Algal Research 9 (2015) 48–54

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

Solid state fermentation (SSF)-derived cellulase for saccharification of the green seaweed *Ulva* for bioethanol production

Nitin Trivedi [a,b], C.R.K. Reddy [a,b,*], Ricardo Radulovich [c], Bhavanath Jha [a,b]

[a] Division of Marine Biotechnology and Ecology, CSIR — Central Salt and Marine Chemicals Research Institute, Bhavnagar 364002, India
[b] Academy of Scientific & Innovative Research (AcSIR), New Delhi, India
[c] Department of Agricultural Engineering, University of Costa Rica, San Jose, Costa Rica

ARTICLE INFO

Article history:
Received 22 August 2014
Received in revised form 12 February 2015
Accepted 14 February 2015
Available online xxxx

Keywords:
Bioethanol
Cellulase
Saccharification
Solid state fermentation
Ulva

ABSTRACT

Cellulase produced from the marine fungus *Cladosporium sphaerospermum* through solid state fermentation (SSF) was investigated for its saccharification potential of seaweed biomass using the common green seaweed *Ulva fasciata*. The seaweed substrate, containing inoculated fungus with 60% moisture content, cultured at 25 °C and pH 4 for four days, showed optimum enzyme production. The enzyme, assayed for carboxymethyl cellulase and filter paper assay, showed an activity of 10.20 ± 0.40 U/g and 9.60 ± 0.64 U/g on a dry weight basis, respectively. Further, ionic liquid tolerance of the enzyme was studied in the presence of 1-ethyl-3-methylimidazolium acetate, 1-butyl-3-methylimidazolium chloride, 1-butyl-3-methylimidazolium trifluoromethanesulfonate and 1-butyl-1-methylpyrrolidinium trifluromethanesulfonate. At 10% v/v concentration, the enzyme retained 72.17 to 85.04% activity in all the ionic liquids. The pre-incubation of enzyme in the same ionic liquids for 24 h, the activity got slightly enhanced and ranged between 73.80 and 93.70%. The hydrolysis of *U. fasciata* feedstock with enzyme (10 U/g) for 24 h at 40 °C and pH 4 gave maximum yield of sugar 112 ± 10 mg/g dry weight. On fermentation, an ethanol yield of 0.47 g/g reducing sugar was obtained, corresponding to 93.81% conversion efficiency. These findings indicate that cellulase produced from a marine fungus can be employed for saccharification of cellulosic feedstock for the production of renewable biofuels from marine macroalgal feedstock. Since bioethanol yields obtained compare very favorably with those from land crops, the strategy employed in this study warrants further exploration.

1. Introduction

According to the U.S. Department of Energy, 30% of petroleum-based transportation fuels would be replaced with biomass-based fuels by 2025 [1]. Worldwide, the demand for renewable fuels, particularly bioethanol, is projected to increase 3.4-fold by 2035 [2]. Cellulose, a structural component of plant biomass, is the most abundant feedstock used for the production of alternative liquid fuels, mainly bioethanol. However, cellulose in terrestrial plants is intertwined with lignin, hemicelluloses and pectin, which require extra energy input as pretreatment for their removal [3,4]. Consequently, due to their high carbohydrate content, high productivity and widespread distribution marine macroalgae (seaweeds) are increasingly gaining prominence as an alternative renewable feedstock for sustainable production of biofuels [5]. Large scale farming of a variety of seaweeds is already practiced in a number of countries. The current cultivated production of seaweeds worldwide is about 24 million tons (fresh weight) per annum [6]. A majority of this produce is used for human consumption as food and the rest for phycocolloid extraction. Since seaweed farming is undertaken directly at sea, it does not clash with terrestrial agricultural crops for land and fresh water resources. Another distinct advantage of macroalgal feedstock for biofuel production is the absence of lignin (occasionally traces), which dispenses the need for energy-intensive pretreatment as part of the hydrolysis process prior to fermentation. However, seaweed polysaccharides are structurally complex and diverse in chemical composition, and differ from land plants with respect to the abundance of matrix and skeletal components. Thus, an efficient hydrolysis for sustainable production of biofuels from different macroalgal feedstocks is required. Recently, Newman et al. [7] and Wargacki et al. [8] genetically re-engineered microbes for efficient hydrolysis and fermentation to achieve higher yields of bioethanol from brown seaweeds.

Earlier studies have employed either chemical or enzymatic processes for the hydrolysis of macroalgal feedstock [7–10]. Chemical hydrolysis (acid hydrolysis) is one of the feasible methods commonly used for the production of fermentable sugars from lignocellulosic biomass. There are also several studies reporting acid hydrolysis of seaweed polysaccharides for bioethanol production. The macroalgal species that have been used include *Kappaphycus alvarezii* [9,10], *Palmaria palmata* [11], *Eucheuma cottonii* [12], *Undaria pinnatifida* [13] and *Gracilaria salicornia*

* Corresponding author at: Seaweed Biology and Cultivation Group, Division of Marine Biotechnology and Ecology, CSIR — Central Salt and Marine Chemicals Research Institute, Bhavnagar 364002, India.
E-mail address: crk@csmcri.org (C.R.K. Reddy).

http://dx.doi.org/10.1016/j.algal.2015.02.025
2211-9264/

[14]. Nevertheless, acid hydrolysis results in the production of some non-sugar by-products such as 5-hydroxymethylfurfural (HMF), formic acid, levulinic acid, acetic acid (organic acid), phenols and heavy metals. These impede subsequent downstream fermentation besides causing environmental hazards [15].

As an alternative, using enzymes obtained from microorganisms could provide new avenues in converting complex seaweed polysaccharides into fermentable sugars. There are a few studies reporting enzymatic saccharification of macroalgal biomass using commercial enzymes [16–18]. Enzymatic hydrolysis indeed presents a green approach but suffers from high cost of commercial hydrolysing enzymes. Since cellulase is widely used in the production of bioethanol from cellulosic biomass, and green seaweeds have higher cellulose content compared to both red and brown seaweeds, they are a clear choice for saccharification.

The success of bioethanol production will greatly depend on the cost of production of cellulase employed in the saccharification process. In 2012, it was reported [19] that cost of cellulosic ethanol production was $0.94 per liter (40% higher than corn ethanol). More recently, Bloomberg New Energy Finance reported that cellulosic ethanol is expected to be cost competitive with corn ethanol by 2016 [19]. It is thus of great importance to isolate cellulase-producing microbial strains for the efficient saccharification of plant biomass of marine origin. Cellulase production through solid state fermentation (SSF) is gaining importance over submerged fermentation. The advantages of SSF include lower processing cost, less energy requirements, production of solid waste, enhanced productivity, improved product stability and lower catabolic repression [20].

To date, a majority of cellulase-producing microbes have been isolated from terrestrial sources. Little effort has been made to date exploring marine based sources for cellulase production. Therefore, an effort was made in this study to isolate marine microbes capable of hydrolyzing cellulose-rich green seaweed *Ulva fasciata* for bioethanol production. The present study reports the cellulase production potential of the marine fungus *Cladosporium sphaerospermum* through SSF and its application in saccharification of the green seaweed *U. fasciata* for bioethanol production.

2. Materials and methods

2.1. Materials

All chemicals, media components and reagents used in this work were purchased from Sigma Aldrich (U.S.A.) and Himedia laboratories (Mumbai, India). All analyses were conducted in laboratory facilities of CSIR-CSMCRI, Bhavnagar, Gujarat, India.

2.2. Microorganism

The cellulase-producing strain of the marine fungus *C. sphaerospermum* was isolated from deteriorated seaweed *Ulva* cultured in the laboratory. Cellulase secretion potential was screened by zone of clearance as qualitative measure of extracellular cellulase activity, after flooding carboxymethyl cellulose (CMC) (1.5%) agar plates with Lugol's iodine solution [21–23]. The molecular identification of the fungal strain was carried out by 18S rDNA sequencing using Internal Transcribed Spacer (ITS) regions. The culture was maintained on potato dextrose agar (PDA) at 4 °C as a pure culture.

2.3. Collection of algal sample

The marine macrophytic green alga *U. fasciata* Delile was collected from Veraval (N 20° 54.87′, E 70° 20.83′), coast of Gujarat, India. Seaweed samples were washed thoroughly with tap water to remove salts, epiphytes and debris and dried to a constant weight at 50 °C. After drying, samples were powdered using a grinder.

2.4. Solid state fermentation and optimization of parameters for cellulase production

The green seaweed *U. fasciata* was used as substrate for solid state fermentation (SSF) for cellulase production. SSF was carried out in 250-mL Erlenmeyer flasks containing 10 g of dried and powdered seaweed in a modified mineral salt medium [24,25]. The mineral salt medium contained (g/L): $(NH_4)_2SO_4$, 1.86; KH_2PO_4, 2.0; urea, 0.3; $CaCl_2$, 0.03; $MgSO_4 \cdot 7H_2O$, 0.3; yeast extract, 4.08; (mg/L): $FeSO_4 \cdot 7H_2O$, 5.0; $MnSO_4 \cdot H_2O$, 1.6; $ZnSO_4 \cdot 7H_2O$, 1.4; $CoCl_2$, 2.0; (w/v): peptone, 0.8%; and, (v/v): Tween 80, 0.1%. The pH was adjusted to 5.0 before sterilization at 121 °C for 15 min. Flasks were inoculated with fungal spore suspension (1×10^9 spores/mL) prepared in sterile 0.1% v/v Triton-X 100. Inoculated flasks were incubated at room temperature for 6 days. In order to achieve optimum enzyme yield through SSF, cellulase production was optimized with respect to different parameters such as moisture content (40–100%), incubation period (2–6 days), pH (2–6) and incubation temperature (25–40 °C). After optimisation, cellulase production was also carried out using cellulose extracted from *U. fasciata* as a substrate to compare enzyme production under optimized conditions.

2.5. Enzyme extraction and assay

To extract the enzyme, fermented substrate was suspended in 150 mL of 50 mM sodium acetate buffer. The broth was mixed properly by gentle shaking before extracting cellulase. The extract was filtered through double layered muslin cloth then centrifuged at 7000 rpm at 4 °C for 15 min. The clear supernatant obtained was considered as crude enzyme mix and used for cellulolytic assay. Total cellulase or filter paper activity (FPase) and carboxymethyl cellulase (CMCase) activity were determined according to Trivedi et al. [23] using filter paper and CMC-Na as a substrate, respectively. The FPA was expressed as FPU/g of dry substrate (FPU/g ds). One unit of enzymatic activity was defined as the amount of enzyme that released 1 μmol of reducing sugar per minute.

2.6. Effect of pH and temperature on cellulase activity and stability

The pH and temperature optima for enzyme activity were assayed at different pH ranging from 2 to 3 (50 mM Glycine–HCl buffer); 4, 5 and 6 (50 mM Sodium acetate buffer); and at different temperatures ranging from 4 °C to 60 °C with increments of 20°. Further, enzyme stability was investigated by estimating the residual enzyme activity after pre-incubation for 1 h at the aforementioned pH and temperature ranges.

2.7. Effect of ionic liquids on cellulase

The ionic liquids (ILs) used in this study include: 1-ethyl-3-methylimidazolium acetate, [EMIM]Ac, (IL1); 1-butyl-3-methylimidazolium chloride, [BMIM]Cl, (IL2); 1-butyl-3-methylimidazolium trifluoromethanesulfonate, [BMIM][OTF], (IL3); and, 1-butyl-1-methylpyrrolidinium trifluromethanesulfonate, [BMPL][OTF], (IL4). Enzyme activity was determined at different IL concentrations (1%, 5%, 10%, 15% and 20% v/v) under optimized assay conditions and was compared with the control reaction carried out in aqueous medium. Further, the IL stability of the enzyme was investigated by estimating enzyme activity after pre-incubation for different time intervals ranging from 24 to 96 h in different ILs (10% v/v). The ILs did not affect pH values in the enzyme assay mix which remained at pH around 5.0.

2.8. Hydrolysis of algal biomass through SSF-derived cellulase

Enzymatic hydrolysis of green seaweed *U. fasciata* mass was optimized with SSF-derived cellulase with respect to enzyme dosage, hydrolysis period, temperature and pH. For this, dried algal powder (1 g) was hydrolysed with different enzyme dosages (5, 10 and 15 U/g