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Purification and characterization of cellulase from a marine *Bacillus* sp. H1666: A potential agent for single step saccharification of seaweed biomass

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ABSTRACT

In this study, 115 marine bacterial isolates were screened for cellulase enzymatic activity and enzyme with a molecular mass of 40 kDa was purified from culture supernatant of the marine bacterium *Bacillus* sp. H1666 using ion exchange and size exclusion chromatography method. Growth of bacterial strain H1666 with efficient cellulase enzyme production was observed on untreated wheat straw and rice bran. The biochemical properties of the extracted cellulase were studied and enzyme was found active over a range of pH 3–9. The optimum cellulase activity was observed at pH 7 and temperature 50 °C. The enzyme was also shown to be slightly thermo-stable with 40% residual activity at 60 °C for 4 h. The potential applicability of enzyme was tested on dried green seaweed (*Ulva lactuca*) and 450 mg/g increase in glucose yield was obtained after saccharification. MALDI TOF–TOF analysis of cellulase peptide fingerprint showed similarity to the sequence of the glycoside hydrolase family protein.

1. Introduction

The combustion of fossil fuels has created a global anxiety for the environment and world economy. Now a days, efforts are made toward renewable resources of energy to overcome environmental issues and also fulfill high energy demand of the world. Lignocellulosic biomass, comprised of waste from agriculture and related industrial processes, has a great potential of renewable source because of abundance and inexpensiveness [1–3] but it is not sufficient to meet the growing energy requirement [4]. There is a new alternative, sustainable and non agricultural feedstock; marine algae, which can provide a high-yield source of cellulosic biomass without compromising food supplies, forest or agricultural land [5,6]. Cellulosic biomass is the most abundant non reducible carbohydrate on the earth comprised of linear polymer of glucose linked with 1,4-β-glycosidic bonds [7]. The cellulose can be broken into reducing sugar using two major saccharification methods namely, dilute acid hydrolysis and enzymatic treatments [8]. Enzymatic saccharification is considered a promising technology compared to other methods. The efficiency and effectiveness of cell wall saccharification are affected by many factors, including feedstock characteristics, pretreatment methods and hydrolysis conditions such as use of enzyme mixtures and type [9]. Enzymatic process to hydrolyze cellulosic materials could be accomplished through a complex reaction of enzymes. Therefore, worldwide research has been focused on isolation and exploration of new microorganisms for the extraction of cellulolytic enzymes with high specific activities and greater efficiency [10]. The majority of the enzymes used in the different industries are microbial in origin and owing specific characteristics like stability, high specific activity, and facilitated mass transfer [11]. Extreme environment such as acidic, thermophilic, alkaline, hyper saline and arid regions are important hot spots of microbial mega diversity [12,13]. These habitats have microorganisms with the genetic and phylogenetic adaptations to survive and grow under the extreme conditions [14]. High saline marine environment have proven to be a rich source of microorganisms harboring industrially important enzymes. Enzymes produced by marine microorganisms can provide numerous advantages over traditional enzymes due to their habitat on wide range of environment [15,16]. Previously, attempts have been made for isolation of cellulase producing marine bacteria [17–19] but quest for bacterial isolates producing enzymes with high efficacy and of commercial value is still going on to exploit enormous marine resources. With the aim to isolate efficient and high specific cellulase enzyme, present study was carried out. In this study a number of marine bacterial isolates were screened, cellulase enzyme was isolated, characterized and further application was also studied. The screened bacterium contained unique characteristic over existing bioresources to produce cellulase enzymes as it does not require environmentally hazardous pre-treatment of lignocellulosic biomass and it is the first report of its kind, so far from the marine environment.

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2. Methods

2.1. Screening of isolates for cellulases enzyme

Marine bacterial cultures, isolated from Indian western coast were screened for cellulase enzyme by plate zymogram assay. Zobell agar media was supplemented with 1% carboxymethylcellulose (CMC) for cellulase production and plates were incubated at room temperature for 48 h. The hydrolysis zones were observed by flooding the plates with an aqueous solution of 0.1% Congo red for 15 min followed by washing with 1 M NaCl [20]. Bacterial isolate exhibiting zone of clearance around colonies were selected and cultivated in the liquid medium containing (%, w/v) 0.1% $(NH_4)_2NO_3$, 0.1% KH_2PO_4, 0.1% K_2HPO_4, 0.005% $FeCl_3$, 0.02% $MgSO_4{\cdot}7H_2O$, 0.002% $CaCl_2$, 3.5% NaCl, 0.5% CMC, pH 7 for the production of cellulase enzyme.

2.2. Quantification of enzyme activities on different carbon sources

Selected marine isolate was grown in liquid medium containing CMC, wheat straw, rice bran, starch, avicell, sucrose and glucose as a carbon source (1%, w/v). Wheat straw and rice bran were used without pre-treatment with acid or base. The isolate was first grown in Zobell Marine Broth and after 12 h of growth (exponential phase), cells were pellet out from 5 ml medium, washed with minimal medium and re-inoculated in 500 ml minimal medium containing one of the carbon sources. The inoculated medium was incubated at 30 °C on shaker at 200 rpm for 10 days. The cellulase activities were measured everyday to check the optimum time period of incubation for each carbon source. The maximum enzyme activity obtained on CMC as a carbon source was considered as 100% and used to calculate the relative enzyme activity for the other carbon sources.

2.3. Determination of optimal salt concentration of growth medium for cellulase activity

In order to find out the effect of salt concentration in growth medium on cellulase activity, bacterial isolate was grown in 100 ml of cellulose containing minimal medium. The salt concentration was kept in the range of 0–7% (w/v). The cellulase assay was carried out [21] and the relative percentage of enzyme activity was calculated for different salt concentrations.

2.4. Fatty acid methyl ester (FAME) analysis for phylogenetic characterization of isolates

Pure bacterial culture was grown on Zobell agar medium for 24 h at 30 °C. About 40 mg wet mass of cells were harvested and placed into reaction tubes. A measure of 1 ml of methanolic sodium hydroxide solution (15%, w/v NaOH in 50%, v/v methanol) was added and the mixture was heated (100 °C) in water bath for 30 min to saponify the cells. The fatty acids were methylated in 2 ml methanolic hydrochloride acid solution (6 N HCl in 46%, v/v methanol) in a water bath at 80 °C for 10 min. The FAMEs were extracted from aqueous phase with 1.25 ml of methyl-terbutyl ether:hexane (1:1, v/v). The FAME extracts were analyzed on gas chromatography (Agilent 6890 GC, Hewlett Packard, Rolling Meadows, IL, USA) and FAME compounds were identified using the microbial identification software (Sherlock aerobe method RTSBA6) developed by MIDI Inc. (Newark, NJ, USA).

2.5. Molecular identification of isolates

Genomic DNA was extracted from bacterial culture using standard phenol chloroform extraction procedure. Amplification of 16S rRNA was carried out using universal primers [22] 27F (5′-GAGAGTTTGATCCTGGCTCAG-3′) and 1495R (5′-CTACGGCTACCTTGTTACGA-3′). The reaction mixture (50 μl) contained primer 0.5 μl each, dNTPs 400 μM, Taq polymerase 2.5 U, $MgCl_2$ 1.5 mM, DNA template 50 ng and 5 μl 10× PCR buffer. The PCR was carried out at initial denaturation 94 °C, 8 min; followed by 28 cycles of 94 °C, 1 min; 58 °C, 1 min, 72 °C, 2 min and final extension at 72 °C for 10 min. The PCR product was purified, analyzed and sequenced (M/s Microgen Inc., Seoul, South Korea). The similarity search was carried out *in-silico* using BLAST of NCBI. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5 [23]. Bootstrap analysis for 100 replicates was performed to estimate the confidence of tree topologies.

2.6. Measurement of cellulase activity and determination of protein concentration

Cellulase activity was measured by 3,5-dinitrosalicylic acid (DNSA) method, through determination of reducing sugars liberated [21]. Two types of enzyme activity were measured (i) CMCase and (ii) cellulose (filter paper cellulase). For the measurement of CMCase activity, the assay mixture contained 0.5 ml of diluted enzyme and 0.5 ml of 1% CMC in 10 mM phosphate buffer (pH 7.0) was used. Measurement of cellulase activity which contains Whatman No. 1 filter paper strip, 1.0 cm × 6.0 cm (= 50 mg) was carried by 0.5 ml of diluted enzyme and 1 ml of 10 mM phosphate buffer (pH 7.0). The enzyme–substrate mixture was incubated at 50 °C for 30 and 60 min, respectively for CMCase and filter paper cellulase assay. The reaction was stopped by addition of DNSA, boiled vigorously for 5 min and cooled in water for color stabilization. The optical absorbance was read at 540 nm against reagent blank using spectrophotometer (T80+ UV/VIS Spectrometer, PG Instruments Ltd., UK). One unit (U) of activity was defined as the amount of enzyme that released 1 μmol of glucose equivalents from the substrate per minute under the define assay condition. The specific activity was expressed in μmol/min/mg protein. Protein concentration was determined by Bradford method using bovine serum (BSA) as the standard.

2.7. Enzyme purification

The crude enzyme extract was purified using ion exchange chromatography followed by size exclusion chromatography. Crude enzyme extract was applied to a DEAE Sephadex A50 column, equilibrated with elution buffer (10 mM phosphate buffer, pH 7). Enzyme extract was eluted by applying a linear gradient of 0.1–0.5 M NaCl in equilibration buffer at a flow rate of 0.3 ml/min. The purified elution was collected as a fraction of 5 ml and each was analyzed for cellulase activity. The active fractions were pooled and concentrated using lyophilizer (VirTis, USA). The concentrated fraction was further processed in a Sephadex G-100 column. The fractions were eluted with 10 mM phosphate buffer (pH 7.0) at a flow rate of 0.5 ml/min and collected as a fraction of 5 ml thereafter each was assayed for cellulase activity.

2.8. Characterization of enzyme

The effect of pH on the cellulase and CMCase activity was studied using buffers of different pH; 10 mM of citrate buffer pH 3–5, sodium phosphate buffer pH 6–8 and NaOH–glycine buffer pH 9–10, in the assay system. Effect of temperature on enzymatic activity was determined by incubating assay mixture of the optimum