center. The retention time inside the reaction chamber was 0.25 min. The reaction was carried out at voltage 8.0 (eV) along with continuous agitation at 100 rpm. The treated cells in the culture were stored in an inverted cone-shaped tank and allowed to settle at the bottom of the clarifier and slurry was collected for drying. The harvested thick microalgae slurry was dried directly under sunlight for 4 days on a tarpaulin sheet and the dried biomass was collected, stored in an air-tight container, and stored under refrigeration conditions for further studies.

Extraction of Total Lipids from Microalgae Biomass

The total lipids were extracted according to Folch et al. (1956) method using chloroform and methanol (2:1 (v/v)). After lipid extraction, the residual spent biomass was dried at room temperature overnight. The spent dried biomass was ground into powder using a pestle and mortar and used for bioethanol production.

Optimization of Acid Pre-treatment Conditions

The spent residual algal biomass of *A. quadricellulare* was subjected to acid pretreatments to produce sugar hydrolysates for ethanol fermentation. Two different acids such as H_2SO_4 and HCL acid were used at various concentrations viz., 0.5, 1.0, 1.5 and 2.0 in the ratio of 1:10 (w/v) for pretreatments. Both acid-treated samples were subjected to different temperatures viz., 30, 40, 60, 80 and 100°C and different time durations viz., 30, 60, 90, 120 and 150 min separately in a shaking condition. After centrifugation, the sugar content in the supernatant was estimated by using the Dubois et al. (1956) method.

Extraction of Sugars from Microalgal Biomass

About 100 g of spent residual biomass powder was treated with the optimized concentration of 500 mL of 2% (v/v) sulphuric acid at 100°C for 3 h with intermittent shaking. The pretreated biomass hydrolysate was centrifuged at 10000 rpm for 15 min. The supernatant containing the sugar content was estimated using Dubois et al. (1956) method.

The sugar filtrate was adjusted to pH 4.8 and autoclaved at 121°C for 15 min.

Bioethanol Production by *Saccharomyces cerevisiae*

Saccharomyces cerevisiae was used for ethanologenic fermentation of *A. quadricellulare* hydrolysates. The commercial yeast cells of *S. cerevisiae* were obtained from the local market and one gram of yeast was resuspended aseptically in 9 mL of warmed distilled water and left for 15 min with proper mixing. Yeast S. *cerevisiae* pre-culture was grown in YPD (1% yeast extract, 2% peptone, and 2% D-glucose) medium at pH 4.8, 30°C for 24 h at 200 rpm. After the aerobically 10% of overnight grown pre-cultured yeast cells were transferred to the liquid fraction of pretreated algal biomass hydrolysate. The airtight fermentation flasks were kept under anaerobic conditions at 30°C for 3 days. The fermentation mixture was distilled and the distillate was analyzed by HPLC for the presence of bioethanol.

RESULTS AND DISCUSSION

Large Scale Cultivation and Harvesting of Microalgae

Large-scale cultivation of *A. quadricellulare* was performed in an open HRP pond system. The maximum accumulation of biomass (total dry weight) was recorded as 1.21 $g.L^{-1}$ on the 25th day. The average pH and TDS values were 8.2 and 1.55 ppm respectively. Electro-clarification is a new technology used for removing solid particles from water in the wastewater treatment process (Phalakornkule et al. 2009). The obtained mass-cultured *A. quadricellulare* biomass from open HRP was subjected to electro-clarification to concentrate the microalgae biomass. About 100 L of algal culture was transferred into the electro-clarification chamber from the open pond and the cells get aggregated under the optimum electrical current. The aggregated microalgae biomass was then transferred into the clarifier with 1.0 ppm of polyacrylamide. The biomass was left overnight. On the next day, a clear supernatant was discarded and the remaining 10 L of concentrated wet algal biomass was recovered from the outlet. *Nannochloropsis sp*. and *Chaetoceros calcitrans* were cultured and electrofloculated with a harvesting efficiency of 95 and 92% respectively observed under the optimum voltage and current (Raut et al. 2017). This technique facilitates the removal of 90% water from the algal culture. The concentrated wet biomass was exposed to direct sunlight for 4 days. A total of 12.091 kg of dry biomass was obtained at the end of the study.

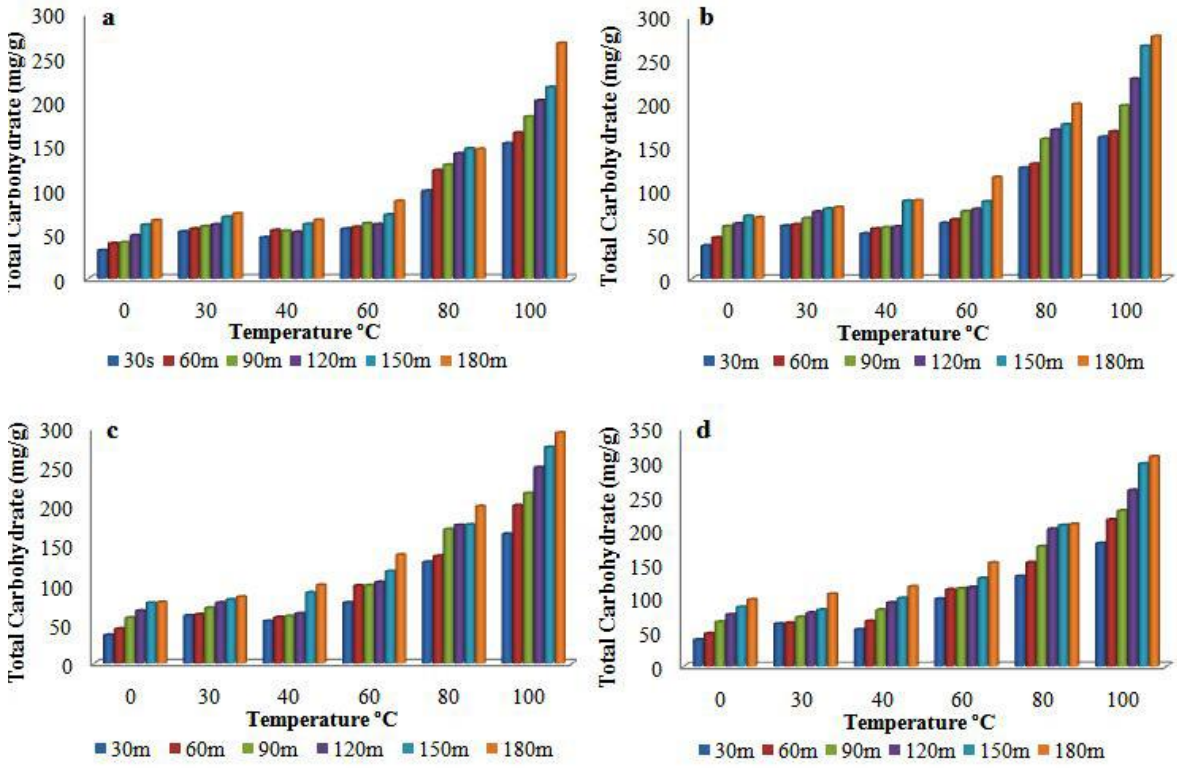

Fig. 1: Effect of Different concentrations of H_2SO_4 on carbohydrate yield (a) 0.5% H_2SO_4 (b) 1.0% H_2SO_4 (c) 1.5% H_2SO_4 (d) 2.0% H_2SO_4.

Optimization of Biomass Pretreatment

Acid hydrolysis is one of the best methods for disturbing the cell wall material and releasing the total carbohydrate content from microalgae biomass to perform fermentation. For the hydrolysis of lipid-extracted microalgae biomass, sulfuric acid (H_2SO_4) and hydrochloric (HCL) acid were used in different concentrations viz., 0.5%, 1.0%, 1.5% and 2.0%. The hydrolysis reaction for each concentration was performed at five different temperatures (30, 40, 60, 80 and 100°C) at different time intervals (30, 60, 90, 120, 150 and 180 min). The effect of H_2SO_4 and HCL acid hydrolysis on carbohydrate extraction was investigated. Among the four different concentrations of H_2SO_4, 2.0% showed the maximum yield of glucose (308.38 $mg.g^{-1}$) at 100°C with 180 min reaction time (Fig. 1). For all the pretreatment time intervals increasing the acid concentration from 0.5% to 2%, accompanied with the enhancement of glucose yield. The lowest amount of glucose yield (266.7 mg/g) was obtained from 0.5% H_2SO_4 pretreated microalgae biomass at 100°C with 180 min reaction time. In the case of HCL acid, among the four different concentrations of HCL, 2.0% showed the maximum yield of glucose (288.15 $mg.g^{-1}$) at 100°C with 180 min reaction time (Fig. 2).

For all the pretreatment time intervals increasing the hydrochloric acid concentration from 0.5% to 2%, accompanied by the enhancement of glucose yield. The lowest amount of glucose yield (143.1 $mg.g^{-1}$) was obtained from 0.5% HCL pretreated microalgae biomass at 100°C with 180 min reaction time. When compared to hydrochloric acid, the 2.0% sulfuric acid pretreated lipid spent microalgae biomass showed a maximum yield of 308.38 mg/g of carbohydrate at 100°C with 180 min reaction time. Acid quantity and strength for a specified amount of microalgae biomass directly influence the hydrolysis reaction and the yield of fermentable sugar (Ho et al. 2013, Khan et al. 2017). High acid strength leads to the degradation of monomeric sugars and conversion to other side products (Miranda et al. 2012). Acid hydrolysis at high temperatures might be more productive. Previous studies reported, the temperature range of 100-200°C required for the pretreatment of microalgae biomass depending on the acid concentration and reaction time to release the monomeric fermentable sugars for fermentation (Harun & Danquah 2011, Khan et al. 2017). This optimized concentration was used for the fermentation of spent microalgae biomass.

Bioethanol Production

Saccharomyces cerevisiae is usually used in fermentations

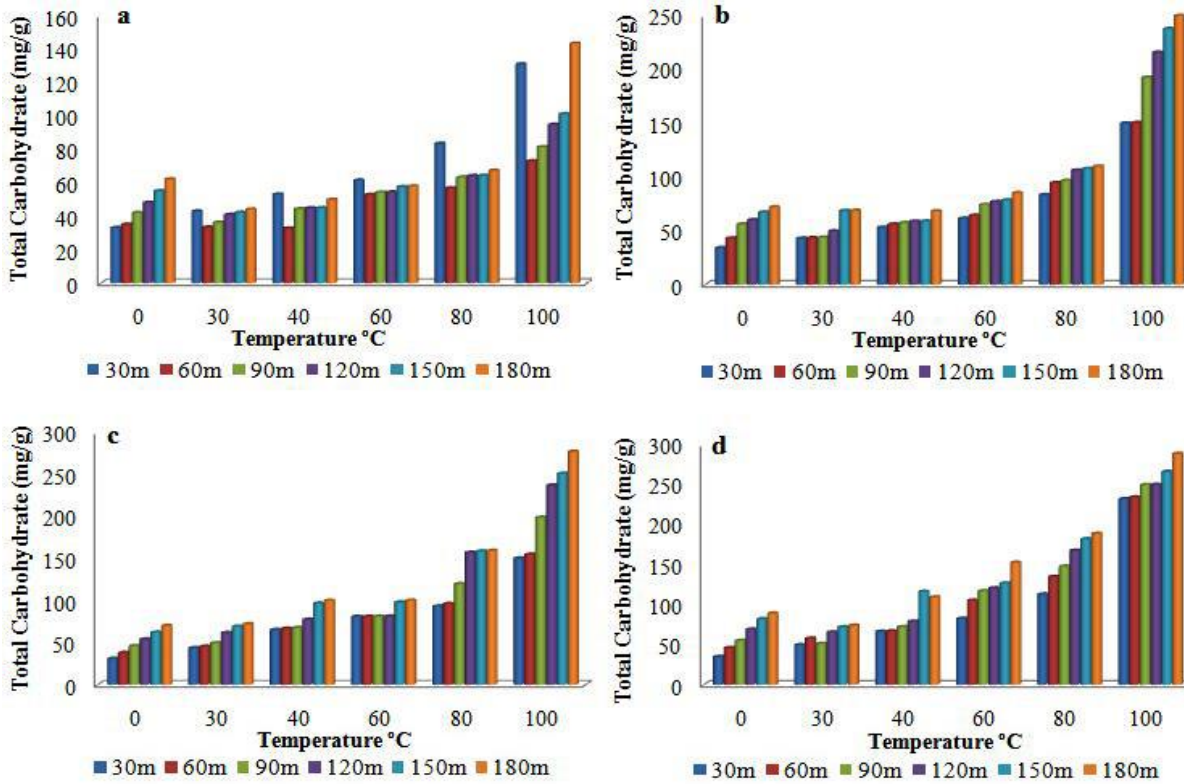

Fig. 2: Effect of different concentrations of HCL on carbohydrate yield (a) 0.5% HCL (b) 1.0% HCL (c) 1.5% HCL (d) 2.0% HCL.

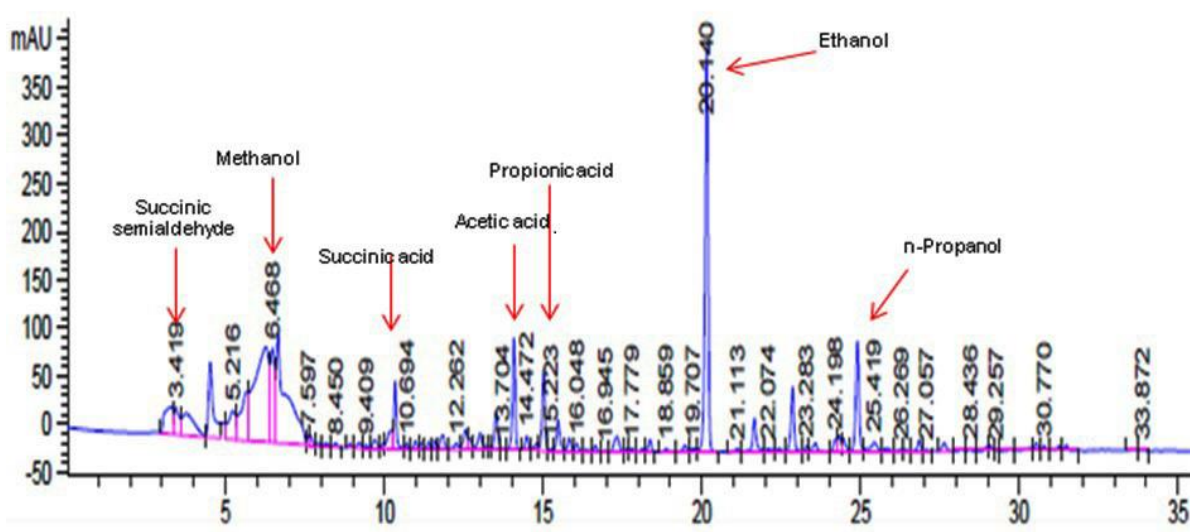

Fig. 3: HPLC analysis of bioethanol obtained from residual biomass of *Asterarcys quadricellulare*.

for the commercial production of bioethanol (Zhou et al 2011). Based on the acid-pretreated studies, for the ethanolic fermentation experiments using *S. cerevisiae*, the hydrolysates derived from the hydrolysis of 100 g of microalgae biomass at 100°C using 2.0% H_2SO_4 with reaction time 180 min was used as substrate. The utilization of acid for the disruption of cell walls was very efficient when compared to other physical methods. These acids pretreatment comparatively yield higher sugar, as well as a lesser amount of inhibitors, was produced (Miranda et al. 2012). The pre-cultured *S. cerevisiae* was inoculated with the microalgae pretreated hydrolysate and kept under anaerobic conditions at 30°C for 3 days. After fermentation, the mixture was filtered and the filtrate was distilled at 70°C. The yield of bioethanol was estimated at 0.192 g ethanol.g^{-1} of biomass. The bioethanol was analyzed using HPLC and the purity of ethanol was 90% (Fig. 3). Choi et al. (2010) reported in general the typical ethanol fermentation yields from microalgae biomass using yeast *S. cerevisiae* do not exceed 0.3 g ethanol.g^{-1} of dry algae biomass.

CONCLUSION

Due to increasing global oil demand and climate change, algal biofuels have gained more attention as an alternative renewable energy source. In this present study, the native microalgae *A. quadricellulare* isolated from freshwater were cultivated in an open pond system with 60% municipal tertiary treated sewage wastewater and 40% modified BBM-containing medium. The microalgae biomass was harvested by electro-clarification method and the total lipid content was extracted from the microalgae biomass. The lipid-extracted carbohydrate-rich biomass was then pretreated with 2.0% sulfuric acid and it showed a maximum yield of 308.38 mg.g^{-1} of carbohydrate at 100°C with 180 min reaction time. The hydrolyzed biomass was fermented with *Saccharomyces cerevisiae* for bioethanol production. The yield of bioethanol obtained was estimated at 0.192 g ethanol.g^{-1} of biomass. The cultivation of these freshwater microalgae offers new opportunities in biofuel production. In the future, the cultivation of microalgae strain KT280061 in wastewater appears to be a low-cost, eco-friendly sustainable, feasible, and scalable approach for large-scale biofuel applications.

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