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ORIGINAL ARTICLE

Saccharification of *Parthenium hysterophorus* biomass using cellulase from *Streptomyces* sp. NAA2

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Abstract

Parthenium hysterophorus biomass can be used as a non-conventional renewable feedstock for the production of bioethanol. Therefore, the present work was designed to hydrolyze *P. hysterophorus* biomass using cellulase enzyme produced from an actinomycete, i.e., *Streptomyces* sp. NAA2 using *P. hysterophorus* biomass as a substrate. The isolate NAA2 was identified by molecular characterization of 16SrDNA. The enzyme production by strain NAA2 was enhanced by optimization studies conducted under submerged fermentation conditions using *P. hysterophorus* as a substrate. The crude enzyme produced under optimized conditions was used to hydrolyze alkali-acid pretreated *P. hysterophorus* biomass. The highest CMCase production was achieved in 4–5 days when steam-pretreated *P. hysterophorus* biomass was used at 1% (*w*/*v*) concentration, using 2 discs (1 disc = 5×10^7 spores/ml) of inoculum, an initial pH 6.5, temperature at 40 °C, an agitation speed of 120–150 rpm, and by supplementing fermentation medium with 1.5% (*w*/*v*) carboxymethyl cellulose (CMC) as additional carbon source. Under optimized conditions, the actinomycete strain NAA2 showed production of 0.967 ± 0.016 U/ml CMCase, 0.116 ± 0.08 FPU/ml FPase, and 0.22 ± 0.012 U/ml β-glucosidase enzymes. On utilizing the cellulase enzyme for biomass hydrolysis, maximum 18.2% saccharification yield (of cellulose 0.202 g/g) was achieved in 96 h when enzyme and substrate levels were 30 FPU/100 ml and 2% (*w*/*v*) respectively. *Parthenium hysterophorus* biomass can be hydrolyzed enzymatically yielding considerable amounts of total reducing sugars. It can, therefore, be used as a feedstock for the production of bioethanol. Also, it has the potential to act as a substrate for the production of cellulases. Furthermore, the improved cellulolytic potential of *Streptomyces* sp. NAA2 can be exploited in various industrial applications.

Keywords Actinomycete · Biomass hydrolysis · Cellulase · *Parthenium hysterophorus* · *Streptomyces*

Introduction

In the past few decades, there has been a growing interest in renewable fuels such as biofuels to meet the energy demands of the rising population in the world (Escobar et al. 2009). The bioethanol produced from lignocellulosic biomass is considered as a sustainable as well as an attractive transport fuel due to its compatibility with the existing engines (Sukumaran et al. 2009). Recent studies are focused on various aspects of the process of bioethanol production from lignocellulose to make its commercialization feasible in the near future. One of the essential factors to be considered for this is the availability and exploitability of diverse lignocellulosic biomass (Sukumaran et al. 2009). Nowadays, various scientific communities, therefore, have started to explore the potential of non-conventional feedstock for its bioconversion to ethanol.

Parthenium hysterophorus, one of the world's most noxious weeds, is found in various countries of Asia, Africa, America, and Australia (Saini et al. 2014). The weed is known for its high adaptability over a wide range of environmental conditions and has been successful in naturalizing various habitats owing to its high reproduction potential and allelopathic effect on many other plant species (Gnanavel 2013). The various physical, chemical, biological, and integrated management strategies employed for its control until now have been unsuccessful in many parts of the world. However, the weed can be used in various other applications. It is known to have various medicinal properties such as cytotoxic, anti-cancer, anti-oxidant, and antimicrobial activities and various other pharmacological effects (Saini et al. 2014).

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Also, the weed biomass can be utilized as a substrate for the production of lignocellulolytic enzymes and as a feedstock for bioethanol production.

The cellulosic ethanol production process involves several crucial steps such as biomass pretreatment for its cell wall deconstruction, hydrolysis of sugar polymers (cellulose and/or hemicellulose) to release fermentable sugars, and finally fermentation of sugars to ethanol (Sarkar et al. 2012). The hydrolysis of cellulose (or saccharification) involves the use of cellulases, a complex of endoglucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.74), and β-glucosidases (EC 3.2.1.21) enzymes (Padilha et al. 2015). The complete cellulose hydrolysis involves synergistic action of all these enzymes, which cleave β-1,4-glycosidic linkages between glucose units in cellulose polymer at different locations. The cellulases are produced by a wide array of microorganisms, including both fungi and bacteria (Sadhu and Maiti 2013; Imran et al. 2016). The Actinobacteria are a group of microorganisms largely known for their secondary metabolite production potential and are also recognized among major lignocellulose decomposers in nature (Bettache et al. 2014).

Keeping the above factors in mind, the present study was designed to enhance cellulase production from *Streptomyces* sp. NAA2 using *P. hysterophorus* biomass as a substrate under submerged fermentation (SmF) conditions and then utilizing the enzyme produced after optimization studies in saccharification of *P. hysterophorus* biomass.

Materials and methods

Microbial isolate

The actinomycete isolate *Streptomyces* sp. NAA2 was originally isolated from waste land soil (Saini et al. 2016). Starch casein agar medium (SCA) containing (*w*/*v*) 1% starch, 0.03% casein, 0.2% KNO_3, 0.2% NaCl, 0.2% K_2HPO_4, 0.005% $MgSO_4{\cdot}7H_2O$; 0.002% $CaCO_3$, 0.001% $FeSO_4{\cdot}7H_2O$, and 2% agar (pH 7.0), was used for maintaining and preserving isolate at 30 °C and 4 °C respectively.

Molecular identification of the isolate NAA2

The isolate was characterized genetically using commercial service provided by Eurofins Genomics India Pvt. Ltd., Bangalore, India. The DNA was extracted using the standard protocol. From the isolated DNA, the fragment of the 16S rDNA gene was amplified by PCR using Act283F (5′-GGGTAGCCGGCCUGAGAGGG-3′) and Act1360R (5′-CTGATCTGCGATTACTAGCGACTCC-3′) primers. Thereafter, the PCR amplicons were purified to remove contaminants, and sequencing was done using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. The 16S rDNA sequence was then compared to available data in EzTaxon e-server (Kim et al. 2012) to determine close relatives of the isolate NAA2. The multiple sequence alignments were performed using CLUSTALX version 2.1 (Thompson et al. 1997). DAMBE (data analysis in molecular biology and evolution) software (Xia and Xie 2001) was used for manual editing of alignment to get unambiguous data. Neighbor-joining end method was used to construct the phylogenetic dendrogram using MEGA 7.0 (Molecular Evolutionary Genetics Analysis, version 7.0) software (Kumar et al. 2016). Bootstrap analysis was done with 1000 replicas. The 853 bp long sequence was deposited in the GenBank (National Centre for Biotechnology Information, NCBI) with accession number MF632248.

Biomass processing and pretreatment

The *Parthenium hysterophorus* biomass, collected from local areas, was first washed thoroughly to remove dirt and then chopped into small pieces. Thereafter, it was dried completely (overnight) at 50–60 °C followed by grinding to powder with a particle size < 1 mm.

For steam pretreatment, powdered biomass was soaked in distilled water and then autoclaved at 121 °C (15 pounds per square inch or psi pressure) for a retention time of 30 min. In alkali-acid (combined) pretreatment, biomass was subjected to alkali pretreatment (using 1.5% *w*/*v* NaOH, at 121 °C for 30 min) followed by acid pretreatment (using 0.5% *v*/*v* H_2SO_4, at 121 °C for 30 min). During both pretreatments, bath ratio was kept as 1:10, and the pressure of the autoclave was released swiftly after completion of retention time in the autoclave. After each pretreatment, the biomass was dried completely (overnight) at 60 °C.

Optimization of enzyme production

The inoculum was developed by cultivating NAA2 isolate on starch casein agar medium at 30–35 °C for 4–5 days till sporulation was completed. The cellulase production was carried out under SmF conditions using *P. hysterophorus* biomass as a substrate. For fermentation, Mandel's medium (pH 7.0) (Mandels and Reese 1957) was used with little modifications. The medium contained (g/l) urea (0.3), ammonium sulfate (1.4), KH_2PO_4 (2.0), $CaCl_2{\cdot}H_2O$ (0.3), $MgSO_4{\cdot}7H_2O$ (0.3), and trace element solution. The trace element solution contained (mg/l) $FeSO_4{\cdot}7H_2O$ (5), $MnSO_4{\cdot}H_2O$ (16), $ZnCl_2{\cdot}7H_2O$ (14), and $CoCl_2{\cdot}6H_2O$ (2.0). For enzyme production, 25 ml of production medium was taken in a 250-ml Erlenmeyer flask and inoculated with 1 disc of inoculum (diameter 8 mm = 5×10^7 spores/ ml). The fermentation was done at 35 °C temperature for 4 days under shaking conditions at 150 rpm.

The enzyme production by *Streptomyces* sp. NAA2 was enhanced by optimization of fermentation conditions using "*one variable at a time*" approach. First, the effect of biomass pretreatment was studied by supplementing fermentation medium with 1% (*w*/*v*) of the *P. hysterophorus* biomass subjected to different pretreatments. Next, the effect of substrate concentration was examined by varying its concentration from 0.5% to 2.5% (*w*/*v*) in the fermentation medium. The influence of inoculum size was evaluated by inoculating production medium with different amounts of the inoculum, i.e., 1 to 5 discs. To know the effect of pH, initial pH of the medium was varied over a range of 5.5 to 9.0. The effect of temperature was observed from 25 °C to 45 °C (varied at an interval of 5 °C). The effect of incubation period was investigated from 2 to 8 days. Furthermore, supplementation of medium (containing biomass substrate) with additional carbon sources may enhance the enzyme production by the microbes. Therefore, the effect of additional carbon source was observed by adding 1% (*w*/*v*) of different carbon sources, viz., glucose, fructose, galactose, cellobiose, lactose, sucrose, CMC, starch, and mannitol, in the fermentation medium. The effect of carbon source concentration was also studied by varying its level in the medium from 0.25% to 2.5% (*w*/*v*). Also, the effect of agitation speed was studied by varying the shaking speed from 100 to 180 rpm.

Biomass saccharification

The *P. hysterophorus* biomass pretreated by alkali-acid pretreatment was used for saccharification by crude enzyme obtained from *Streptomyces* sp. NAA2. The crude enzyme was first concentrated by acetone precipitation method (Choudhary et al. 2014). For the hydrolysis, the biomass was mixed with 100 mM citrate buffer (pH 4.8) and crude enzyme and was further added with 0.03% sodium azide to avoid microbial contamination. The incubation was done at 50 °C at 120 rpm. The effect of substrate concentration was studied by varying the amount of pretreated biomass from 1.0% to 7.0% (*w*/*v*). To study the effect of enzyme loadings, the amount of the enzyme was varied in reaction volume (100 ml) in FPU and total volume was made with citrate buffer. The progress of hydrolysis was monitored for 4 days (96 h) at an interval of 24 h. After completion of the reaction, the hydrolysate was subjected to centrifugation at 10,000 rpm for 15–20 min and the supernatant was assayed for total reducing sugars (TRS) released in hydrolysis process (Miller 1959). The saccharification yield was calculated using the formula:

$$\text{Saccharication yield}(\%) = \frac{\text{Reducing sugars released}}{\text{Cellulose content in biomass}} \times 0.9 \times 100$$

where 0.9 is the conversion coefficient from glucan to reducing sugars.

Enzyme assays

The extracellular enzyme was extracted by separating liquid medium from solid biomass by filtration through muslin cloth followed by centrifugation at 4 °C at 10,000 rpm for 20 min. The supernatant was filtered through Whatman filter paper no. 1 to obtain crude enzyme extract.

The carboxymethyl cellulase (CMCase or endoglucanase activity) and FPase (Filter paper activity) were assayed using the method by Ghose (1987). For CMCase assay, 0.5 ml of the appropriately diluted enzyme was mixed with 0.5 ml of 1% (*w*/*v*) carboxymethyl cellulose (prepared in 100 mM sodium acetate buffer, pH 5.0). The reaction mixture for FPase assay consisted of 0.5 ml of crude enzyme mixed with 0.5 ml of the acetate buffer containing 50 mg (1 × 6 cm) of Whatman filter paper no. 1. The incubation was done at 50 °C in a thermostat water bath for 10 min and 30 min for CMCase and FPase assays respectively. The glucose (reducing sugar) liberated in the reaction was measured by DNS (dinitrosalicylic acid) method (Miller 1959). β-Glucosidase assay was carried out according to the method by Ng et al. (2010) with some modifications. The reaction mixture (1 ml) was prepared by mixing 200 μl of the appropriately diluted enzyme with 800 μl of substrate solution (2 mM pNPG or 4-nitrophenyl-β-D-glucopyranoside), and the incubation was done at 50 °C for 30 min in dark. The reaction was terminated by adding 1 ml of 1 M Na_2CO_3 solution. The color developed during the assay, as a result of the liberation of pNP, was observed at 405 nm and quantified using the pNP as a standard.

One unit of enzyme activity is defined as the amount of enzyme required for producing 1 μmol of glucose per ml per minute under assay conditions. The enzyme activity was expressed as units per ml (U/ml) for CMCase and β-glucosidase, and as FPU/ml for filter paper activity.

Determination of cellulose content

The cellulose content was determined using the method by Ahmed et al. (2010). One gram of the oven-dried biomass (untreated or pretreated) was fluxed with 10 ml of 80% acetic acid and 1.5 ml of nitric acid for 20 min. Then, contents were filtered using a vacuum pump and solid residues were oven dried at 105 °C until constant weight. The difference in initial and final weights was used to calculate the cellulose content of biomass.

Statistical analysis

All the experiments were performed in triplicates. The data points were expressed as mean value ± SD of three replicates. In addition, analysis of variance was performed statistically using SPSS 16 according to Tukey's test at 5% probability

($p \leq 0.05$). For each series of values, the significant differences were labeled by lowercase superscript letter.

Results and discussion

Molecular identification of the cellulolytic strain NAA2

The comparison of 16S rDNA sequence of strain NAA2 (853 bp) with those of close homologs in the EZTaxon database revealed that isolate was closely related to members of the genus *Streptomyces* in the family Streptomycetaceae within the class Actinobacteria and order Actinomycetales (classification based on RDP database). The strain NAA2 showed highest 98% similarity with *Streptomyces atrovirens* (NRRLB-16357). Also, it exhibited 97–98% similarity with other *Streptomyces* strains. Figure 1 depicts the phylogenetic tree showing the evolutionary relationship of *Streptomyces* sp. NAA2 with closely related strains. Thus, based on nucleotide homology and phylogenetic analysis, the isolate was designated as *Streptomyces* sp. NAA2.

Optimization of process parameters for carboxymethyl cellulase production

Effect of pretreatment

The effect of biomass pretreatment was studied by observing enzyme production using native *P. hysterophorus* biomass, and biomass subjected to steam and alkali-acid pretreatment (Fig. 2). The cellulose content of biomass pretreated using different methods has been given in Table 1. It was found that *Streptomyces* sp. NAA2 produced the highest amount of CMCases (0.273 ± 0.015 U/ml) in the presence of steam-pretreated biomass. However, a reduction in enzyme production was recorded when alkali-acid-pretreated biomass was used in production medium despite higher cellulose content in it. The cellulose has inductive effects on the synthesis of cellulases (Todero Ritter et al. 2013), but its accessibility is important during induction. The pretreatment alters the structure of biomass and cellulose accessibility, which may have varying effects on the enzyme yields. Similar to our study, Brijwani and Vadlani (2011) also observed enhanced CMCase production in *Trichoderma reesei* and *Aspergillus oryzae* when cultivated on steam-pretreated soybean hulls compared to native, acid-, and alkali-pretreated biomass. The steam pretreatment is known to increase the porosity of the biomass (Brijwani and Vadlani 2011), which could account for increased accessibility of cellulose, consequently increasing production of cellulolytic enzymes. The findings indicate that the structural attributes of biomass are important determining factors for the production of hydrolytic enzymes.

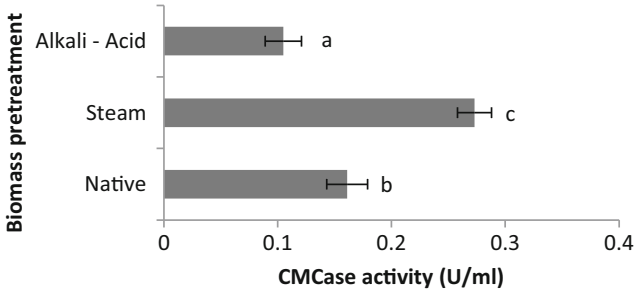

Fig. 2 Effect of biomass pretreatment on CMCase production by *Streptomyces* sp. NAA2 under SmF conditions

Effect of substrate concentration

The concentration of cellulosic biomass may have a significant effect on the induction of cellulolytic enzymes. Hence, the effect of substrate (steam pretreated biomass) level was studied by varying its concentration in production medium from 0.5% to 2.5% (*w*/*v*). The results depicted in Fig. 3 indicate that maximum CMCase production (0.292 ± 0.014 U/ml) by *Streptomyces* sp. NAA2 was observed when the

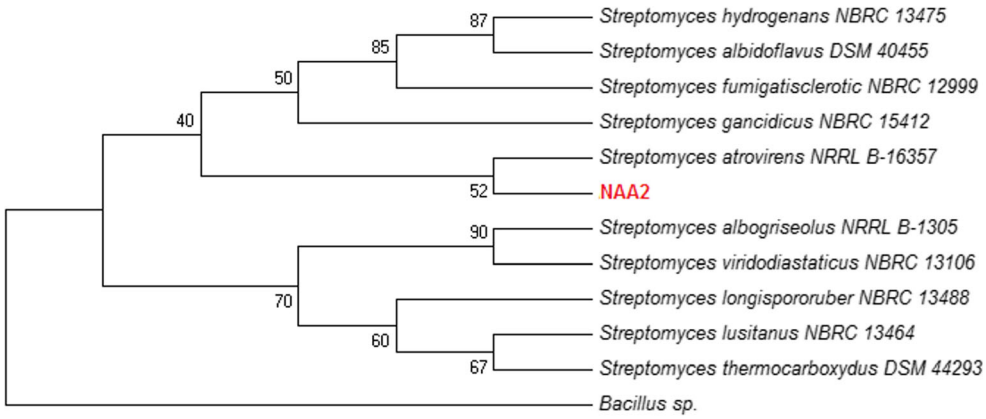

Fig. 1 Neighbor-joining phylogenetic tree of *Streptomyces* sp. NAA2 (Bootstrap values have been expressed as percentages of 1000 replications. The sequence of *Bacillus* sp., NCBI GenBank accession no. EU754025, has been used as an out group)

Table 1 Cellulose content of untreated and pretreated *P. hysterophorus* biomass

Sample (biomass)	Cellulose content (% *w/w*)
Untreated	36.11 ± 0.39[a]
Steam pretreated	38.13 ± 0.17[b]
Alkali-acid pretreated	56.00 ± 0.38[c]

Values are mean ± SD and the values with different superscripts differ significantly ($p < 0.05$; Tukey test)

production medium was containing 1% of biomass. The biomass levels higher or lower than the optimum produced lesser amounts of enzyme. The amount of required substrate varies depending on the type of biomass, microorganisms as well as fermentation conditions. Da Vinha et al. (2011) and El-Naggar and Abdelwahed (2012) have reported that 2% (*w/v*) of steam pretreated wheat bran and 1.5% (*w/v*) of dilute acid pretreated rice straw were suitable for maximum CMCases production by *Streptomyces viridobrunneus* SCPE-09 and *Streptomyces* sp. NEAE-D under SmF conditions, respectively.

Effect of inoculum level

The amount of inoculum is another important parameter governing enzyme production. In the present study, an increase in inoculum level from 1 disc to 2 discs in the medium caused 1.36-fold increase in CMCase production by *Streptomyces* sp. NAA2 (Table 2). However, the further rise in inoculum level resulted in a decrease in enzyme activity. This could be attributed to the increased competition between microbial cells (Sabu et al. 2005). Studies have revealed that cellulase production was highest by *Cellulomonas* sp. (Safdar et al. 2013) and *Streptomyces* sp. DSK29 (Budihal and Agsar 2015) when 2% (*v/v*) and 1×10^8 spores/ml inoculum were used in medium containing potato waste and sorghum stover respectively.

Table 2 Effect of inoculum level, Incubation time and shaking speed on CMCase production by *Streptomyces* sp. NAA2 under SmF conditions

Parameter	CMCase activity (U/ml)
Inoculum level (no. of discs)	
1	0.287 ± 0.008[a]
2	0.392 ± 0.015[d]
3	0.325 ± 0.015[b]
4	0.331 ± 0.011[b]
5	0.326 ± 0.006[b]
Incubation time (days)	
2	0.134 ± 0.011[a]
3	0.291 ± 0.011[b]
4	0.453 ± 0.011[d]
5	0.451 ± 0.009[d]
6	0.389 ± 0.005[c]
7	0.393 ± 0.005[c]
8	0.387 ± 0.005[c]
Shaking speed (rpm)	
100	0.439 ± 0.014[a]
120	0.943 ± 0.006[c]
150	0.967 ± 0.016[c]
180	0.636 ± 0.017[b]

Values are means ± standard deviation and the values with the different superscripts differ significantly ($p < 0.05$; Tukey test)

Effect of initial pH

The production of enzymes is strongly influenced by pH of fermentation medium. Therefore, the effect of initial pH was determined on carboxymethyl cellulase production by *Streptomyces* sp. NAA2. The actinomycete exhibited maximum CMCase activity, i.e., 0.388 ± 0.008 U/ml when the initial pH of the fermentation medium was 6.5 (Fig. 4a). Very high or low pH values, however, caused a significant reduction in enzyme activity. The pH requirements for obtaining the

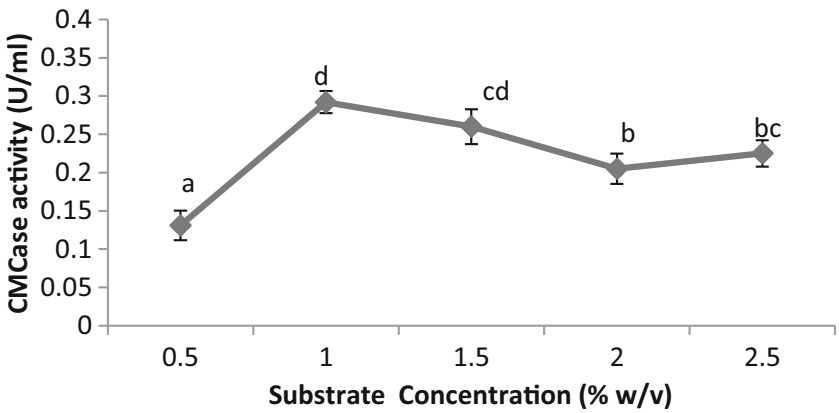

Fig. 3 Effect of substrate concentration on CMCase production by *Streptomyces* sp. NAA2 under SmF conditions

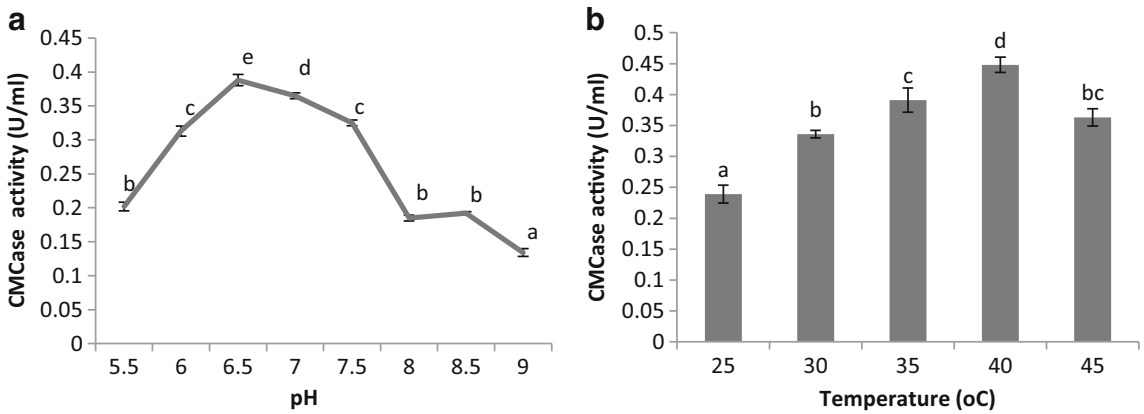

Fig. 4 Effect of **a** initial pH and **b** temperature on CMCase production by *Streptomyces* sp. NAA2 under SmF conditions

highest levels of extracellular cellulases vary with the microorganisms and substrates used for their cultivation. Highest enzyme production was detected at pH 7.0 in *Streptomyces actuosus* (Murugan et al. 2007) and *Streptomyces* sp. J2 (Jaradat et al. 2008) in a medium containing CMC, and at pH 6.0 in *Streptomyces ruber* in a rice straw-supplemented medium (El-sersy et al. 2010). The pH exerts its effect by inducing changes in cell morphology and also has a role in the transport of the enzymes (Mrudula and Murugammal 2011).

Effect of temperature

The excretion of extracellular cellulases is affected by the permeability of the cell membrane, and temperature has a profound effect on permeability (Behera et al. 2016). Therefore, the effect of incubation temperature was evaluated over a range of 25 °C to 45 °C (Fig. 4b). The results demonstrated a gradual increase in CMCase production with the increase in temperature from 25 °C (0.239 ± 0.015 U/ml) to 40 °C (0.448 ± 0.015 U/ml). Thereafter, a decline in enzyme production was detected beyond optimum temperature. Similar to our observations, 40 °C temperature was found ideal for highest carboxymethyl cellulase production by *Streptomyces ruber* (El-sersy et al. 2010) and *S. albidoflavus* strain SAMRC-UFH5 in media containing rice straw and CMC, respectively (Fatokun et al. 2016). A decline in enzyme activity recorded beyond optimum temperature could result from denaturation of enzymes at higher temperatures (Karim et al. 2015; Behera et al. 2016).

Effect of incubation time

The optimum incubation time for enzyme production varies with the type of microorganisms as well as the substrate. To analyze the effect of time course on CMCase production by *Streptomyces* sp. NAA2, flasks were incubated for different time periods, i.e., 2 to 8 days. A nearly constant increase in enzyme production was recorded up to the 4th day (0.453 ± 0.011 U/ml), and the production remained unchanged on day 5 (0.451 ± 0.009 U/ml), whereas a decline was observed on increasing incubation time to 6 days (0.389 ± 0.005 U/ml) (Table 2). Further incubation, however, did not result in significant variation in enzyme production from 6 to 8 days. A

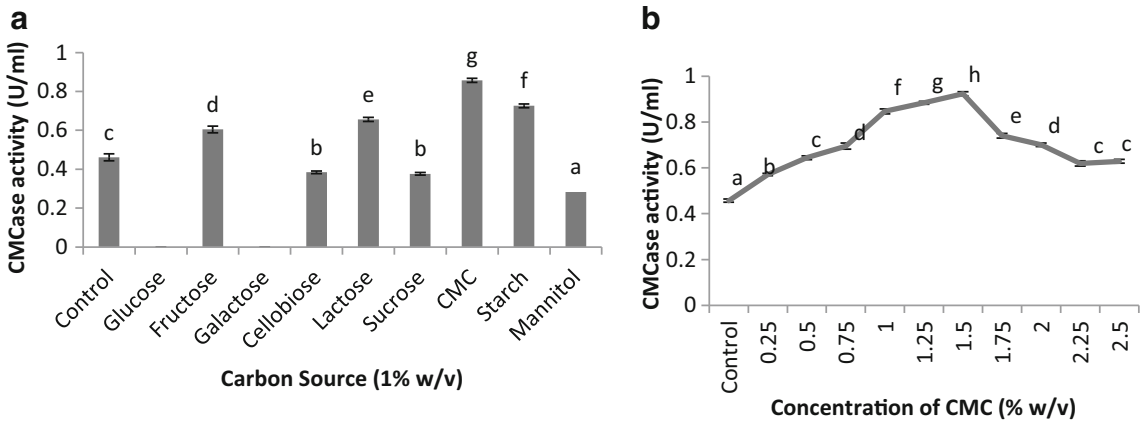

Fig. 5 Effect of **a** different sources of carbon and **b** CMC concentrations on CMCase production by *Streptomyces* sp. NAA2 under SmF conditions

Table 3 Effect of substrate concentration on the TRS yield from hydrolysis of pretreated *P. hysterophorus* biomass using cellulase activity of *Streptomyces* sp. NAA2

Time (h)	Total reducing sugars (g/g biomass) at different substrate concentrations (%w/v)				
	1.0	2.0	3.0	5.0	7.0
24	0.031 ± 0.0013^{a}	0.045 ± 0.0012^{c}	0.042 ± 0.0014^{c}	0.038 ± 0.0014^{b}	0.035 ± 0.0011^{b}
48	0.048 ± 0.0011^{a}	0.062 ± 0.0013^{c}	0.059 ± 0.0011^{c}	0.052 ± 0.0011^{b}	0.048 ± 0.0016^{a}
72	0.073 ± 0.0014^{a}	0.094 ± 0.0011^{e}	0.086 ± 0.0010^{d}	0.081 ± 0.0010^{c}	0.077 ± 0.0013^{b}
96	0.099 ± 0.0014^{b}	0.113 ± 0.0009^{d}	0.103 ± 0.0010^{c}	0.098 ± 0.0013^{b}	0.094 ± 0.0013^{a}

Values are means ± standard deviation. The values with similar superscripts in each row do not differ significantly ($p < 0.05$; Tukey test)

decrease in enzyme yields beyond optimum time is associated with increased biomass production and depletion of nutrients in the medium. The carboxymethyl cellulase titers were highest in *Streptomyces* sp. BRC1 and *Streptomyces* sp. BRC2 at 72–88 h (Chellapandi and Jani 2008), and on day 6 in *Streptomyces viridobrunneus* SCPE09 when cellulose and wheat bran were used as the substrates respectively (Da Vinha et al. 2011).

Effect of carbon supplement and its concentration

The supplementation of additional carbon source (other than the cellulosic substrate) may have an inductive effect on cellulases synthesis. Therefore, production medium containing steam-pretreated substrate was supplemented with different carbon sources. It was found that CMC caused the highest enzyme production in *Streptomyces* sp. NAA2, i.e., 0.857 ± 0.010 U/ml compared to 0.461 ± 0.010 U/ml in control (without any carbon supplement) (Fig. 5a). The fructose, lactose, and starch also showed a positive effect on CMCase production by *Streptomyces* sp. NAA2 (0.605 ± 0.017 U/ml, 0.656 ± 0.011 U/ml, and 0.726 ± 0.010 U/ml CMCase activity, respectively). On the other hand, glucose and galactose were strongly repressive, while cellobiose, sucrose, and mannitol also showed inhibitory effect. Several studies have also documented an inhibitory effect of glucose and several other simple sugars on induction of cellulases in *T. reesei* (Dashtban et al. 2011). Amore et al. (2013) have reported repression of several cellulases in the presence of mannitol. Furthermore, while evaluating the effect of CMC levels, 1.5% (*w*/*v*) concentration was found most suitable for obtaining highest CMCase titers (0.924 ± 0.009 U/ml), followed with a drop on a further rise in its concentration (Fig. 5b).

Effect of shaking speed

The agitation speed has a significant effect on enzyme production by bacteria (Haritha et al. 2012). It influences the growth of cellulase-producing microbes by governing levels of dissolved oxygen in the fermentation medium (Deka et al. 2013) and also ensures uniform mixing of medium contents (Srilakshmi et al. 2017). In *Streptomyces* sp. NAA2, the study on the effect of agitation speed indicated that the highest CMCase production (0.967 ± 0.016 U/ml) was recorded at 120 or 150 rpm (Table 2). Very high speeds, on the other hand, cause lysis of microbial cells (Haritha et al. 2012) consequently reducing the

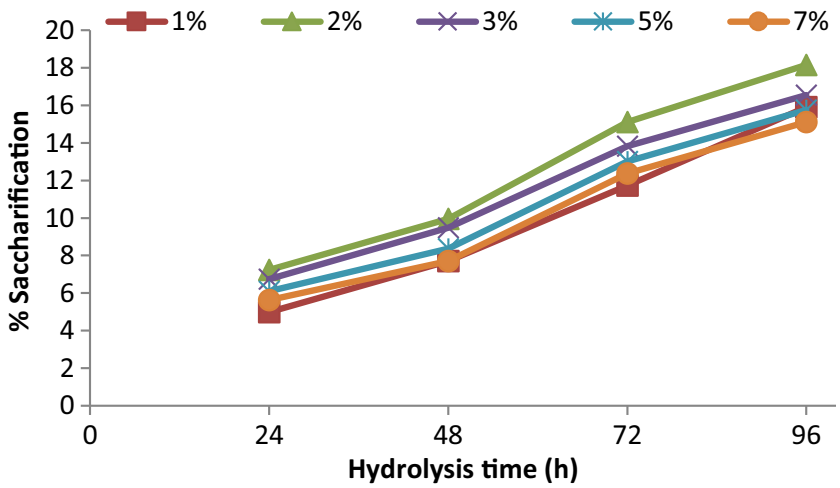

Fig. 6 Effect of substrate concentration on saccharification of pretreated *P. hysterophorus* biomass with cellulase enzyme from *Streptomyces* sp. NAA2

Table 4 Effect of enzyme concentration on the TRS yield from hydrolysis of pretreated *P. hysterophorus* biomass using cellulase activity of *Streptomyces* sp. NAA2

Time (h)	Total reducing sugars (g/g biomass) at different enzyme loadings (FPU/100 ml)			
	10.0	20.0	30.0	40.0
24	0.031 ± 0.0013^{a}	0.035 ± 0.0017^{b}	0.045 ± 0.0012^{d}	0.041 ± 0.0011^{c}
48	0.048 ± 0.0011^{a}	0.051 ± 0.0016^{a}	0.064 ± 0.0013^{c}	0.058 ± 0.0017^{b}
72	0.073 ± 0.0014^{a}	0.081 ± 0.0014^{b}	0.094 ± 0.0011^{d}	0.090 ± 0.0016^{c}
96	0.089 ± 0.0014^{a}	0.100 ± 0.0016^{b}	0.113 ± 0.0009^{d}	0.109 ± 0.0011^{c}

Values are means ± standard deviation and the values with similar superscripts in each row do not differ significantly ($p < 0.05$; Tukey test)

enzyme titers. Fatokun et al. (2016) observed the highest cellulase production by *Streptomyces* sp. at 100 rpm.

Under optimized conditions (i.e., in 4–5 days, using 1% *w/v* steam-pretreated *P. hysterophorus* biomass as substrate, 2 discs of inoculum, initial pH 6.5, temperature 40 °C, 120–150 rpm, and 1.5% CMC as additional carbon supplement), *Streptomyces* sp. NAA2 exhibited 0.967 ± 0.016 U/ml CMCase activity, 0.116 ± 0.08 FPU/ml total cellulase (FPase) activity, and 0.22 ± 0.012 U/ml β-glucosidase activity.

Biomass saccharification

The saccharification study was carried out to maximize production of sugars from pretreated biomass by optimizing the levels of the substrate as well as the enzyme produced from *Streptomyces* sp. NAA2. While studying the effect of biomass concentration, it was observed that saccharification yield increased initially with the rise in substrate levels. Further increase in substrate concentration, however, led to a decrease in hydrolysis yield. Highest levels of saccharification yield, i.e., 18.2% of biomass (TRS = 0.113 g/g biomass, Table 3) was obtained in 96 h when substrate level was 2% (*w/v*) (Fig. 6). The initial rise in TRS levels could be attributed to the requirement of an optimum amount of substrate proportional to enzyme levels present in the reaction mixture. The decrease in the hydrolysis yield at high substrate loading might occur due to adsorption of enzymes on to unproductive sites in the biomass, mass transfer limitations, or end-product inhibition of enzyme due to higher sugar levels (Gama et al. 2017).

The study of the effect of enzyme loadings on saccharification of biomass with actinomycete cellulase enzyme indicated that the rise in enzyme loading from 10 FPU to 30 FPU in reaction volume resulted in an increase in hydrolysis yield. The maximum level of TRS (0.113 g/g biomass, or 0.202 g/g cellulose, Table 4) and saccharification yield (18.2%) was achieved in 96 h when enzyme level was 30 FPU (Fig. 7). However, a further rise in the enzyme concentration resulted in a decrease in saccharification yield.

Hsu et al. (2011) have reported production of 53.1 g/100 g TRS from acid-pretreated corncob using *Sterptomyces* sp. T3–1, when sacchrification was carried out by cultivating it directly in the medium containing biomass. Kshirsagar et al. (2015) have shown enhanced production of cellulase from *Nocardiopsis* sp. KNU and achieved the release of >30 mg/g sugars when crude enzyme from this strain was used for hydrolysis of NaOH-pretreated rice husk, at 15% biomass loading in the hydrolysis time of 96 h. Ventorino et al. (2016) have also isolated several actinomycetes strains from degrading biomass and used cellulases from the strains exhibiting high cellulolytic potential for saccharification of pretreated biomass of *Arundo donax*. The

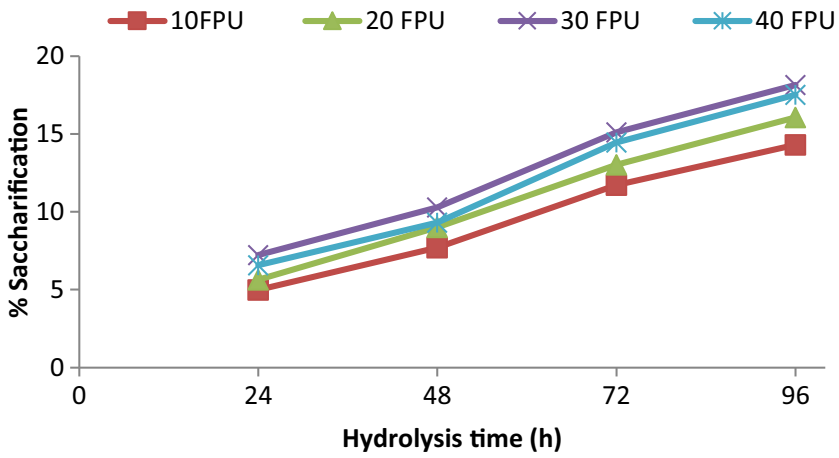

Fig. 7 Effect of enzyme concentration on saccharification of pretreated *P. hysterophorus* biomass with cellulase enzyme from *Streptomyces* sp. NAA2

results indicated that endo-cellulase from *Streptomyces argenteolus* AE58P yielded 76% of glucose when biomass was pretreated with Accellerase 1500. Adiguzel and Tuncer (2017) produced endoglucanase and endoxylanase from *Actinomadura geliboluensis*, partially purified the enzymes and applied their mixture for saccharification of alkali-pretreated wheat straw. They achieved production of 265.12 mg/g sugars using the cocktail of hemicellulolytic and cellulolytic enzyme from the actinomycete.

Conclusion

The search for cellulase-producing isolates has been an interest of researchers for many years. Despite exploring a wide range of microbial species, studies are still continued and new microbes are being tapped for their cellulolytic potential. The present study reveals the cellulase production potential of *Streptomyces* sp. NAA2 and the optimization study resulted in 6-fold increase in CMCase production by it. The saccharification study showed that cellulases produced from strain NAA2 could effectively hydrolyze alkali-acid pretreated *P. hysterophorus* biomass resulting in the production of the considerable amount of TRS. In future, the study can be extended for bioethanol production from *P. hysterophorus.* The study also indicates that *P. hysterophorus* can be used as an effective substrate for bioethanol production or biosynthesis of cellulases. Its availability and abundance can be leveraged as an economical substrate for applications at industrial scales. Also, the hydrolytic potential of actinomycete NAA2 can be used in many other industrial applications including cellulosic bioconversions of diverse biomass.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals This article does not contain any studies with human or animal subjects.

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