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Short Communication

Direct utilization of waste water algal biomass for ethanol production by cellulolytic *Clostridium phytofermentans* DSM1183

Anwar Aliya Fathima [a], Mary Sanitha [a], Thangarathinam Kumar [b], Sellamuthu Iyappan [a], Mohandass Ramya [a,*]

[a] Molecular Genetics Lab, Department of Genetic Engineering, School of Bioengineering, SRM University, Kattankulathur, Kancheepuram District, Tamil Nadu 603203, India
[b] Analytical Research and Development Division, Piramal Enterprises Limited, Chennai 600057, India

HIGHLIGHTS

- Ethanol production from non-pretreated and pretreated algal biomass using *Clostridium phytofermentans* DSM1183.
- The ethanol production achieved was 4.6 g L^{-1} with acid pretreated algal biomass (2%, w/v) with solvent yield of 0.26 g/g.
- Fermentation of 2% (w/v) non-pretreated algal biomass produced 0.52 g L^{-1} of ethanol with solvent yield of 0.19 g/g.

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ABSTRACT

Direct bioconversion of waste water algal biomass into ethanol using *Clostridium phytofermentans* DSM1183 was demonstrated in this study. Fermentation of 2% (w/v) autoclaved algal biomass produced ethanol concentration of 0.52 g L^{-1} (solvent yield of 0.19 g/g) where as fermentation of acid pretreated algal biomass (2%, w/v) produced ethanol concentration of 4.6 g L^{-1} in GS2 media (solvent yield of 0.26 g/g). The control experiment with 2% (w/v) glucose in GS2 media produced ethanol concentration of 2.8 g L^{-1} (solvent yield of 0.25 g/g). The microalgal strains from waste water algal biomass were identified as *Chlamydomonas dorsoventralis*, *Graesiella emersonii*, *Coelastrum proboscideum*, *Scenedesmus obliquus*, *Micractinium* sp., *Desmodesmus* sp., and *Chlorella* sp., based on ITS-2 molecular marker. The presence of glucose, galactose, xylose and rhamnose were detected by high performance liquid chromatography in the algal biomass. Scanning Electron Microscopy observations of fermentation samples showed characteristic morphological changes in algal cells and bioaccessibility of *C. phytofermentans*.

1. Introduction

The depletion of petro-based fuels has led to greater concern for alternative fuel technology. Plant and microalgal biomass rich in carbohydrates can be used as the source for conversion of carbohydrate into biofuels. The requirement of arable land and water for cultivation of plant biomass proves it to be a less economical source for production of biofuels (Ho et al., 2013). The research on producing biofuels using microorganisms has been extensively carried out recently to find a viable and excellent substitute for petroleum fuel. The important aspect being the source from which carbohydrates are used should be inexpensive and easily accessible. Lignocellulose (comprising cellulose, hemicelluloses, pectin and lignin) obtained from plant biomass is widely available and most renewable source of carbohydrates for solvent production. A number of plant sources such as cassava, maize stalk, oil palm and sugar molasses have been used as a source for carbohydrate fermentation (Ibrahim et al., 2015). However, the presence of lignin and hemicellulose greatly hinders the hydrolysis of cellulose into monosaccharides. Microalgae can be considered as an alternative source because they lack lignin and more than 40% of dry weight of microalgae accumulates carbohydrates which can be used as fermentable sugars (John et al., 2011). Cellulolytic bacteria capable of degrading a wide range of polysaccharides have been used for production of ethanol. In the present study *Clostridium phytofermentans* was used for the direct fermentation of algal biomass for ethanol production. *C. phytofermentans*, a mesophilic anaerobe isolated from forest soil converts cellulosic biomass into ethanol (Warnick et al., 2002). It secretes glycoside hydrolases that cleave cellulose, starch and hemicellulose into hexose and pentose sugars that are converted to ethanol (Tolonen et al., 2011). In this

* Corresponding author at: Department of Genetic Engineering, School of Bioengineering, SRM University, Kattankulathur, Kancheepuram District, Tamil Nadu 603203, India. Tel.: +91 9442044277.
E-mail address: ramya.m@ktr.srmuniv.ac.in (M. Ramya).

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study, cellulolytic *C. phytofermentans* was used for direct conversion of waste water algal biomass into ethanol. The micro algal strains isolated from sewage water were identified based on ITS-2 molecular marker. The monomeric sugar content of algal biomass was determined by HPLC and the total soluble carbohydrate in algal biomass was determined by phenol sulfuric acid assay. The growth characteristics, total soluble sugar utilization and ethanol production was analyzed at regular intervals (0,24,48,72,96,120 h) during fermentation. Scanning Electron Microscopy (SEM) analysis was performed to observe the morphological changes in the algal cells and bioaccessibility of *C. phytofermentans* during fermentation.

2. Methods

2.1. Strains and culture media

C. phytofermentans DSM1183 was purchased from DSMZ, Germany. The culture was grown in GS2 medium (Zuroff et al., 2013) with the following composition per liter: 6 g Yeast Extract, 2.9 g K_2HPO_4, 2.1 g Urea, 1.5 g KH_2PO_4, 3 g Tri-Na Citrate H_2O, 2.33 g Cysteine HCl, 1 g MOPS, 0.1 g $MgCl_2$ $6H_2O$, 0.0113 g $CaCl_2$ Anhydrous, and 0.000125 g $FeSO_4$ $7H_2O$ and 0.01% resazurin. The salts solution and resazurin solution were prepared separately and added after autoclave. The media was sparged with nitrogen before and after autoclave. The microalgal strains isolated from Sewage Treatment Plant (STP) located at SRM University, Tamil Nadu were grown on BG11 medium. The BG11 media comprises of following components per liter: 1.5 g Sodium Nitrate, 0.0314 g Dipotassium Hydrogen Phosphate, 0.036 g Magnesium Sulfate, 0.0367 g Calcium Chloride dihydrate, 0.02 g Sodium Carbonate, 0.001 g Disodium Magnesium EDTA, 0.0056 g Citric Acid, 0.006 g Ferric Ammonium Citrate and the pH was adjusted to 7.1. All chemicals used in this study were purchased from Himedia Chemicals Pvt. Ltd. India.

2.2. Algal biomass

BG11 media was inoculated with 10 mL of sewage water sample and incubated at 25 ± 1 °C at a light intensity of 100 μmol photons m^{-2} s^{-1} and 16:8 light/dark cycles to allow the growth of mixed algae. After appearance of visibly dense growth, the algal culture was centrifuged and the harvested algal biomass was mixed with fermentation media and sterilized by autoclave (121 °C for 15 min). For fermentation of pretreated algal biomass the harvested biomass was mixed with 1 M sulfuric acid and autoclaved (121 °C for 15 min). The slurry was cooled and centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant collected was neutralized with solid sodium bicarbonate. The pH of the hydrolysate collected was adjusted to 6.5 before it was added to fermentation media.

2.3. Determination of carbohydrate composition of algal biomass

The dried algal biomass was used for analysis of carbohydrate composition. The starch content according to Hirokawa et al. (1982) and cellulose content according to Updegraff (1969) was estimated. The xylan content in the algal biomass was estimated as the total xylose in the algal biomass. In brief, the algal biomass was treated with 10 mg/mL xylanase enzyme (lyophilized powder, SRL Chemicals, India) and incubated at 50 °C for 15 min. The reaction was carried out in 96 well microtitre plate. The amount of reducing sugar (xylose) released was determined by anthrone method (McCready et al., 1950). The absorbance was read at 620 nm using ELISA reader (Biotek ELx800 MS, USA) and values were plotted against xylose standard curve. The total soluble sugar content was estimated by colorimetric method using phenol and sulfuric acid. The dried algal biomass was acid hydrolyzed and the hydrolysate collected was used for analyzing the monomeric carbohydrate composition by high performance liquid chromatography. The algal hydrolysate was precolumn derivatized for determination of monosugars (Templeton et al., 2012). For precolumn derivatization, freshly prepared 0.35 M *p*-aminobenzoic acid (w/v) in DMSO/acetic acid (70:30 v/v) was used. A 200 μL aliquot of standard solution and algal hydrolysate were mixed with 500 μL of 0.35 M *p*-aminobenzoic acid and 10 mg of sodium borohydride followed by heating at 60 °C for 15 min. After cooling, the solution was made up to 10 mL with HPLC eluent and preceded for analysis using Shimadzu Prominence with PDA detector. The column used was Xterra C18 (250 mm × 4.6 mm) with flow rate of 1.0 mL/min (Isocratic) and wavelength 290 nm. The sample injection volume was 20 μL with oven temperature 40 °C and cooler temperature 15 °C. The mobile phase used consists of 7.8 g of monobasic sodium phosphate and 1 g of sodium decane-1-sulfonate in 1000 mL of water and the pH is adjusted to 3.0 with phosphoric acid. To 850 mL of the resulting solution 150 mL of acetonitrile was added.

2.4. Screening and molecular characterization of microalgae from mixed algal biomass

The mixed algal biomass was serially diluted and plated onto BG11 medium to obtain individual colonies. The colonies obtained from various dilutions were maintained in BG11 medium as individual isolates and subsequently used for molecular identification based on ITS-2 molecular marker. Colony PCR was performed using 2 mL algal cells from individual isolates (Radha et al., 2013). The cells were pelleted by centrifugation at 10,000 rpm for 5 min and resuspended in 30 μL Tris–EDTA buffer (pH 8.0). The primer pair for internal transcribed spacer region included sequences forward 5′-AGGAGAAGTCGTAACAAGGT-3′ and reverse 5′-TCCTCCGCTTATT GATATGC-3′. The 30 μL PCR reaction mix included the following components 1 μL algal lysate, 1 U Taq DNA polymerase (New England Biolabs), 3 μL 10X PCR buffer, 1 μL dNTP mix (10 mM each nucleotide) (New England Biolabs) and 1 μL primer (5 pmol of each, forward and reverse primer) (Bioserve, India). The conditions for PCR used were initial denaturation at 95 °C for 5 min, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min for 35 cycles. The PCR reaction was carried out using Thermal Cycler (Applied Biosystems, USA) and the PCR products were examined on 1% (w/v) agarose gel. The amplified ITS-2 DNA fragments were purified using EZ-10 column purification kit (Biobasic Inc., USA) and subjected to sequencing using automated DNA sequencer AB1 3130 XL (Genetic Analyzer, Applied Biosystems, USA). The resultant sequences were analyzed using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and submitted to Genbank.

2.5. Batch fermentation with C. phytofermentans

C. phytofermentans was initially grown on GS2 media with 0.5% (w/v) glucose. The fermentation media was inoculated with 2% (v/v) (2×10^8 CFU/mL) from freshly grown overnight culture of *C. phytofermentans*. The fermentation experiments were performed in 100 mL serum vials fitted with rubber stopper. The experiments were conducted with modified GS2 media containing – 2% (w/v) glucose (set A), 2% (w/v) algal biomass (set B) and acid pretreated algal biomass (2%, w/v) (set C) as carbohydrate sources. The media was sparged with nitrogen prior to inoculation and the pH was adjusted to 6.5. The vials were incubated at 30 °C throughout the fermentation experiment for 120 h with occasional shaking. All fermentation experiments were performed in triplicates and