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Utilization of sea water based media for the production and characterization of cellulase by *Fusarium subglutinans* MTCC 11891

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ABSTRACT

The present study focuses on isolation, screening and identification of fungal strain capable of producing cellulase enzyme in sea water based media from paddy fields of Rourkela, Odisha, India. The filamentous fungi isolated were identified as *Fusarium subglutinans* MTCC 11891. Cellulase enzyme was characterized for its optimal pH and temperature and also studied for the effect of metal ions on enzyme activity. Comparative studies were carried out using both fresh and sea water based media in order to investigate the salt tolerance level of cellulase produced by the fungal strain. The fungal strain was found to be halotolerant with optimal pH of 5.0 with cellulase activity of 292.53 U/mL and 184 U/mL in fresh and sea water based Mandel's media, respectively. The optimal temperature for *F. subglutinans* MTCC 11891 cellulase was recorded at 80 °C with 347.43 U/mL for fresh water and 232 U/mL for sea water based Mandel's media. Manifold increase in cellulase activity was evidenced in the presence of 5 mM Mn^{2+} and Fe^{2+} concentration in both fresh and sea water based Mandel's media suggesting these two cations as key catalytic molecules. Partial purification of the cellulase produced in fresh water based Mandel's media was performed using diethylaminoethanol (DEAE)-Sepharose column and the fraction with enzyme activity of 298.68 U/mL was recorded as fraction with highest cellulase concentration. Halotolerant cellulases would be more useful in future for the development of sea water based systems to produce bioethanol.

1. Introduction

Lignocelluloses are the most abundant biomass available on earth with immense potential to meet global energy demands in sustainable manner (Payne et al., 2015). Few key factors are involved in order to accomplish the same and cellulases play a pivotal role in this task. Cellulases are critical enzymes in biofuel and food industries. There has been significantly higher number of research papers published to reveal the production of cellulases by unexplored microorganisms isolated from different sources including both bacterial and fungal species. Several bacterial and fungal species have been reported to be the cellulase producer using different cellulose sources. Utilization of fungal species has some advantages over bacterial species as cellulase producers. Fungi being major organisms responsible for biomass degradation in nature plays cardinal role in recycling of carbon. Filamentous fungi characterized as soft rots and white rots are well known for their enzymatic degradation of biomass. Various filamentous species of fungi are capable of producing large volume of cellulase applying free cellulase paradigm (Himmel et al., 2007; Zhang et al., 2006) whereas in some anaerobically growing fungal and bacterial strains cellulases are secreted applying cellulosomal paradigm, making free enzyme emerge as the foundation of industrial biofuel research and development (Himmel et al., 2007; Lynd et al., 2002). Isolation and characterization of soft-rot actinomycetes *Trichoderma resei* in south pacific and Natick Research Laboratories beckoned the interest in utilization of filamentous fungi in biomass conversion process (Reese, 1976). In addition, fungal species produce higher quantity of enzymes than bacterial species due to the large accumulation of mycelium. Fungal mycelium is easy to separate from the fermentation medium than bacterial cells, which reduces the cost of separation process. Stability of enzymes of microbial origin over a wide range of temperature and pH make them a preferable choice over their plant or animal counterparts. Especially in case of fungi it is easier as they grow over a variety of substrate and production of enzyme in large titer is less expensive in biotechnological industries (Dashtban et al., 2009). Likewise

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there are many reports published which studied the utilization of cellulases for saccharification of biodegradable resources to produce fuels. However, all these works have been carried out using fresh water as a source of medium. Recent public threats about the fresh water depletion signify the exploration of non-freshwater medium for the production of fuels. Among the non-freshwater sources, sea water is the best source to be studied as medium for biomass conversion due to its abundant availability in India. Utilization of halotolerant microorganisms capable of producing salt tolerant enzymes will be a major breakthrough in this field as they can tolerate high salt levels and ionic liquids better than current fungal cellulases. Further, there will be advancement in use of sea and brackish water for biomass conversion. In the present study, a cellulase was partially purified from filamentous fungi *Fusarium subglutinans* MTCC 11891, isolated from paddy fields and its enzymatic properties were characterized using alkali treated rice straw as cellulose source. Rice straw being rich source of carbohydrate including cellulose (40%), hemicellulose (26%), and lower lignin (9%) is preferred as substrate for cellulase action (Rahnama et al., 2013). Comparative studies were carried out using Mandel's media prepared with fresh and sea water in order to investigate the salt tolerance level of cellulase produced by the fungal strain.

2. Methods

2.1. Isolation of fungal strains

Six soil samples from randomly chosen locations were collected from the agricultural fields of Rourkela city (22.24 °N, 84.88 °E), Sundargarh District, Odisha, India from a depth of 8–10 cm in the sterile polyethylene bags in the month of September 2013 and brought to the lab for further studies. Equal amount (W) of six different samples was mixed homogeneously, 20 g of it was diluted in 100 mL of sterile distilled water and soil was allowed to settle down. The supernatant collected was subjected to serial dilution using pre-sterilized sea water and plated on Potato Dextrose Agar (PDA) [Himedia, Mumbai, India] of pH 5.66 ± 0.2. The plates were incubated for 5 days at room temperature (25 ± 2 °C).

2.2. Screening and identification of cellulase producing fungi

The isolated sporulating fungal cultures were screened for the potential to produce cellulases using congo red staining method. The fungal cultures were grown in a petri plate using PDA with 1% carboxymethylcellulose (CMC) (Himedia, Mumbai, India) for seven days at 37 °C. After incubation, the plates were flooded with congo red solution (1 mg/mL) and left for fifteen minutes, followed by destaining with 1 M NaCl was for 15 min. CMC degradation around the colonies appeared as a yellow opaque zone against a red color for undergraded CMC. The most potent fungal strain capable of producing cellulase at sea water was identified at the Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India.

2.3. Collection of sea water and determination of its composition

Sea water was collected from Gopalpur, Odisha (19.27°N, 84.92°E) on the Bay of Bengal coast during the month of July 2013 using 50 mL ficol tube (Tarsons, India) and stored at 4 °C. The stored water was filtered using vacuum filtration unit (Millipore, India) fitted with 0.45 μm membrane filter (HAWP04700, Millipore, India) and used for cellulase production. Atomic absorption spectroscopy (AAS) (AANALYST200, PerkinElmer) was used to determine concentration of metal ions in sea water. The standard solution for required metal ions was prepared in three different concentrations (1 ppm, 2 ppm and 3 ppm). The sea water was diluted in 5× concentration and passed through the sample holder. The atomization of flame takes place through oxygen-nitrous oxide gas and according to flame the concentration of metal ions is estimated.

2.4. Screening of fungal culture for its growth in Mandel's media with sea water

F. subglutinans MTCC 11891 was inoculated in Mandel's media prepared with sea water and added with CMC (2%) as sole carbon source and analyzed for its growth. After incubation of 5 days the broth checked for growth of the fungal strain and sampling was done after 10 days to assay the production of cellulase.

2.5. Pretreatment of rice straw (biomass)

Rice straw was considered as a biomass feedstock and collected from the agricultural fields located on the outskirts of Rourkela City (22.2492°N, 84.8828°E), Sundergarh district of Odisha, India. The biomass feedstock weighed 20 g and washed thoroughly with distilled water and dried overnight at 70 °C in a hot air oven until the moisture was less than 10%. Dried rice straw was milled to reduce the size as 1–2 mm, prior to pretreatment aimed to remove the lignin, hemicellulose and xylan. Rice straw was heated along with alkali to increase the biodegradability. The dried biomass was treated with 1% NaOH and subjected to autoclave at 121 °C, 15 psi for 20 min followed by cooling down to the room temperature. The samples were washed several times in running tap water to neutralize the pH and dried at 65 °C (Zhu et al., 2005). Structural changes in the sample due to alkali pretreatment were observed under FESEM (Field Emission Scanning Electron Microscope) (Nova Nanosem 450). The pretreated feedstock was either used immediately for hydrolysis experiments or stored in airtight containers at 4 °C for further use.

2.6. Production of cellulase

Enzyme production assay was performed using pretreated rice straw as substrates in Mandel's Media (Table 1) prepared with fresh water and sea water. For cellulase production, 1 g (wet weight) of freshly grown *F. subglutinans* MTCC 11891 mycelium was added to 100 mL of Mandel's media with 2% pretreated rice straw prepared in both fresh and sea water. The preparations were incubated for 21 days at pH of 5.8, 37 °C in an orbital shaker set at 100 rpm. Then, the broth was centrifuged at 7000 rpm at 4 °C and the supernatant was collected as crude enzyme extract, following which cellulase activity was assayed. Determination of enzyme activity was measured using methods suggested by International Union of Pure and Applied Chemistry (IUPAC) (Ghose, 1987).

Table 1.
Composition of Mandel's media.[a]

Components	Quantity (g/L)
KH_2PO_4	2.0
$CaCl_2 \cdot H_2O$	0.4
$MgSO_4 \cdot 7H_2O$	0.3
$FeSO_4 \cdot 7H_2O$	5.0
$MnSO_4 \cdot 4H_2O$	1.6
$CoCl_2 \cdot 6H_2O$	1.4

[a] Mandel's media was prepared either with distilled water or sea water.