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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.

2.7. Characterization of cellulase activity

Estimation of reducing sugar was performed using DNS (3, 5-dinitrosalicylic acid) reagent (DNS-10 g, sodium sulfite-0.5 g, and sodium hydroxide-10 g in 1 L of distilled water) and 40% potassium sodium tartrate (Miller, 1958). To 1 mL of substrate (2% CMC in 10 mM citrate buffer), 1 mL of crude cellulase extract was added and incubated at 50 °C for 15 min. After 15 min of incubation, 3 mL of DNS reagent was added to the reaction mixture and incubated at 100 °C in boiling water bath for 5 min. The reaction mixture was then cooled down to room temperature and 1 mL of 40% Rochelle's salt solution was added to terminate the reaction. The amount of reducing sugar i.e. glucose produced was estimated spectrophotometrically by recording optical density of complex formed at 560 nm (UV/VIS Spectrophotometer, Lambda 35, PerkinElmer).

2.7.1. Effect of pH on cellulase activity

Crude enzyme extract (1 mL) along with substrate (2% CMC) was taken in phosphate buffer (10 mM $Na_2HPO_4 \cdot 2H_2O$, 1.8 mM KH_2PO_4) with different pH values (4.0, 5.0, 6.0, 7.0, 8.0 and 9.0) are incubated at 50 °C for 15 min. The test samples were then assayed for reducing sugar content as mentioned in 2.7 and enzyme activity was calculated (Miller, 1958).

2.7.2. Effect of temperature on cellulase activity

Crude enzyme extract (1 mL) along with the substrate (2% CMC in 10 mM citrate buffer) was incubated for 15 min at different temperatures (37, 50, 60, and 80 °C) to study their effect on cellulase activity. After 15 min of incubation, the test samples were assayed for the content of reducing sugar as mentioned in 2.7 and enzyme activity was calculated (Miller, 1958).

2.7.3. Effect of metals on cellulase activity

After getting optimum temperature and pH value, different metal ions ($MgCl_2$, $ZnCl_2$, $MnCl_2$, $FeCl_2$ and $CuSO_4$) in 5 mM concentration were subjected to study their effect on enzyme activity. Crude enzyme extract (1 mL) along with substrate (2% CMC in 10 mM citrate buffer) in the presence of 5 mM concentration of different metal ions ($MgCl_2$, $ZnCl_2$, $MnCl_2$, $FeCl_2$ and $CuSO_4$) individually was incubated at optimum temperature and pH found in the 2.7.1 and 2.7.2 for 15 min. After 15 min of incubation, the test samples were analyzed for the concentration of reducing sugar as mentioned in 2.7 and enzyme activity was calculated (Miller, 1958).

2.8. Partial Purification of cellulase

The crude enzyme extract produced in Madel's media with fresh water was used for partial purification of cellulase using anion exchange chromatography. The crude enzyme extract was subjected to ammonium sulfate precipitation at (0–80%) saturation. The precipitate was subjected to centrifugation at 10,000 g and the pellet was dissolved in tris-HCl buffer (pH 8.0) with 5 mM ethylenediaminetetraacetic acid (EDTA). Resulting sample was dialyzed using dialysis membrane with 14 kDa cut-off value (Sigma Aldrich, USA) against the same buffer overnight. The dialyzed sample was applied to DEAE-Sepharose (Sigma Aldrich, USA) column pre-equilibrated with (5 mM EDTA, 20 mM tris base (Himedia, Mumbai, India), pH 8.0). Column was run with same buffer; elution was done using linear gradient of 0.2–1.2 M NaCl. Fractions were collected at the rate of 10 mL/h. Fractions were analyzed for their cellulase activity as mentioned in 2.7 and those with maximum activity were pooled.

2.9. Statistical analysis

All the treatments and enzyme assays were performed in triplicate and the data was presented as mean ± S.D. (standard deviation). Mean and S.D. values were calculated using Microsoft Excel 10 software. S.D. values were shown as Y error bars.

3. Results and discussion

3.1. Isolation, screening and characterization of fungi

Three fungal strains were isolated from the soil samples plated on saline PDA plates. Out of the three fungal isolates one isolate was confirmed for cellulolytic ability on CMC agar plates on incubation of 48 h at 37 °C by production of a clear halo zone which was identified using the congo red assay. The isolated fungal culture was identified by performing traditional method of classification and identification of organisms based on morphological, physiological, biochemical, and nutritional characteristics performed at the Microbial Type Culture Collection Center and Gene Bank (MTCC & GB), Institute of Microbial Technology (IMTECH), Chandigarh, India. The potent fungal isolate was identified as *Fusarium subglutinans* and was deposited in the MTCC & GB of IMTECH, India with MTCC No. 11891.

3.2. Screening of fungal cultures for its growth in sea water

An attempt was made to explore the possibilities of cultivating *Fusarium subglutinans* on sea water based Mandel's media. Significant growth of the inoculated strain along with increased glucose concentrations were observed in CMC broth. The subject showed faster growth by depleting the cellulose and converting it to glucose and enzyme activity was recorded as 130 U/mL, substantial spreading of the mycelium was also observed with this fungal strain. This particular characteristic reveals that the strain could have acclimatized to the saline conditions and it might have aided in sustaining the salinity environment. This further reaffirmed that *F. subglutinans* MTCC 11891 could be the promising candidate for cellulase productions even with sea water.

3.3. Composition of sea water

Sea water was analyzed for its ionic content using AAS. The water sample collected was having a higher concentration of sodium (5.496 ppm), magnesium (3.837 ppm), calcium (3.785 ppm), manganese (1.25 ppm), copper (1.248 ppm), which is more than 1 ppm and thereby considered as major elements in sea water. Other minor elements detected include potassium (0.02 ppm), cobalt (0.012 ppm) and iron (0.0056 ppm), with concentration below 1 ppm (Fig. 1).

3.4. Effect of alkali pretreatment on rice straw

Pretreatment for rice straw using dilute alkali hydrolysis is an effective method of pretreatment of biomass. The optimum concentration of NaOH used for pretreatment was 1 M at 121 °C for 15 min. Alkali pretreatment aids in depletion of moisture and lignin contents and thereby expanding the amount of cellulose and hemicellulose in the treated biomass (Zhu et al., 2005). Solubilization of lignin in NaOH is due to fracture of ester bond between hydroxycinnamic acids, *p*-coumaric and ferulic acids and α-benzyl ether linkages between hemicelluloses and lignin (Lam et al., 2001; Xiao et al., 2001). The rate of increase in depletion of ester bond is directly proportional to factors like temperature, concentration of base and incubation time. Digestion of lignin

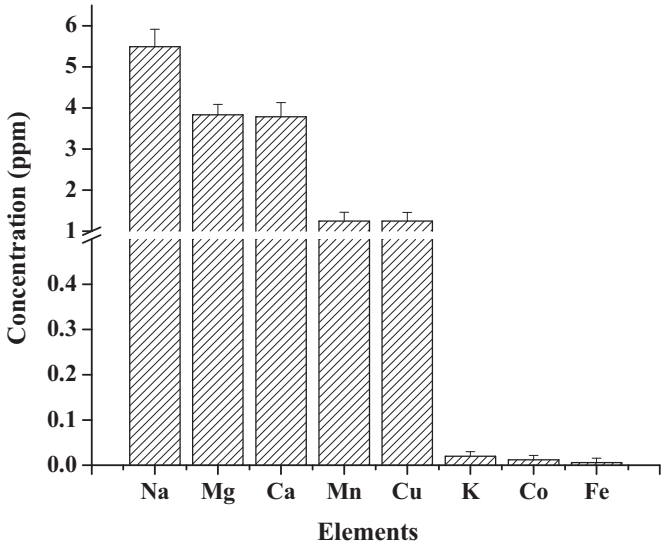

Fig. 1. Composition of sea water analyzed by AAS.

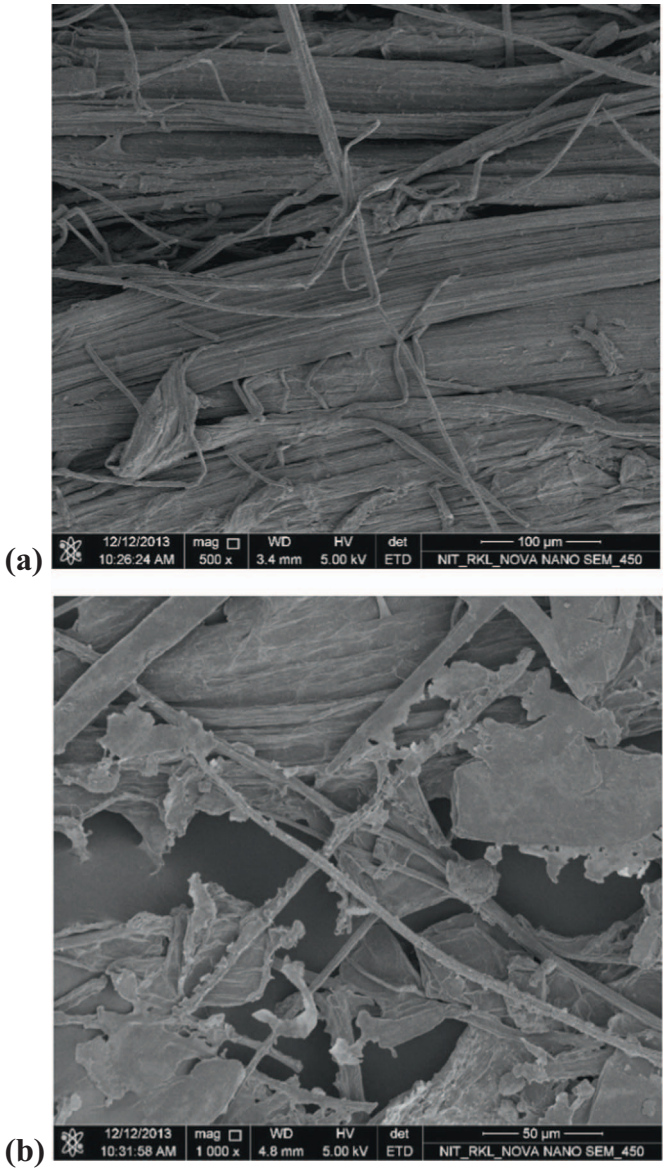

Fig. 2. FE-SEM images of rice straw (a) before pretreatment, (b) after pretreatment of rice straw.

results in increase in pore size of rice straw which facilitate moisture loss and increasing accessibility to cellulases for further hydrolysis (Zhu et al., 2005). Fig. 2 shows the FESEM images of rice straw before and after pretreatment with NaOH.

3.5. Cellulase production using pretreated rice straw in Mandel's media and sea water

Enzyme production was carried out using pretreated rice straw in Mendel's media prepared with fresh water and in sea water simultaneously to assess the ability of the fungus to grow and produce cellulase in sea water. The growth initiated at the end of the 3rd day and process extended up to 21 days, though further studies were halted due to decrease in the volume of media. Hydrolysis of cellulose to glucose was assayed at regular intervals to check the production of cellulase. After 21 days cellulase activity was recorded as 277.5 U/mL and 126.72 U/mL in basal media prepared with fresh water and sea water, respectively (Fig. 3). The activity of cellulase in sea water was certainly less as compared to that of Mandel's media prepared with sea water but it was adequately more. The production of enzyme could be increased by giving alternate stress, nutrient deprivation stress, etc. The fast depletion of cellulose was due to the pretreatment because it decreased the crystallinity and increased the porosity in rice straw cellulose fiber. A steady increase in cellulase activity was significant due to the improved cellulase production. At the end of fermentation (21 days), enzyme production was observed to be increased. Diminished production volume aids the availability of an increased surface area, a factor which is difficult to achieve using conical flasks.

3.6. Parameters influencing cellulolytic potential

3.6.1. Effect of pH on cellulase activity

Effect of pH on cellulolytic activity of *F. subglutinans* was studied over a wide range of pH ranging from 4.0 to 9.0. Cellulolytic activity of enzyme produced in media prepared with sea water was almost half as compared to that of the enzyme produced in freshwater but in both cases the enzyme follows the similar trend over the pH range. The maximum activity was observed at pH 5.0 as 292.53 U/mL and 184 U/mL for basal media prepared with fresh water and sea water produced cellulase, respectively (Fig. 4). With the increasing pH the freshwater cellulase activity decreases indicating the active sites are not compatible with the pH change. It is noteworthy that the activity of sea water cellulase was stable over an expansive range of pH 5.0–9.0 with more than 85% activity, which makes the enzyme suitable for industrial usage where maintenance of pH plays a crucial role.

3.6.2. Effect of temperature on cellulase activity

The effect of temperature on cellulolytic activity of *F. subglutinans* was analyzed over a range from 37 °C to 80 °C. The optimum temperatures for cellulase produced using fresh water and sea water incorporated Mandel's media between 75 and 80 °C. It was revealed that there was a gradual increase in the activity of crude enzyme from 260 U/mL for basal media prepared with fresh water and 143.28 U/mL for sea water at 37 °C to 347.43 U/mL for basal media prepared with fresh water and 232 U/mL for sea water at 80 °C (Fig. 5). Increase in temperature would increase the kinetic energy of the enzyme system so that the number of collisions per unit volume increases and thereby increasing the binding of substrate to the active site of enzyme. Therefore, it is highly possible that enzyme is thermostable and is stable as well as active at

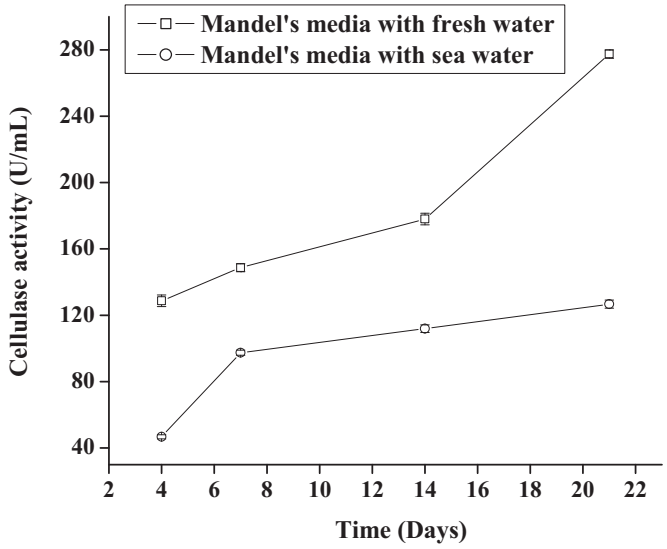

Fig. 3. Cellulase production by *Fusarium subglutinans* MTCC 11891 in shake flask using Mandel's media prepared with fresh water and sea water.

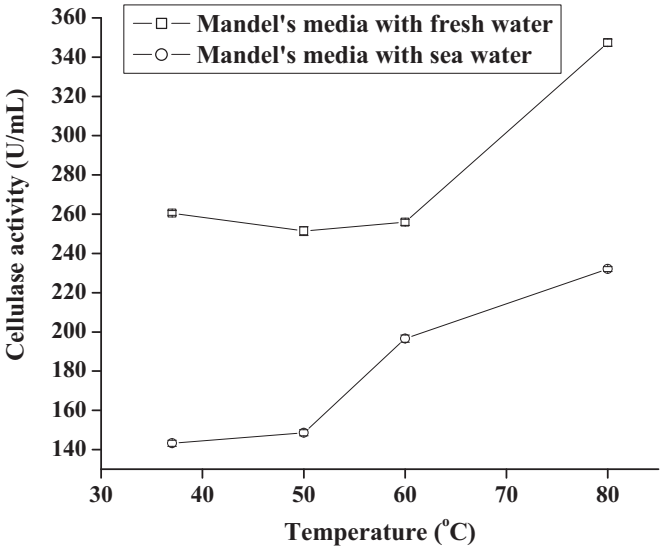

Fig. 5. Effect of temperature on cellulase produced in fresh water and sea water based Mandel's media.

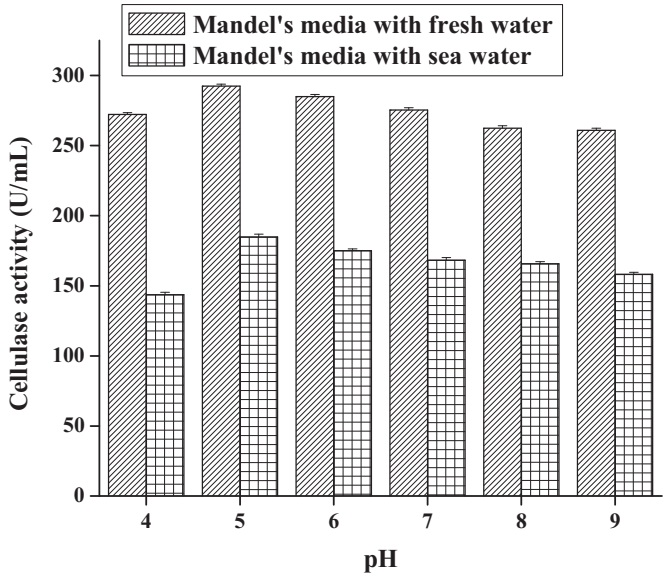

Fig. 4. Effect of pH on cellulase produced in fresh water and sea water based Mandel's media.

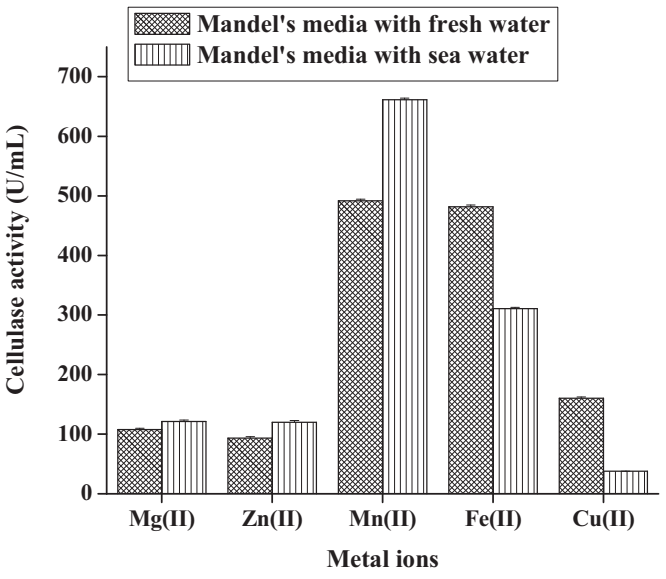

Fig. 6. Effect of metal ions on cellulase produced in fresh water and sea water based Mandel's media.

temperatures as high as 80 °C. The above results indicated that the enzyme extract can be directly utilized in industries where high temperature plays a vital role.

3.6.3. Effect of metal ions on cellulase activity

Effect of metal ions was analyzed using five metal ions which were regularly used as catalyst in many chemical reactions. Mn^{2+} and Fe^{2+} showed increased enzyme activity in both enzymes. Two fold increase in cellulase activity in basal media prepared with fresh water and four folds in sea water media was evidenced in presence of Fe^{2+}. In presence of Mn^{2+} threefold increase in activity of basal media produced cellulase and four fold increase in sea water media produced cellulase was recorded (Fig. 6). Mg^{2+}, Zn^{2+} and Cu^{2+} significantly lowered the cellulase activity than the normal. From the above study it can be clearly explained that Mn^{2+} and Fe^{2+} are activator molecules and their presence enhances the enzyme activity by manifolds whereas Mg^{2+}, Zn^{2+} and Cu^{2+} can be termed as inhibitors of enzyme activity. Previous studies on *Fusarium oxysporum* reported Mn^{2+} to be a activator molecule (Dar et al., 2013).

3.7. Partial purification cellulase enzyme

Partial purification of cellulase produced in fresh water based Mandel's media was performed using anion exchange chromatography. After elution using various linear gradient of NaCl (0.2–1.2 M), the fractions were assayed for cellulase activity and from the assay it was confirmed that cellulases was eluted at 0.8 and 1 M fractions of NaCl (Fig. 7). Further work has to be carried out to complete characterization of cellulase produced by *F. subglutinans* MTCC 11891.

4. Conclusion

Rice straw is an abundant lignocellulosic biomass, which can be used as renewable energy source. Alkaline pretreatment of rice straw improves the utilization of sugars by increasing access of

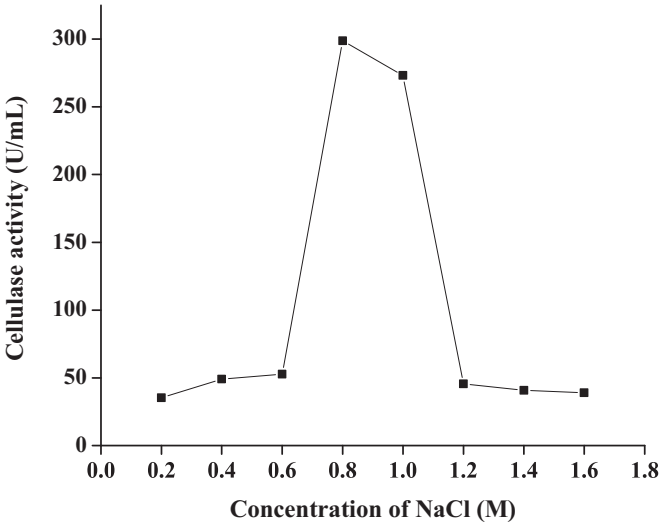

Fig. 7. Fractions of cellulase collected at different concentration of NaCl.

hydrolytic enzymes to the complex polysaccharides. *Fusarium subglutinans* MTCC 11891 was found to be eminent filamentous fungi for the production of cellulase. The cellulase showed activity at a wide range of pH and it is also capable of sustaining a high temperature of 80 °C and hence can be categorized as thermostable enzyme. Cellulase from *F. subglutinans* MTCC 11891 was found to be halotolerant in nature i.e. the enzyme produced in sea water based system was active over a range of pH from 5.0 to 9.0 and stable at a higher temperature. This is the first time when a halotolerant as well as thermotolerant enzyme is reported in *F. subglutinans*. Our study demonstrates that the isolated fungus *F. subglutinans* MTCC 11891 is capable of producing halotolerant and thermostable cellulase which can be used in pretreatment of lignocellulosic biomass in biofuel industries, in sea water based systems which can a major approach towards conservation of freshwater resources. Metal ions, particularly Mn^{2+} and Fe^{2+} can play major catalytic roles in enhancing the enzyme activity by many folds. Further studies on structure and feature of active site of enzyme and its catalytic potential will be carried over in order to demonstrate the full potential of enzyme in industrial scale.

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