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ORIGINAL PAPER

Production of thermotolerant and alkalotolerant cellulolytic enzymes by isolated *Nocardiopsis* sp. KNU

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Abstract A novel cellulolytic bacterium was isolated from the forest soil of KNU University campus. Through 16S rRNA sequence matching and morphological observation it was identified as *Nocardiopsis* sp. KNU. This strain can utilize a broad range of cellulosic substrates including: carboxymethyl cellulose (CMC), avicel, xylan, cellobiose, filter paper and rice straw by producing a large amount of thermoalkalotolerant endoglucanase, exoglucanase, xylanase and glucoamylase. Optimal culture conditions (Dubos medium, 37°C, pH 6.5 and static condition) for the maximal production of the cellulolytic enzymes were determined. The activity of cellulolytic and hemicelluloytic enzymes produced by this strain was mainly present extracellularly and the enzyme production was dependent on the cellulosic substrates used for the growth. Effect of physicochemical conditions and metal additives on the cellulolytic enzymes production were systematically investigated. The cellulases produced by *Nocardiopsis* sp. KNU have an optimal temperature of 40°C and pH of 5.0. These cellulases also have high thermotolerance as evidenced by retaining 55–70% activity at 80°C and pH of 5.0 and alkalotolerance by retaining >55% of the activity at pH 10 and 40°C after 1 h. The efficiency of fermentative conversion of the hydrolyzed rice straw by *Saccharomyces cerevisiae* (KCTC-7296) resulted in 64% of theoretical ethanol yield.

Keywords *Nocardiopsis* sp. KNU · Cellulose hydrolysis · Thermoalkalotolerant cellulase · Xylanase · Rice straw · Ethanol fermentation

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Introduction

Fossil fuels are the main global energy resources for the industrialization and economic growth of countries during the past century. Depending on the production and consumption rates, the presently known reserves of fossil fuels will not appreciably run out for at least 100 years or more, but demand for oil is expected to exceed production from known and anticipated oil reserves 10 or 20 years from now (Goldemberg 2007). In addition, the unfettered use of fossil fuels shows negative impact on the environment because of emission of greenhouse gases (CO_2, CH_4 and CO) resulting in global warming and pollution (Saratale et al. 2008). Thus an energy paradigm based on the fossil fuel dependency, leading to economic and environmental challenges (Demirbas 2007; Lo et al. 2009). For these reasons, in

this century large efforts are being conducted worldwide in order to develop technologies that generate clean, sustainable energy sources in particular, lignocellulosic biomass to ethanol which could substitute to fossil fuels and to achieve energy independence (Ragauskas et al. 2006).

Lignocellulosic biomass is composed of cellulose (insoluble fibers of β-1,4-glucan), hemicellulose (noncellulosic polysaccharides, including xylans, mannans, and glucans) and lignin (a complex polyphenolic structure) (Mabee et al. 2005). Moreover agricultural residues or byproducts are annually renewable, abundantly available, account for more than 180 million tons per year which is energetically equivalent about two-third of the world's energy requirement (Kim and Yun 2006). Thus lignocellulosic biomass is being considered as the largest renewable energy resource all over the world and being promising and economically feasible carbohydrate source for producing the new generation of biofuels (Kapdan and Kargi 2006; Saratale et al. 2010). However, cellulosic materials are usually not readily fermentable by the microorganisms because many factors affect the hydrolysis of cellulose including porosity (accessible surface area) of the waste materials and crystallinity of cellulose fiber (Zhang et al. 2006). The molecular organization of the plant fiber cell wall, i.e. cellulose, hemicellulose, and lignin which are jointly termed as the lignocellulose complex, also limits the accessibility of microorganisms and their enzymes to its fiber components (Lo et al. 2008). For that purpose pretreatment is required to get rid of lignin and hemicellulose, to reduce the crystallinity of cellulose and increase the surface area of materials which can improve the formation of fermentable sugars for the production of bioenergy products (Kumar et al. 2008). Formation of soluble sugars from cellulose in agricultural residues relies on the sequential/coordinated action of individual components of cellulase enzyme system derived from the cellulolytic microorganisms (Zhang et al. 2006; Adsul et al. 2007). Xylan is the major constituent of hemicellulose, the second most abundant renewable resource with a homopolymeric backbone of β-D-xylose. The microorganisms that produce xylanases have attracted considerable research interest because of their potential industrial applications (Tsujibo et al. 1990; Lo et al. 2009). In addition starch is also abundant carbon source in nature so glucoamylase production by cellulolytic microorganisms also becomes beneficial (Stamford et al. 2001).

Actinomycetes are gram-positive mycelial soil bacteria having ability to synthesize a wide variety of antibiotics and biologically active compounds as well as they produce extracellular hydrolytic enzymes to obtain nutrients and energy by solubilizing polymeric compounds in soil (Stamford et al. 2001). These enzymes include proteases, nucleases, lipases and a variety of enzymes that hydrolyze different types of polysaccharides, such as chitin and cellulose (McCarthy 1987). *Nocardiopsis* species are mainly studied for the production of alkaline proteases (Moreira et al. 2003; Dixit and Pant 2000), endo β-1-4 D-glucanase (Walker et al. 2006), keratinolytic enzymes (Mitsuiki et al. 2002), xylanase (Tsujibo et al. 1990), thermostable amylase (Stamford et al. 2001) and chitinase (Tsujibo et al. 2003). However, no work has been done on the production of multiple cellulolytic enzymes and their characterization by using this species. In the present investigation, we have determined the ability of isolated *Nocardiopsis* sp. KNU in the hydrolysis of different cellulosic substrates and the production of multiple cellulolytic and hemicelluloytic enzymes at different cellular locations (extracellular, intracellular and cell bound) under static condition. Various physicochemical parameters were optimized to achieve maximum enzymes and reducing sugar production. To our knowledge this could be the first report on *Nocardiopsis* sp. for the hydrolysis of different cellulosic substrates by producing multiple cellulolytic enzymes having stability at extremely thermophilic and alkaline conditions which increases the industrial applicability of this strain. Finally, the rice straw hydrolysate was used as the carbon substrate for fermentative ethanol production using *Saccharomyces cerevisiae*.

Materials and methods

Cellulosic substrates

Cellulosic materials such as carboxymethyl cellulose (CMC), avicel, birch wood xylan, cellobiose, Whatman filter paper and rice straw were chosen as the carbon substrates in this study. Coverage of the huge

extensive area of cultivable land with rice crop in South Korea generates high volumes of rice straw as a vegetative biomass which is abundance in the local area. The rice straw was collected from a local farmer. The commercial cellulosic materials, such as CMC, birch wood xylan, cellobiose and avicel were obtained from Sigma chemical company (USA). The raw materials were air dried, milled and sieved through a 0.2 mm screen before storing at room temperature prior to usage. All other chemicals used were of the highest purity available and of the analytical grade.

Bacteria isolation and morphological tests

To isolate the microorganisms producing cellulases, soil sample was collected from the forest area of Kangwon National University, South Korea and used as a screening source. The cellulose-hydrolytic bacteria were isolated by using modified Dubos salt medium amended with carboxymethyl cellulose (CMC) as the sole carbon source. The CMC-amended Dubos salt medium consisted of (g l^{-1}): CMC, 10; $NaNO_3$, 0.5; K_2HPO_4, 1.0; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; $FeSO_4.7H_2O$, 0.001. For isolation, 2 g of different soil samples were transferred to the fresh 400 ml Dubos medium containing CMC as the sole carbon source in 500 ml sealed bottles for incubation at 30°C for 7 days. After enrichment in CMC-amended medium for more than five times, the inoculum (0.1 ml; successively diluted to 10^{-5} times) was repeatedly streaking on Dubos agar plates containing CMC as a sole carbon source. After certain incubation the plates were stained by Congo red to see the cellulolytic activity of isolated strains (Lo et al. 2009). The cellulase activity of each culture was determined by measuring the zone of clearance on agar plate. The individual colony KNU which showed better growth and higher degradation ability in cellulosic material was selected and used for the further experiments. The isolated microorganism was further identified on the basis of morphological and biochemical characteristics.

16S rRNA gene sequencing and phylogenetic analysis

The analysis of 16S rRNA genes was conducted as follows: Genomic DNA was extracted from the isolated cellulolytic bacteria using the Genomic DNA extraction kit (SolGent, Daejeon, Korea). The extracted DNA was then used as a template for PCR to amplify the 16S rRNA gene. The 16S rRNA gene was amplified from the chromosomal DNA using the universal bacterial primer set 27F (5′-AGAGTTTG ATCMTGGCTCAG) and 1525R (5′-AAGGAGGT GWTCCARCC).The PCR product was then purified using a SolGent PCR purification kit (SolGent, Daejeon, Korea) according to the manufacturer's instructions. The amplified 16S rRNA gene was sequenced using an ABI BigDye Terminator v3.1 cycle sequencing kit and an ABI 3730XL DNA analyzer (Applied Biosystems, Foster City, Cal., USA). The 16S rRNA gene sequences of related taxa were obtained from GeneBank. Multiple alignments were performed with the program CLUSTAL X (version 1.83) (Thompson et al. 1997). Gaps were edited using the BIOEDIT program (Hall 1999). Evolutionary distances were calculated using the Kimura two-parameter model. Phylogenetic trees were constructed using the neighbour-joining method with the program MEGA (version 3.1) (Kumar et al. 2004). Bootstrap values were calculated on the basis of 1000 replications. The sequence was refined manually after cross checking with the raw data to remove ambiguities and submitted to the GenBank database under accession number HQ433551.

Microorganism and culture conditions

The isolated *Nocardiopsis* sp. KNU growth study was carried out using modified Dubos salt medium and BHM medium consisting of (g l^{-1}): CMC, 10.0; $MgSO_4{\cdot}7H_2O$, 0.2; K_2HPO_4, 1.0; KH_2PO_4, 1.0; NH_4NO_3, 1.0; $FeCl_3{\cdot}6H_2O$, 0.05; $CaCl_2$, 0.02. The optimum environmental conditions such as agitation, initial pH of the media, incubation temperature and selection of the medium for better cellulolytic enzymes production by *Nocardiopsis* sp. KNU were determined.

Preparation of enzyme source

The isolated *Nocardiopsis* sp. KNU was grown in modified Dubos salt medium containing CMC as the sole carbon source at initial pH, 6.5, 37°C for 3 days, and then subcultured once a month and stored at 4°C. *Nocardiopsis* sp. KNU was grown in Dubos salt medium with carbon sources including CMC, birch

wood xylan, avicel, cellobiose, small strips of Whatman filter paper and rice straw (10 g l^{-1} each) at 37°C for 8 days under static condition, centrifuged at 4000×*g* for 20 min. The culture supernatant obtained after centrifugation during the harvesting of cell biomass was directly used as a source of extracellular enzymes in order to determine the enzymatic status directly contact with target. The harvested cells were re-suspended in McIlvaine's 0.1 mol l^{-1} citric acid 0.2 mol l^{-1} phosphate buffer, pH 5.0, for sonication (Sonics-Vibracell ultrasonic processor, Model-BCX-750, USA), keeping sonifier output at 40 amp and giving 10 stroke of 40 s with 2 min time interval each at 4°C. Supernatant from this disruption mixture was used as the intracellular source of enzyme. The particulate or cell associated fraction obtained as a pellet after centrifugation (4000×*g* for 20 min) was suspended in McIlvaine's buffer and used for cell bound enzyme study. Protein concentration of each enzyme source was kept constant (1.0 mg ml^{-1}) for the enzymatic studies.

Enzyme assay

Endoglucanase activity was determined according to the method described by Nitisinprasert and Temmes (1991) using a reaction mixture containing 1 ml of enzyme solution with 1 ml of 1% carboxymethyl cellulose (CMC) in McIlvaine's buffer (0.1 mol l^{-1} citric acid-0.2 mol l^{-1} phosphate buffer; pH 5), and incubated at 40°C for 30 min. Exoglucanase (avicelase) activity was also performed by using the method (Nitisinprasert and Temmes 1991). In this assay method, the reaction mixture containing 2 ml enzyme solution with 1 ml of 1% avicel in McIlvaine's buffer was incubated at 40°C for 2 h. The reaction was terminated by filtration through a 0.45 μm membrane filter (Millipore) and adding of 2 ml of dinitrosalicylic acid reagent. Glucoamylase activity was determined according to the method of (Swan and Dekker 1996) with some modification. In this assay method, reaction mixture containing 1 ml of enzyme solution appropriately diluted in McIlvaine's buffer, pH 5, with 1 ml of aqueous suspension of 1% starch at 40°C for 10 min. In these enzymes test the reaction was terminated by adding 2 ml of dinitrosalicylic acid reagent and heating in boiling water bath for 10 min. One unit of enzyme activity in each case was defined by the amount of enzyme that produces one micromole of reducing sugar from the substrate per minute. Xylanase activity was determined according to the method (Saratale et al. 2010) using a reaction mixture containing 1 ml of enzyme solution appropriately diluted in McIlvaine's buffer with 1 ml of aqueous suspension of 1% xylan at 50°C for 10 min. One unit (U) of xylanase activity is defined as the amount of enzyme that releases one micromole of xylose from the substrate per minute under the above mentioned conditions. β-glucosidase activity was determined by incubating 1 ml enzyme solution with 1 ml of 0.2% cellobiose in McIlvaine's buffer (pH 5) at 40°C for 1 h. The glucose released was measured by using HPLC to calculate the β-glucosidase activity.

Establishment of optimum operational conditions for cellulolytic enzyme activity

The optimum temperature and pH of the cellulolytic enzymes (endoglucanase, exoglucanase, xylanase and glucoamylase) by *Nocardiopsis* sp. KNU in the presence of CMC, xylan and rice straw were determined by following the procedure (described in above section) for the enzyme assay. The optimum temperature was determined by incubating the enzyme and 1% (w/v) substrate at different temperature ranging from 25 to 80°C by keeping constant pH 5.0 for 1 h. The effects of different pH range from 3 to 10 at 40°C on the cellulolytic enzymes activities were determined. The hydrolytic efficiency of *Nocardiopsis* sp. KNU in the presence of different cellulosic substrates (CMC, avicel, birch wood xylan, cellobiose, Whatman filter paper and rice straw; each 10 g l^{-1}) was determined by measuring the reducing sugar and cellulolytic enzymes production. The effect of increasing substrate concentrations (5–25 g l^{-1}) using CMC in the production of reducing sugar and cellulolytic enzymes by *Nocardiopsis* sp. KNU were investigated in the Dubos medium.

Effect of various metal additives on cellulolytic enzyme activity

The effect of various metal ions on the cellulolytic enzyme activity was determined. The additives used in this study were $MnCl_2$, KCl, $CaCl_2$, $ZnCl_2$, $FeCl_3$, NH_4Cl, $COCl_2$, $PbNO_2$, $HgCl_2$, $MgCl_2$, and $CdSO_4$. The concentration of each metal additive used was 5 mM. For all enzyme assays the reaction mixture

with various metal additives (0.25 ml) was incubated under optimum temperature at 40°C and pH of 5.0 and the residual activity of each sample was then quantified with control containing no metal ion in the reaction mixture.

Fermentation medium and conditions for ethanol production

Saccharomyces cerevisiae (KCTC 7296), cultured in the medium containing 40 g of glucose, 5.0 g of yeast extract and 5.0 g of peptone per liter of deionized water was used. After 36 h incubation at 30°C the cells were centrifuged and washed with 0.1% peptone water to remove the residual media. The mean yeast inoculum count was 6.3×10^7 colony forming units (cfu) ml^{-1} corresponding to 11 g of dry cell weight per liter. A total of 0.25 ml of yeast inoculum was added to the tube containing 19 ml of sterile rice straw hydrolysate for fermentation in an incubator at 30°C for 48 h. Fermented samples were centrifuged at 4°C and 4000 rpm for 10 min; supernatant was filtered through 0.2 μm syringe filters for determination of ethanol and residual sugar contents.

Analytical methods

The protein concentration was measured by the dye binding method of Bradford using the Bio-Rad dye reagent concentrate (500-0006, Bio-Rad) in microtiter plates. A standard curve was generated using solutions containing 0.1 g l^{-1} bovine serum albumin (BSA). Absorbances were measured in triplicate at 595 nm after 20 min of incubation at room temperature. Standard methods were used to determine the biomass concentration (APHA 1995). Reducing sugars concentration was determined by using the dinitrosalicylic acid (DNS) method (Miller 1959). The residual sugar contents (glucose, cellobiose and xylose) in cellulose hydrolysates and soluble metabolites in fermented broth were analyzed using HPLC LC-20AT (RID-10A, Shimadzu, Japan) for the presence of residual sugar and ethanol in the filtered (0.2 mm) supernatant of culture broth. The column used in HPLC analysis was Phenomenex Rezex-ROA with 0.005 M H_2SO_4 as eluent with flow rate of 0.6 ml min^{-1} with RI detectors. The injection sample volume was 20 μl and the column temperature was controlled at 60°C. The yield of ethanol after fermentation, expressed as a percentage of theoretical maximum ethanol yields, was calculated as follows (Kim and Lee 2005):

$$\%\ \text{Ethanol yield} = \frac{\text{Ethanol produced(g) in fermented broth}}{\text{Initial glucose in fermented broth} \times 0.511} \times 100$$

Scanning electron microscopy (SEM) observation

After 8 days of incubation with *Nocardiopsis* sp. KNU the hydrolyzed rice straw biomass was separated then washed with deionized water and kept in oven at 70°C for 24 h. The samples were fixed in 2.5% glutaraldehyde for 12 h at 4°C. After fixation, samples were dehydrated gradually after successive immersions in ethanol solutions of increasing concentration. Drying was completed by incubating the samples for 24 h at 50°C. The particles were then coated with platinum and attached on to the microscope supports with silver glue. A scanning electron microscope (S-3500 N Hitachi, Japan) was used to image the biomass samples.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) with Tukey–Kramer multiple comparisons test.

Results and discussion

Isolation and identification

Isolated KNU strain grows well at 37°C on CMC-amended Dubos medium under static conditions (no aeration and agitation). Colonies on CMC agar plates are circular colonies that had yellow substrate mycelia and white aerial mycelia within 3 days of incubation. Microscopic examination showed that the isolate was Gram-positive, non-acid-fast and catalase-positive. The nearly full length sequence of 16S rRNA gene for isolate KNU was determined. Based on the sequence identity of 16S rRNA gene against the GeneBank database indicates that the isolate was closely related to the members of the genus

Nocardiopsis. The highest similarity (99.7%) towards the type strain of *Nocardiopsis synnemataformans* IMMIB D-1215 T (Yassin et al. 1997) and *Nocardiopsis dassonvillei* sub sp. *albirubida* DSM 40465 T (Evtushenko et al. 2000) was observed. A phylogenetic tree illustrating the relationship of strain KNU to other *Nocardiopsis* species is depicted in Fig. 1. Selected strain was identified as *Nocardiopsis* sp. KNU on the basis of 16S rRNA sequence and biochemical characteristics (Table 1).

Optimization of culture conditions

The *Nocardiopsis* sp. KNU was incubated in two different medium (BHM and Dubos) containing CMC as a sole carbon source. The strain can grow in both medium however higher cellular growth, cellulolytic enzymes and reducing sugar production was observed in Dubos medium so this media was selected for the further study. Better growth of *Nocardiopsis* sp. KNU was observed under static condition (no aeration and agitation) compared with under shaking condition (100 rpm). Similar results were observed in case of *Streptomyces lividans* where static condition found to be better for xylanase enzyme production when grow on agricultural waste materials (Abd El-Nasser et al. 2010). Thus static condition was necessary for the better reducing sugar production as well as for the cellular growth, and was adopted to investigate the cellulolytic enzymes production by *Nocardiopsis* sp. KNU in the following experiments. The optimum temperature and pH for the better growth and cellulolytic enzyme production was observed at 37°C and initial Dubos medium pH of 6.5 in all cellulosic substrates used in this study.

Effect of incubation time on cellulase production

Under the optimum culture conditions, the detailed growth study of *Nocardiopsis* sp. KNU and the pro-

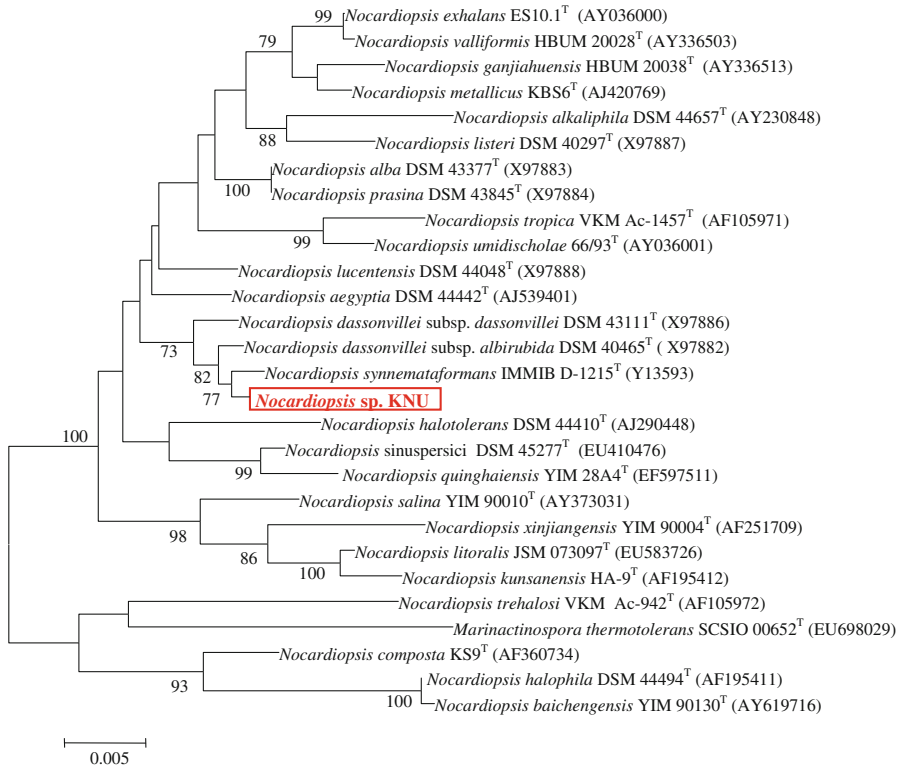

Fig. 1 Neighbor-joining showing phylogenetic positions of *Nocardiopsis* sp. KNU and *Nocardiopsis* species based on 16S rRNA gene sequence comparisons. *Marinactinospora thermotolerans* SCSIO 00652 was used as an out group. Bootstrap values are indicated at nodes. Only bootstrap values >50% are shown. Scale bar, 1% sequence dissimilarity (one substitution per 100 nt). Representative sequences in the dendrogram were obtained from GeneBank (accession number in parentheses)

Table 1 Biochemical characteristics of isolated bacterial strains *Nocardiopsis* sp. KNU

	Nocardiopsis sp. KNU
Characters	
Gram staining	Gram positive
Motility	Motile
Beta-galactosidase	+
Arginine dihydrolase	−
Lysine decarboxylase	−
Ornithine decarboxylase	−
Urease	−
Tryptophane deaminase	−
Gelatinase	+
H_2S production	−
Indole production	−
Acetoin production	−
Utilization of the following compounds as a carbon source	
D-Glucose	+
D-Galactose	+
D-Xylose	+
D-Cellobiose	+
L-Rhamnose	+
Sucrose	+
Mannitol	−
Inositol	−
Sorbitol	−
Citrate	−
Melibiose	−
Amygdalin	−
Arabinose	−

+ Positive, − Negative

duction of cellulolytic and hemicellulolytic enzymes were characterized in Dubos medium containing different cellulosic substrates (mainly, CMC, xylan and rice straw) at different incubation time. Maximum expression of multiple cellulolytic enzyme activities by *Nocardiopsis* sp. KNU in Dubos medium containing different cellulosic substrates were obtained when it entered in the late logarithmic growth phase (after 8 days of incubation) and continued to secrete enzyme well into stationary phase (Fig. 2a). In addition the reducing sugar production and production rate by *Nocardiopsis* sp. KNU in each cellulosic substrate was also maximum after 8 days incubation (data not shown). The foregoing results suggest that *Nocardiopsis* sp. KNU produced different cellulolytic enzyme activities which work synergistically on the hydrolysis of cellulosic substrates. The decrease in the cellulolytic enzymes production after 8 days of incubation (Fig. 2b–d) may be due to the catabolite repression by the metabolites (glucose or cellobiose) released after the hydrolysis (Jang and Chen 2003). Similarly the isolated *Streptomyces* sp. shows maximum production of cellulase enzymes after 5 days of incubation and afterwards reduction in the enzyme activities was observed (Alani et al. 2008).

Effect of cellulosic substrates on the production of reducing sugar and cellulolytic enzyme activities by *Nocardiopsis* sp. KNU

Nocardiopsis sp. KNU could utilize various cellulosic substrates including; CMC, xylan, rice straw, cellobiose, avicel, and filter paper. It was observed that the hydrolytic activity of cellulolytic microorganisms varies on different cellulosic materials due to different composition of cellulose, hemicellulose and lignin (Zhang et al. 2006). We have determined the hydrolytic efficiency of *Nocardiopsis* sp. KNU grown on Dubos medium by taking CMC, xylan, rice straw, cellobiose, avicel, and filter paper as the carbon source (all at an initial concentration of 10 g l^{-1}) to explore the effect of carbon sources on cellulose hydrolysis. All carbon sources could be hydrolyzed significantly by *Nocardiopsis* sp. KNU and produces reducing sugars. Hydrolysis of xylan, rice straw and cellobiose exhibited maximum reducing sugar production and production rate than that with crystalline cellulose or more complex structure (CMC, avicel and filter paper) (Fig. 3). Among the cellulosic materials examined, hydrolysis of xylan gave the best hydrolysis efficiency with a maximum reducing sugar production quantity (1697 mg l^{-1}) and reducing sugar production rate (10.10 mg h^{-1} l^{-1}) (Fig. 3). Moreover, hydrolysis of cellobiose and rice straw exhibited moderate reducing sugar production (735 and 715 mg l^{-1}) and reducing sugar production rate (4.37 and 4.25 mg h^{-1} l^{-1}), respectively. In the presence of CMC, the reducing sugar production (215 mg l^{-1}) and reducing sugar production rate (1.27 mg h^{-1} l^{-1}) was observed (Fig. 3).

Enzymatic hydrolysis of cellulosic feedstock has several advantages over chemical processes because it requires mild experimental conditions, less energy

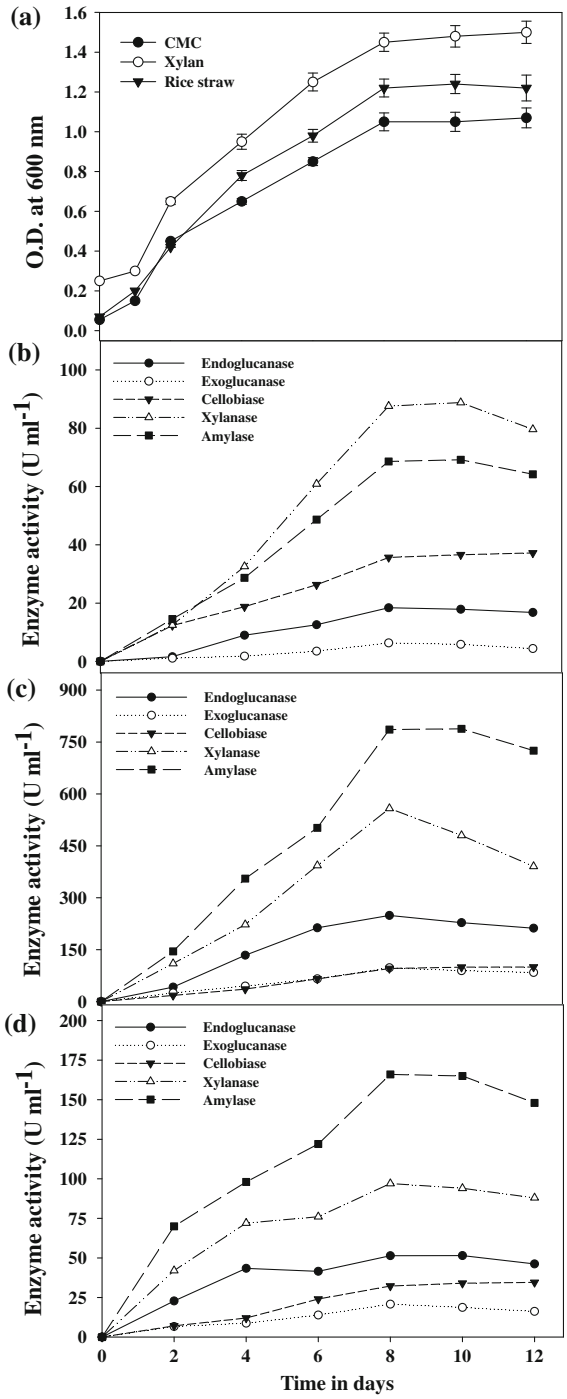

Fig. 2 **a** Growth profile and volumetric activity of excreted polysaccharidases of *Nocardiopsis* sp. KNU in Dubos medium containing different cellulosic substrate (10 g l^{-1}, at 37°C, initial pH 6.5); **b** CMC; **c** Xylan and **d** Rice straw at different incubation period

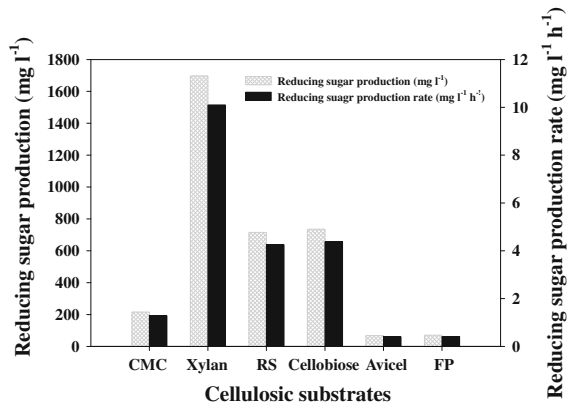

Fig. 3 Effect of cellulosic substrate on cellulose hydrolysis by *Nocardiopsis* sp. KNU in Dubos medium supplemented with 10 g l^{-1} of each cellulosic substrate at 37°C, initial pH 6.5 under static condition after 8 days incubation

consumption and avoidance of pollution (Saratale et al. 2008; Zhang et al. 2006). In the presence of all cellulosic substrates, *Nocardiopsis* sp. KNU has ability to metabolize these cellulosic substrates for their growth by expressing multiple cellulolytic enzyme activities (e.g., endoglucanase, exoglucanase, β-glucosidase, glucoamylase and xylanase) mainly at extracellular location. All cellulolytic enzyme activities by *Nocardiopsis* sp. KNU were higher in the presence of xylan and moderate induction was observed in the presence of cellobiose, rice straw and CMC. Low activities were obtained in the presence of avicel and filter paper possibly due to the poor growth of *Nocardiopsis* sp. in the presence of these substrates (Table 2). The maximum activity of endoglucanse (249 U ml^{-1}) was observed in the presence of xylan whereas moderate activity (72.64 and 18.4 U ml^{-1}) was observed in the presence of cellobiose and CMC. The observed endoglucanse enzyme activity in *Nocardiopsis* sp. KNU is several folds higher than the activity of *Nocardiopsis* sp. SES28 reported earlier (Walker et al. 2006). Similarly higher exoglucanse, β-glucosidase, xylanase and glucoamylase enzyme activities were observed in the presence of xylan and cellobiose (Table 2). From the economic point of view, there is a need to increase the cellulase enzyme mass productivity by using cheaper substrates (i.e., lignocellulosic biomass), with higher stability and specificity (substrates) for

Table 2 Multiple cellulolytic enzyme activity profiles by *Nocardiopsis* sp. KNU extracellularly after 8 days incubation in Dubos medium containing different cellulosic substrates (10 g l^{-1} each) at 37°C under static condition

Cellulosic substrate	Endoglucanase[a]	Exoglucanase[a]	β-glucosidase[a]	Xylanase[a]	Glucoamylase[a]
CMC	18.4 ± 0.40	6.34 ± 0.35	35.65 ± 1.24	87.6 ± 0.30	68.6 ± 0.42
Xylan	249 ± 2.56	97.83 ± 3.34	95.62 ± 2.23	558.16 ± 2.56	786.2 ± 3.23
Rice straw	51.4 ± 0.85	20.8 ± 0.68	32.2 ± 1.64	97.06 ± 0.77	166.2 ± 1.20
Avicel	2.47 ± 0.04	0.93 ± 0.003	2.32 ± 0.65	19.6 ± 3.27	8.2 ± 0.03
Cellobiose	72.64 ± 0.67	56.54 ± 1.40	98.2 ± 2.21	165.9 ± 1.8	145.5 ± 1.15
Filter paper	2.56 ± 0.07	0.87 ± 0.002	1.8 ± 0.004	12.1 ± 0.34	2.81 ± 0.005

Values are mean of three experiments, SEM (±), and by one-way ANOVA with Tukey–Kramer Multiple Comparisons Test

[a] Enzyme activity (U ml^{-1})

the specific processes (Lo et al. 2009). Therefore, in this study, a natural agricultural residue (rice straw) was used as a carbon source for cellulase production by *Nocardiopsis* sp. KNU. It was observed that during the hydrolysis of lignocellulosic biomass, disaccharides and oligosaccharides were liberated and found to be strong inducers of cellulases (Adsul et al. 2007; Saratale et al. 2010). Using rice straw as a carbon source, significant induction in the endoglucanase (51.4 U ml^{-1}), exoglucanase (20.8 U ml^{-1}), xylanase (97 U ml^{-1}), β glucosidase (32.2 U ml^{-1}) and glucoamylase (166 U ml^{-1}) was observed which increases the applicability of this strain (Table 2).

Multiple cellulolytic enzyme production by *Nocardiopsis* sp. KNU using CMC, xylan and rice straw at different cellular location

Studies on the production and secretion of cellulases are important to develop enzyme systems which could be directly used for converting lignocellulosic biomass into safe alternative energy sources (Kapdan and Kargi 2006). In the presence of CMC, xylan and rice straw higher secretion of cellulolytic enzyme activities (endoglucanase, exoglucanase, glucoamylase and xylanase) by *Nocardiopsis* sp. KNU were observed at extracellular location. Endoglucanase activity was observed at all cellular locations in the presence of all cellulosic substrates. In the presence of xylan and rice straw the xylanase activity was observed at all cellular location but in CMC it observed only at extracellular and intracellular location. Moreover, exoglucanase and glucoamylase activity was observed only at extracellular and intracellular location in all substrates except in rice straw where glucoamylase activity was observed at all cellular location.

In the presence of CMC, significant production of endoglucanase (18.1 U ml^{-1}), exoglucanase (5.85 U ml^{-1}), glucoamylase (68 U ml^{-1}) and xylanase (88.8 U ml^{-1}) observed extracellularly, whereas moderate production of endoglucanase (1.45 U ml^{-1}), exoglucanase (1.23 U ml^{-1}), glucoamylase (10 U ml^{-1}) and xylanase (22.5 U ml^{-1}) activity was observed at intracellular location (Table 3). The exoglucanase activity of *Nocardiopsis* sp. KNU in the presence of CMC which is higher than the *Streptomyces* transformant T3-1 (3.2 U ml^{-1}) reported earlier (Jang and Chen 2003). In the presence of xylan, significant induction in xylanase (558 U/ml) and endoglucanase (250 U ml^{-1}), was observed extracellularly whereas moderate production of xylanase (98.5 and 22.5 U ml^{-1}) and endoglucanase (54 and 22 U ml^{-1}) was observed at intracellular and cell bound locations, respectively (Table 3). In contrast maximum expression of exoglucanase and glucoamylase activities was observed at extracellular (97.8 and 786 U ml^{-1}) and intracellular (12.3 and 65 U ml^{-1}) location but no activity was observed at cell bound location (Table 3). The maximum production of glucoamylase by *Nocardiopsis* sp. KNU (786 U ml^{-1}) was superior to the results obtained by *Nocardiopsis* sp. (39.2 U ml^{-1}) (Stamford et al. 2001), *Bacilllus liqueniformis* (15.6 U ml^{-1}) and *Bacillus coagulans* (14.5 U ml^{-1}) (Medda and Chandra 1980). Using rice straw as a carbon source, significant induction in all enzyme activities was observed extracellularly (Table 3). Moderate production of xylanase (12.0 and 6.4 U ml^{-1}) and glucoamylase (12.7 and 4.0 U ml^{-1}) was observed at intracellular and cell bound locations,

Table 3 Effect of different cellulosic substrates (CMC; xylan and rice straw) on the production of endoglucanase, exoglucanase, glucoamylase and xylanase by *Nocardiopsis* sp. KNU at different cellular locations

Cellulosic substrate	Cellular location	Endoglucanase[a]	Exoglucanase[a]	Xylanase[a]	Glucomylase[a]
CMC	Extracellular	18.10 ± 0.49	5.85 ± 0.37	68.0 ± 1.12	88.8 ± 1.22
	Intracellular	1.45 ± 0.008	1.23 ± 0.006	10.0 ± 0.88	22.5 ± 0.89
	Cell bound	1.30 ± 0.006	NA	NA	NA
Xylan	Extracellular	250 ± 2.34	97.8 ± 1.46	786 ± 4.23	558 ± 4.87
	Intracellular	54.0 ± 1.12	12.3 ± 0.89	65.0 ± 1.45	98.5 ± 2.21
	Cell bound	22.0 ± 0.89	NA	NA	22.5 ± 1.09
Rice straw	Extracellular	50.7 ± 1.40	20.5 ± 0.76	166 ± 3.21	97.0 ± 1.24
	Intracellular	2.4 ± 0.08	0.9 ± 0.005	12.7 ± 0.78	12.0 ± 0.98
	Cell bound	1.9 ± 0.006	NA	4.0 ± 0.44	6.4 ± 0.42

Values are mean of three experiments, SEM (±), and by one-way ANOVA with Tukey–Kramer Multiple Comparisons Test

NA No activity

[a] Enzyme activity (U ml^{-1})

respectively. Likewise, endoglucanase production was observed at intracellular (2.4 U ml^{-1}) and cell bound (1.9 U ml^{-1}) locations (Table 3). The foregoing results suggest that production of cellulolytic enzymes at different cellular locations were influenced by cellulosic substrates used for the bacterial growth.

Effect of the temperature on the activity and stability of multiple cellulolytic enzymes produced by *Nocardiopsis* sp. KNU

Microbial cellulase production has been influenced by a number of factors including the type of strain used, reaction conditions (temperature, pH, etc.) and inducer/substrate types. The relationship between these variables has a marked effect on the production of the cellulase enzymes (Zhang et al. 2006). Moreover, cellulases are relatively costly enzymes, and a significant reduction in the cost will be important for their commercial use in the preparation of cellulosic feedstock. The cellulolytic enzymes produced (exoglucanase, endoglucanase, xylanase and glucoamylase) by *Nocardiopsis* sp. KNU in the presence of different cellulosic substrates (CMC, xylan and rice straw) was studied at different temperatures (25–80°C). Multiple cellulolytic enzymes activities by *Nocardiopsis* sp. KNU showed higher performance at 40–50°C by keeping the constant pH 5.0 in the presence of all cellulosic substrates. Thermostable enzymes are stable and active at temperatures which are even higher than the optimum temperatures for the growth of the microorganisms (Haki and Rakshit 2003). The thermostability of all enzymes was assessed by incubating the enzyme at different temperatures for 1 h of incubation. The enzymes produced in the presence of xylan are found to be less effective at higher temperature since all enzymes retained (65–70%) initial activity whereas, in the presence of CMC and rice straw all enzymes produced are able to maintain more than 55% initial activity at higher temperature (80°C). In this study, particularly endoglucanase thermal stability of *Nocardiopsis* sp. KNU is higher than the isolated *Streptomyces* sp. and *Streptomyces drozdowiczii* in which it retained only (58% activity at 80°C) and (50% activity at 70°C), respectively (Alani et al. 2008; Lima et al. 2005). The glucoamylase activity produced by our strain is also comparable with isolated *Nocardiopsis* sp. in which 50% of residual activity obtained at 90°C after 10 min incubation (Stamford et al. 2001). Likewise the temperature profile of xylanase activity found to be better than *Thermomonospora curvata* MT815 and *Sacharomonospora viridis* NCIB9602 in which a marked decrease in the activity at 75°C was observed (McCarthy et al. 1985). The thermostability of cellulolytic enzymes offer advantages in the lignocellulose bioconversion processes which are operated at high temperature and requiring viable enzyme recovery. Stability of the enzymes at higher operation temperature has also a significant influence on the bioavailability and solubility of organic compounds and thereby provides efficient hydrolysis of cellulosic biomass and increased flexibility with respect to process configuration, all contributing towards the

overall improvement of the economy of the process (Viikari et al. 2007). In addition it is also useful to various industrial applications such as in the food, sugar, fuel ethanol and agricultural industries that process cellulose-derived materials, where higher temperature process conditions are applied (Jang and Chen 2003).

Effect of pH on the activity and stability of multiple cellulolytic enzymes produced by *Nocardiopsis* sp. KNU

The effect of pH for endoglucanase, exoglucanase, xylanase and glucoamylase enzyme activity was studied at different pH (3–10) by employing the standard assay conditions. The optimum pH for endoglucanase, exoglucanase, xylanase and glucoamylase activity by *Nocardiopsis* sp. KNU was found pH 5 at temperature 40°C in the presence of all cellulosic substrates. The highest stability of the enzyme was at pH 5, but all cellulolytic enzymes retained more than 75% of the initial activity at pH 8, and more than 50% at pH 10, after incubation at 40°C for 1 h. The enzymes produced in the presence of xylan shows higher alkalotolerance by retaining more than 60% and in the presence of CMC and xylan more than 50% initial activity of each enzyme at higher pH 10. The endoglucanase enzyme alkalotolerance produced in the presence of all cellulosic substrates found to be more active than the cellulase produced by the alkalophilic *Streptomyces* that retained only 31% of the activity at pH 7 (George et al. 2001) and *Streptomyces drozdowiczii* that retained 50% activity at pH 10 (Lima et al. 2005). Likewise it is more active than the *Streptomyces* transformant T3-1 which retained 65% of the activity at pH 9 (Jang and Chen 2003). Actinomycete xylanase was found to be effective for the saccharification of lignocellulose. Alakalotolerance of xylanase activity in this study was also comparable with *Thermomonospora species and Saccharomonospora viridis* (McCarthy et al. 1985). The optimal pH for the maximum production of glucoamylase by *Nocardiopsis* sp. KNU was observed at pH 5.0. Similarly in isolated *Nocardiopsis* sp. the optimal pH was 5.0 for better amylase production which is similar to the value obtained in the present work (Stamford et al. 2001). The ability to retain high activity at elevated pH is a potentially useful property in the processes employing alkaline delignification.

Effect of different metal additives on cellulolytic enzymes production

Studies concerning metal ions influence are very important for industrial enzyme applications. It was reported that the metal additives acts as a cofactor which induce or inhibit the amino acids present at the active site of the enzymes (Haki and Rakshit 2003). Cellulolytic enzyme activities produced by *Nocardiopsis* sp. KNU were assayed under standard optimal conditions in the presence of several metal supplements (at concentration 5 mM each) (Table 4). With the addition of 5 mM $CaCl_2$, all cellulolytic enzyme activities were sharply induced in the presence of all cellulosic substrates. In contrast addition of $FeCl_3$ shows inductive performance in all enzyme activities in the presence of CMC and rice straw whereas it shows inhibitory performance in the presence of xylan (Table 4). Similarly it was reported that supplementation of 1 mM calcium chloride shows an improvement in the β-1, 4-glucanase production (about 200%) by *Nocardiopsis* sp. SES28 and supplementation of iron chloride increases the biomass which supports our results (Walker et al. 2006). In the presence of 10 mM of iron 135% induction in the cellulase activity from *Streptomyces drozdowiczii* has been reported earlier (Lima et al. 2005). In addition it was observed that activities of a number of starch hydrolyzing enzymes are calcium dependent requires different concentration of Ca^{2+} for the higher expression (Haki and Rakshit 2003). Moreover addition of NH_4Cl induces enzyme system in the presence of all cellulosic substrates (except endoglucanase and exoglucanase in the presence of CMC). When CMC was used as a carbon source, induction in all enzyme activities was observed in the presence of $MgCl_2$ but shows inhibitory effect when the strain was grown on xylan and rice straw (Table 4). The foregoing results suggest that the uses of these metals for stabilization experiments are required for industrial formulations. However addition of $MnCl_2$, KCl, $ZnCl_2$, $COCl_2$, $PbNO_2$, $HgCl_2$, and $CdSO_4$ showed substantial inhibitory effect on all enzyme activities in the presence of all cellulosic substrates (Table 4).

Effect of cellulose concentration on cellulolytic enzyme production

The effect of increasing cellulose concentration on cellulose hydrolysis by *Nocardiopsis* sp. KNU was

Table 4 Effect of different metal additives on endoglucanase, exoglucanase, glucoamylase and xylanase enzyme production by *Nocardiopsis* sp. KNU in the presence of different cellulosic substrates

Cellulosic substrate	Enzymes	Metal ion concentration (5 mM)										
		Control	$MnCl_2$	KCl	$CaCl_2$	$ZnCl_2$	$FeCl_3$	NH_4Cl	$COCl_2$	$PbNO_2$	$HgCl_2$	$MgCl_2$	$CdSO_4$
CMC	Endoglucanase	100	74	69	215	69	128	69	00	78	75	137	68
	Exoglucanase	100	72	66	245	63	126	56	00	82	65	129	62
	Xylanase	100	68	64	215	62	123	124	00	75	56	121	45
	Glucoamylase	100	76	68	284	72	108	256	00	00	60	108	24
Xylan	Endoglucanase	100	80	80	158	89	88	111	66	75	73	93	73
	Exoglucanase	100	78	78	145	82	81	118	62	72	66	84	58
	Xylanase	100	76	72	142	84	79	124	58	66	69	65	54
	Glucoamylase	100	74	76	148	78	90	127	58	74	75	72	62
Rice straw	Endoglucanase	100	88	65	168	84	133	149	14	85	79	75	63
	Exoglucanase	100	92	88	195	75	120	185	18	66	72	66	62
	Xylanase	100	185	96	242	79	135	218	15	72	46	69	66
	Glucoamylase	100	106	105	150	74	105	190	00	69	79	72	65

further investigated. The reducing sugar production rate and the amount of reducing sugar produced increased when the CMC carbon source concentration was increased from 5 to 25 g l^{-1}. The maximum reducing sugar production (595 mg l^{-1}) and the maximum reducing sugar production rate (3.10 mg h^{-1} l^{-1}) were achieved when CMC concentration was 25 g l^{-1} (Table 5). The results show that there was essentially no signal of substrate inhibition on cellulose hydrolysis of CMC when the concentration range was within 5–25 g l^{-1} (Table 5). Similar results were observed in the hydrolysis of CMC at a concentration range of 5–50 g l^{-1} by cellulolytic mixed culture (Lo et al. 2008). Likewise, the effect of carbon source concentration of CMC (from 5 to 25 g l^{-1}) on the production of cellulolytic enzymes was investigated. All cellulolytic enzyme activities increased when the substrate concentration increased from 5 to 20 g l^{-1}, but slight elevation or no change in the enzyme activities was observed at higher concentration (25 g l^{-1}) (Table 5). Thus, the maximal production of all cellulolytic enzyme activities was observed at a 20 g l^{-1} CMC concentration by *Nocardiopsis* sp. KNU.

Table 5 Effect of increasing CMC concentration (5–25 g l^{-1}) in Dubos medium on the production of reducing sugar and endoglucanase, exoglucanase, glucoamylase and xylanase enzyme activity by *Nocardiopsis* sp. KNU after 8 days incubation at 37°C under static condition

	Increasing CMC concentration (g l^{-1})				
	5	10	15	20	25
RS production[a]	159 ± 2.88	232 ± 3.43	387.8 ± 4.36	560.6 ± 4.89	595 ± 2.88
RS production rate[b]	0.83 ± 0.02	1.21 ± 0.02	2.12 ± 0.16	2.92 ± 0.3	3.10 ± 0.5
Endoglucanase[c]	17.2 ± 0.8	18.6 ± 0.86	25.7 ± 0.99	34.0 ± 0.89	38.1 ± 1.80
Exoglucanase[c]	2.86 ± 0.14	5.92 ± 0.42	5.72 ± 0.38	10.1 ± 0.42	9.98 ± 0.25
Xylanase[c]	57.06 ± 1.45	88.8 ± 1.20	90.6 ± 0.85	105.4 ± 1.03	112.8 ± 1.31
Glucoamylase[c]	51.92 ± 0.72	69.4 ± 0.46	76.6 ± 0.68	96.2 ± 0.94	107.3 ± 0.87

Values are mean of three experiments, SEM (±), and by one-way ANOVA with Tukey–Kramer Multiple Comparisons Test

[a] Reducing sugar production (mg l^{-1})

[b] Reducing sugar production rate (mg l^{-1} h^{-1})

[c] Enzyme activity (U ml^{-1})

Scanning electron microscopic observation

The morphological changes of the rice straw after 8 days of incubation with *Nocardiopsis* sp. KNU were examined by scanning electron microscopy (SEM) to obtain insight into the structural modification in the rice straw. Figure 4 shows SEM micrographs of untreated and microbial treated rice straw. After microbial treatment the width and length of the rice straw fiber was decreased and deformation of structural morphology was observed (Fig. 4a and b). The SEM observations showed that the treatment with *Nocardiopsis* sp. KNU resulted in partial degradation of rice straw.

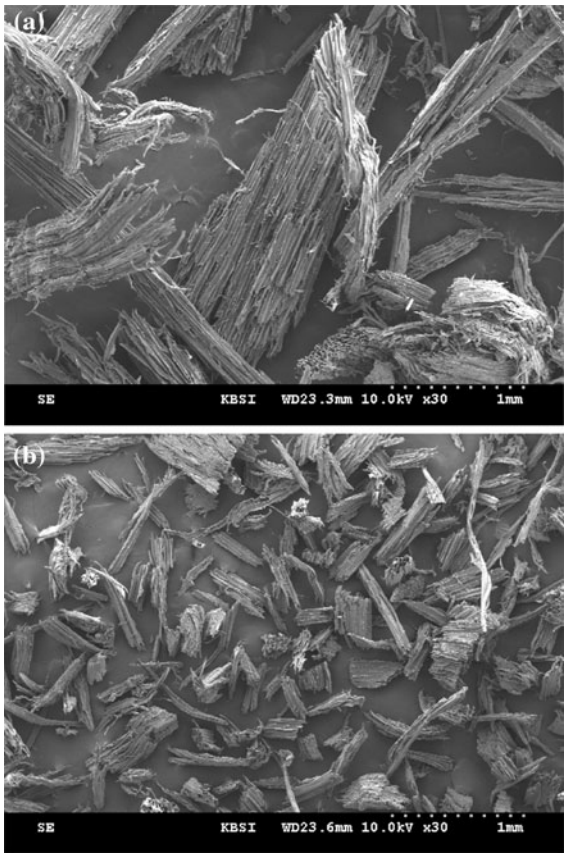

Fig. 4 Scanning electron micrograph of rice straw biomass before and after incubation with *Nocardiopsis* sp. KNU **a** before; **b** after microbial treatment

HPLC analysis and ethanol fermentation

Identification of the composition of natural cellulosic feedstock (rice straw hydrolysate) is useful for the utilization in ethanol fermentation. Hence, HPLC analysis of rice straw was conducted to determine the composition of reducing sugars (e.g., glucose, cellobiose, cellotriose, xylose, maltose and L-arabinose etc.) as well as other fermentation products (such as lactic acid, acetic acid and ethanol) obtained after incubation with *Nocardiopsis* sp. KNU at 37°C for 8 days under static condition. HPLC results show the formation of saccharides and acidogenic secondary metabolites, indicating cellular metabolism of the rice straw by *Nocardiopsis* sp. KNU (Table 6). Still some complex components such as larger oligosaccharides seemed to be present in the hydrolysate in HPLC results presented as unidentified peaks (data not shown). The formation of glucose and cellobiose suggests the sequential action of endoglucanase, exoglucanase and glucoamylase, whereas formation of xylose and L-arabinose suggests the involvement of xylanase in the degradation of these cellulosic materials. The efficiency of fermentative conversion of rice straw hydrolysate into ethanol was carried using *Saccharomyces cerevisiae* strain. Ethanol yields based on glucose available in fermentation broth was 64% after 48 h of fermentation (Table 6). During hydrolysis of rice straw large amounts of hemicellulose sugars (mainly xylose), making up about two-third of the total sugars were produced (Table 6). The *Saccharomyces*

Table 6 HPLC analysis of rice straw hydrolysate and soluble metabolites produced after fermentation by *Saccharomyces cerevisiae* KCTC 7296

	Hydrolysate composition (mg l^{-1})							
	Glucose	Cellobiose	Cellotriose	Xylose	Arabinose	Lactate	Acetate	Ethanol
Hydrolyzed rice straw[a]	75	58	00	258	21	14	24	00
Fermented broth[b]	00	00	00	254	19	16	00	86

[a] Rice straw hydrolysate after 8 days incubation with *Nocardiopsis* sp. KNU at 37°C under static condition

[b] Soluble metabolites of rice straw produced after fermentation by *Saccharomyces cerevisiae* KCTC-7296

strain used in this study lacks the ability to ferment xylose to ethanol. There is need to develop/use stable derivatives of xylose fermenting recombinant yeast to enhance the ethanol yield.

Conclusion

The present study reports a newly isolated and identified *Nocardiopsis* sp. KNU which has ability to decompose different cellulosic substrates by producing substantial multiple cellulolytic and hemicellulolytic enzymes (endoglucanse, exoglucanase, xylanase and glucoamylase) mainly at extracellular location. SEM and HPLC analysis confirmed the hydrolysis of rice straw and formation of soluble sugars production. Regardless of the type of cellulosic substrate used, Ca^{2+} and Fe^{2+} ions significantly stimulated the activity of all cellulolytic enzymes produced by *Nocardiopsis* sp. KNU. All cellulolytic enzymes in the presence of different cellulosic substrates show thermal stability and alkalotolerance which increases the applicability of this strain in the lignocellulosic waste treatment. Using natural cellulosic feedstock (i.e., rice straw), ethanol fermentation was carried out low ethanol yield was obtained may be due to limited cellulose conversion. This work may be important for progress towards utilization and bioconversion of lignocellulosic biomass as well as reduction in environmental problem associated with such wastes. It may also help in dropping the cost of enzyme production. Further studies will be directed towards the purification and characterization of the cellulolytic enzyme complex of *Nocardiopsis* sp. KNU and direct application of purified enzymes for the hydrolysis of lignocellulosic biomass.

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References

Abd El-Nasser NH, Ali AM, Keera AA (2010) Xylanase production by *Streptomyces Lividans* (NRC) and it's application on waste paper. Aust J Basic Appl Sci 4:1358–1368

Adsul MG, Bastawde KB, Varma AJ, Gokhale DV (2007) Strain improvement of *Penicillium janthinellum* NCIM-1171 for increased cellulase production. Bioresour Technol 98:1467–1473

Alani F, Anderson WA, Moo-Young M (2008) New isolate of *Streptomyces* sp. with novel thermoalkalotolerant cellulases. Biotechnol Lett 30:123–126

APHA (1995) Standard methods for the examination of water and wastewater. American Public Health Association, New York, USA

Demirbas A (2007) Progress and recent trends in biofuels. Prog Energy Combustion Sci 33:1–18

Dixit VS, Pant A (2000) Hydrocarbon degradation and protease production by *Nocardiopsis* sp. NCIM 5124. Lett Appl Microbiol 30:67–69

Evtushenko LI, Taran VV, Akimov VN, Kroppenstedt RM, Tiedje JM, Stackebrandt E (2000) *Nocardiopsis tropica* sp. nov., *Nocardiopsis trehalosi* sp. nov., nom. rev. and *Nocardiopsis dassonvillei* subsp. *albirubida* subsp. nov., comb. nov. Int J Syst Evol Microbiol 50:73–81

George S, Ahmad A, Rao M (2001) Studies on carboxymethyl cellulase produced by an alkalothermophilic actinomycete. Bioresour Technol 77:171–175

Goldemberg J (2007) Ethanol for a sustainable energy future. Science 315:808–810

Haki GD, Rakshit SK (2003) Developments in industrially important thermostable enzymes: a review. Bioresour Technol 89:17–34

Hall TA (1999) BioEdit: a user friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41:95–98

Jang HD, Chen KS (2003) Production and characterization of thermostable cellulases from Streptomyces transformant T3-1. World J Microbiol Biotechnol 19:263–268

Kapdan LK, Kargi F (2006) Biohydrogen production from waste materials. Enzyme Microbial Technol 38:569–582

Kim TH, Lee YY (2005) Pretreatment of corn stover by soaking in aqueous ammonia. Appl Biochem Biotechnol 124:1119–1132

Kim J, Yun S (2006) Discovery of cellulose as a smart material. Macromolecules 39:4202–4206

Kumar S, Tamura K, Nei M (2004) MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform 5:150–163

Kumar R, Singh S, Singh OV (2008) Bioconversion of lignocellulosic biomass: Biochemical and molecular perspectives. J Ind Microbiol Biotechnol 35:377–391

Lima A, Nascimento R, Bon E, Coelho R (2005) *Streptomyces drozdowiczii* cellulase production using agro-industrial by-products and its potential use in the detergent and textile industries. Enzyme Microbial Technol 37:272–277

Lo YC, Bai MD, Chen WM, Chang JS (2008) Cellulosic hydrogen production with a sequencing bacterial hydrolysis and dark fermentation strategy. Bioresour Technol 99:8299–8303

Lo YC, Saratale GD, Chen WM, Bai MD, Chang JS (2009) Isolation of cellulose-hydrolytic bacteria and applications of the cellulolytic enzymes for cellulosic biohydrogen production. Enzyme Microbial Technol 44:417–425

Mabee WE, Gregg DJ, Saddler JN (2005) Assessing the emerging biorefinery sector in Canada. Appl Biochem Biotechnol 123:765–778

McCarthy AJ (1987) Lignocellulose degrading actinomycetes. FEMS Microbiol Rev 46:145–163

McCarthy AJ, Edwin P, Paul B (1985) Studies on the extracellular xylanase activity of some thermophilic actinomycetes. Appl Microbiol Biotechnol 21:238–244

Medda A, Chandra AK (1980) New strains of *Bacillus licheniformis* and *Bacillus coagulans* producing thermostable alpha amylase at active and alkaline pH. J Appl Bacteriol 48:47–58

Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 31:426–428

Mitsuiki S, Sakai M, Moriyama Y, Goto M, Furukawa K (2002) Purification and some properties of a keratinolytic enzyme from an alkaliphilic *Nocardiopsis* sp. TOA-1. Biosci Biotechnol Biochem 66:164–167

Moreira KA, Porto TS, Teixeira MFS, Porto ALF, Filho JLL (2003) New alkaline protease from *Nocardiopsis* sp.: partial purification and characterization. Process Biochem 39:67–72

Nitisinprasert S, Temmes A (1991) The characteristics of a new non-spore-forming cellulolytic mesophilic anaerobe strain CMC126 isolated from municipal sewage sludge. J Appl Bacteriol 71:154–161

Ragauskas AJ, Williams CK, Davison BH, Britovsek G, Cairney J, Eckert CA et al (2006) The path forward for biofuels and biomaterials. Science 311:484–489

Saratale GD, Chen SD, Lo YC, Saratale RG, Chang JS (2008) Outlook of biohydrogen production from lignocellulosic feedstock using dark fermentation—a review. J Sci Ind Res 67:962–979

Saratale GD, Saratale RG, Lo YC, Chang JS (2010) Multicomponent cellulase production by *Cellulomonas biazotea* NCIM-2550 and their applications for cellulosic biohydrogen production. Biotechnol Prog 26:406–416

Stamford TLM, Stamford NP, Coelho LCBB, Araújo JM (2001) Production and characterization of a thermostable α-amylase from *Nocardiopsis* sp. endophyte of yam bean. Bioresour Technol 76:137–141

Swan RR, Dekker EG (1996) Seed germination studies. Purification and properties of amylase from cotyledons of germinating peas. Biochim Biophys Acta 122:77–86

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL X windows interface: exible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882

Tsujibo H, Sakamoto T, Nishino N, Hasegawa T, Ina Y, Mor I (1990) Purification and properties of three types of xylanases produced by an alkalophilic actinomycete. J Appl Bacteriol 69:398–405

Tsujibo H, Kubota T, Yamamoto M, Miyamoto K, Inamori Y (2003) Characterization of chitinase genes from an alkaliphilic actinomycete, *Nocardiopsis prasina* OPC-131. Appl Environ Microbiol 69:894–900

Viikari L, Alapuranen M, Puranen T, Vehmaanperä J, Siika-Aho M (2007) Thermostable enzymes in lignocellulose hydrolysis. Adv Biochem Eng Biotechnol 108:121–145

Walker D, Ledesma P, Delgado OD, Breccia JD (2006) High endo-β-1, 4-D-glucanase activity in a broad pH range from the alkali-tolerant *Nocardiopsis* sp. SES28. World J Microbiol Biotechnol 22:761–764

Yassin F, Rainey FA, Burghardt J, Gierth D, Ungerechts J, Lux I et al (1997) Description of Nocardiopsis synnemataformans sp. nov., Elevation of Nocardiopsis alba subsp. prasina to Nocardiopsis prasina comb. nov., and Designation of Nocardiopsis antarctica and Nocardiopsis alborubida as Later Subjective Synonyms of Nocardiopsis dassonvillei. Int J Syst Bacteriol 47:983–988

Zhang YHP, Himmel ME, Mielenz JR (2006) Outlook for cellulase improvement: screening and selection strategies. Biotechnol Adv 24:452–481