Agriculture and Natural Resources 52 (2018) 399–406

Contents lists available at ScienceDirect

Agriculture and Natural Resources

journal homepage: http://www.journals.elsevier.com/agriculture-and-natural-resources/

Original Article

Utilization of agricultural waste biomass by cellulolytic isolate *Enterobacter* sp. SUK-Bio

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ARTICLE INFO

Article history:
Received 2 March 2017
Accepted 30 August 2017
Available online 3 November 2018

Keywords:
Cellulolytic enzyme
Congo red
Dubos media
Enterobacter sp. SUK-Bio
Sorghum husk

ABSTRACT

A cellulolytic bacterium was isolated from plant litter soil and identified as *Enterobacter* sp. SUK-Bio. This isolate was investigated for its utilization of different cellulosic materials (carboxymethyl cellulose, sugarcane trash, grass powder, sorghum husk, wheat straw and water hyacinth). Utilization of sorghum husk was comparatively more than for the other cellulosic materials used, producing higher cellulolytic and hemicellulolytic enzymes (filter paperase (0.15 U/mL), β-glucosidase (37.10 U/mL), endoglucanase (12.24 U/mL), exoglucanase (2.52 U/mL), xylanase (26.26 U/mL) and glucoamylase (33.26 U/mL)) on day 8 of incubation. Furthermore, it produced the maximum reducing sugar production (554 mg/L) at a rate of 3.84 mg/h/L. Fourier-transform infrared spectroscopy analysis of sorghum husk revealed functional groups changes and a decrease in the total crystallinity ratio after microbial degradation. The effects of supplementation of different metals additives, thermal stability and pH on cellulolytic enzymes were also studied.

Introduction

The developed world's energy economy is highly dependent on oil resources as a primary source of energy (Sarkar et al., 2012). The uncontrolled usage of fossil fuels is leading to their depletion in the future as they have limited nonrenewable reserves. These fossil fuels also release harmful combustion products in the atmosphere which cause environmental challenges such as global climate change (El-Dalatony et al., 2016; Salama et al., 2017). Thus, most countries have started investment into searching for alternative energy sources which can help to resolve the issues of environmental safety and energy security, and to slow down climate change (El-Dalatony et al., 2017). The production of second-generation biofuels by utilizing promising feedstock like lignocellulosic biomass is considered as a good alternative to the present source of energy (Harding et al., 2016). The use of agricultural crop residues for biofuels production can resolve the problem of food insecurity generated because of limited first-generation biofuels (Papini and Simeone, 2010).

The lignocellulosic biomass produced by the photosynthesis process is composed of mainly cellulose (homopolysaccharide of β-1, 4-glucan), hemicelluloses (heteropolysaccharides of xylans, mannans) and lignin (complex polyphenolic structure) (Pérez et al., 2002). The biomolecular cellulose is associated with hemicellulose through cross-linking of lignin. The decomposition of lignocellulosic material is a complex process as it passes through intermediate steps such as saccharification of cellulosic material to reducing sugar and sugar fermentation (Ghatge et al., 2014). Cellulose, a renewable carbohydrate polymer is the major component of plant biomass and it is also most abundant polymer on Earth (Sandgren et al., 2005).

More than 180 million t of agricultural wastes are produced annually that could fulfill two-thirds of the energy needs of the world (Kim et al., 2006). The availability of lignocellulosic biomass is now considered as the largest renewable energy resource globally; thus, it can be considered as the most promising and

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https://doi.org/10.1016/j.anres.2018.10.019

economically feasible carbohydrate source for producing the new generation of biofuels (Kapdan and Kargi, 2006). The most abundant lignocellulosic agricultural crop residues produced every year are corn cobs, corn stover, wheat straw, rice straw, barley straw, sorghum husk, coconut husk, sugarcane bagasse, switch grass, pineapple and banana leaves (Demain et al., 2005). Zhang et al. (2006) reported processing factors such as porosity of the waste materials and crystallinity of cellulosic fiber, which affect biodegradation of lignocellulosic materials by microorganisms. Production of clean biofuels by utilizing agricultural waste materials is a novel and promising approach for achieving the increasing demand for an energy substitute for fossil fuels.

The objective of present study was to isolate cellulolytic bacteria from soil samples and to investigate the cellulolytic and hemicellulolytic activities of isolated *Enterobacter* sp. SUK-Bio by using different agricultural wastes (sorghum husk, grass powder, sugarcane trash, wheat straw, water hyacinth). Various physicochemical parameters were optimized to achieve maximum production of enzymes and reducing sugars. To the authors' knowledge, this is the first report on *Enterobacter* sp. for the hydrolysis of different agricultural lignocellulosic substrates which can produce multiple cellulolytic enzymes.

Materials and methods

Cellulosic substrates

The commercially available chemicals, carboxymethyl cellulose (CMC) and birch wood xylan were procured from Hi-Media (Mumbai, India), while Avicel and phosphomolybdic acid solution were procured from Sigma-Aldrich (St Louis, MO, USA). All other chemicals were of the highest purity or of analytical grade. Agricultural waste residues (sugarcane trash, grass powder, sorghum husk, wheat straw and water hyacinth) were chosen for this study as a carbon source and were collected from local farmers cultivating agricultural land near Kolhapur city, India. The agricultural waste residues were washed, air dried, milled and sieved through a 0.2 mm sieve screen and stored at room temperature under moisture-free conditions until used.

Isolation of bacteria and morphological tests

The plant litter soil samples were collected from sites containing dead, decaying plant material on the Shivaji University campus, Kolhapur, India for the isolation of cellulolytic microorganisms using modified Dubos salt medium with CMC. The CMC-amended Dubos salt medium (pH 6.5) was composed of: CMC, 10 g/L, $NaNO_3$, 0.5 g/L, K_2HPO_4, 1.0 g/L, $MgSO_4.7H_2O$, 0.5 g/L, KCl, 0.5 g/L and $FeSO_4.7H_2O$, 0.001 g/L. At the initial step of isolation, 2.0 g of different soil samples were inoculated in fresh 400 mL of 1% CMC Dubos medium in a 500 mL flask at 30 °C for 7 d for enrichment. After enrichment in the CMC-amended medium for more than five times, the inoculum (0.1 mL serially diluted to 1×10^{-5}) was repeatedly streaked on Dubos agar plates containing CMC as a sole carbon source. The well-grown bacterial colonies on Dubos agar were further screened for cellulolytic activity using the Congo red agar method (Lo et al., 2009). Bacterial colonies showing a large zone of clearance on Congo red agar were further sub-cultured on Dubos agar slants and stored at 4 °C. Isolated colonies were subjected to morphological and biochemical characteristics.

16S rRNA gene sequencing and phylogenetic analysis

The genomic DNA of isolated cellulolytic bacteria was extracted according to the method described by Ausubel et al. (1994). The extracted whole DNA was then used as a template for polymerase chain reaction to amplify the 16S rRNA gene using the conditions: initial denaturation at 95 °C for 5 min (1 cycle); subsequent denaturation at 94 °C for 15 s, annealing at 57 °C for 15 s, extension at 72 °C for 15 s (34 cycles), final extension at 72 °C for 10 min (one cycle) and finally holding at 4 °C. The 16S rRNA gene was amplified from the chromosomal DNA, using the universal bacterial primer set 27F (5′-AGAGTTTGATCMTGGCTCAG) and 1525R (5′-AAGGAGGTGWTCCARCC). The 16S rRNA gene sequence obtained after DNA sequencing was blasted with 16S rRNA sequences available in the NCBI (https://www.ncbi.nlm.nih.gov/nuccore/KC825345.1). The obtained sequence was deposited in NCBI. Multiple alignments were performed with version 1.83 of the program CLUSTAL X (Thompson et al., 1997). Evolutionary distances were calculated using the Kimura two-parameter model. The phylogenetic tree was constructed using the neighbor joining method with the program MEGA 4 (Tamura et al., 2007). Bootstrap values were calculated based on 1000 replications.

Microorganism and culture conditions

The study of the isolated *Enterobacter* sp. SUK-Bio growth was carried out using the modified Dubos salt medium containing 1% CMC as a carbon source. For the production of cellulolytic enzymes by *Enterobacter* sp. SUK-Bio, optimum conditions (agitation, initial pH of the media, incubation temperature) were determined.

Preparation of enzyme source

Enterobacter sp. SUK-Bio was cultured under submerged growth conditions using Dubos salt medium with carbon sources consisting of: CMC, sugarcane trash, grass powder, sorghum husk, wheat straw and water hyacinth (10 g/L), at 30 °C, for 8 d under static conditions. The culture supernatant obtained after centrifugation (4000× *g* for 20 min) during the harvesting of cell biomass was directly used as a source of extracellular enzymes for the determination of the enzyme activities.

Enzyme assay

Endoglucanase activity was determined according to the method described by Saratale et al. (2012), using a reaction mixture containing 1 mL of enzyme solution with 1 mL of 1% CMC in McIlvaine's buffer (0.1 mol/L citric acid-0.2 mol/L phosphate buffer; pH 5) and incubated at 50 °C for 30 min. Exoglucanase (avicelase) activity was determined in the reaction mixture containing 1 mL of enzyme solution with 0.5 mL of 1% Avicel cellulose in McIlvaine's buffer, incubated at 50 °C, for 2 h, and then a further 1 mL of dinitrosalicylic acid reagent was added (Waghmare et al., 2014b). The β-glucosidase activity was determined by monitoring the release of *p*-nitrophenol from *p*-nitrophenyl-β-glucoside (PNPG). The enzyme (200 μL) was incubated with 5 mM PNPG in 1 mL of 50 mM citrate buffer, pH 4.5, at 50 °C for 10 min. The enzyme reaction was stopped by the addition of 2 mL of 1 M sodium carbonate. One unit of β-glucosidase activity was defined as that amount of enzyme which will hydrolyze 5 mM of PNPG per min (Lymar et al., 1995). Filter paperase (FPU) activity was measured according to IUPAC (Andney and Baker, 1996). FPU activity was determined by measuring the reducing sugars produced on Whatman no. 1 filter paper (50 mg, 1 cm × 6 cm). The reaction was carried out in 50 mM citrate buffer at pH 4.5. The reaction mixture was incubated at 50 °C for 1 h (Adney and Baker, 1996).

Xylanase activity was determined in a reaction mixture containing 1 mL of enzyme solution diluted in McIlvaine's buffer with 1 mL of an aqueous suspension of 1% xylan at 50 °C for 10 min

(Saratale et al., 2010). Glucoamylase activity was determined in a reaction mixture containing 1 mL of enzyme solution appropriately diluted in McIlvaine's buffer with 1 mL of an aqueous suspension of 1% starch at 50 °C for 10 min (Anto et al., 2006). In these enzyme tests, the reaction was terminated by adding 1 mL of dinitrosalicylic acid reagent and heating in a boiling water bath for 10 min. One unit of enzyme activity in each case was defined by the amount of enzyme that produced 1 μg of reducing sugar from the substrate per minute.

Optimization of operational conditions for cellulolytic enzyme activity

The optimum temperature and pH of the cellulolytic enzymes (endoglucanase, exoglucanase, FPU, β-glucosidase, xylanase, glucoamylase) of *Enterobacter* sp. SUK-Bio in the presence of agricultural wastes (10 g/L) was determined through the incubation of the enzyme and 1% (w/v) substrate at different temperatures (60 °C, 70 °C, 80 °C, 90 °C) at constant pH 5.0 for 1 h. The effects of different pH ranges were determined at 2–10 °C at 50 °C. The hydrolytic efficiency of *Enterobacter* sp. SUK-Bio in the presence of different agricultural cellulosic substrates (sugarcane trash, grass powder, sorghum husk, wheat straw, water hyacinth at about 10 g/L) was determined by measuring the reducing sugar produced due to the bacterial cellulolytic activity.

Effects of metal additives on cellulolytic enzyme activity

The effect of various metal ions on the cellulolytic enzyme activity was determined in the presence of 5 mM of $MnCl_2$, $CaCl_2$, $ZnSO_4$, $CoCl_2$, $FeCl_3$ and $HgCl_2$. For all enzyme assays, the reaction mixture with 0.5 mL of metal ion solution was incubated at optimum temperature (30 °C) and pH 5.0. The residual activity of each sample was quantified relative to the control containing no metal ions in the reaction mixture.

High performance thin-layer chromatography analysis

High performance thin-layer chromatography (HPTLC) analysis was used for qualitative determination of sugars produced from sorghum husk by *Enterobacter* sp. SUK-Bio after 8 d of incubation. The activation of HPTLC silica gel 60 F_{254} (Merck; Mumbai, India) plate was performed using impregnation with 0.02 M sodium acetate. Ten μl of hydrolysate samples and sugar standards of glucose and xylose (0.2 μg/mL in hydro-methanolic solution) were applied on the activated plate using a sample applicator (Linomat V; CAMAG; Muttenz, Switzerland). The experiment was performed in a twin-trough chamber 10 cm × 10 cm (CAMAG; Muttenz, Switzerland) using the mobile phase acetone-water 9:1 (volume per volume). Sugar detection was done using phosphomolybdic acid solution (20% in ethanol) for derivatization of the developed HPTLC plate (Waghmare et al., 2014a). The winCATS 1.4.4.6337 software (CAMAG; Muttenz, Switzerland) was used for data processing.

Fourier-transform infrared spectroscopy analysis

Fourier-transform infrared spectroscopy (FTIR) analysis was carried out for determination of changes in the functional group due to the cellulolytic degradation of sorghum husk after 8 d of incubation. FTIR spectra were recorded on an FTIR spectrometer (Spectrum one B; Perkin Elmer; Shelton, WA, USA). The cellulolytically degraded sorghum husk was collected and washed with distilled water for removal of bacterial cells, bound enzymes and reducing sugars. The washed sorghum husk was dried overnight in an oven at 60 °C. The dried samples of sorghum husk material were embedded in KBr pellets with approximately 1% of sample in KBr. The background FTIR spectra were obtained using a pure KBr pellet without sorghum husk. The FTIR spectra were recorded in the absorption band mode in the range 4000–400 cm^{-1} with a resolution of 4 cm^{-1} and 32 scans. The change in the total crystallinity index (TCI) of the cellulose in sorghum husk after microbial degradation was also determined (Nelson and O'Connor, 1964).

Statistical analysis

Data were analyzed using one-way analysis of variance with the Tukey-Kramer multiple comparisons test.

Results

Isolation and identification

The isolated strain produced white, shiny, circular colonies on CMC agar. Microscopic examination of isolates revealed their Gram-negative nature, rod shape, and non-motility. This strain showed a zone of clearance on the Congo red agar plate indicating cellulolytic activity (Fig. 1). Partial sequencing of the 16S rRNA gene of the isolated bacterial strain and the phylogenetic tree showed its closeness to *Enterobacter* sp., hence further named as *Enterobacter* sp. SUK-Bio (Fig. 2). The partial sequence of 16S rRNA gene was deposited to GeneBank with the accession number KC825345.1. The biochemical characterization of the bacterial isolate is shown in Table 1.

Optimization of growth conditions

Enterobacter sp. SUK-Bio incubated in Dubos supplemented with 1% CMC as a sole carbon source displayed a higher cellular growth, cellulolytic enzyme activity and production of reducing sugar after 8 d of incubation. *Enterobacter* sp. SUK-Bio showed better growth under static conditions compared to shaking conditions. Thus, static growth conditions were continued to produce cellulolytic enzymes using *Enterobacter* sp. SUK-Bio. The best growth was observed in Dubos medium pH 6.5 at 30 °C using sorghum husk compared to the other lignocellulosic substrates used in this study (Fig. 3).

Effects of incubation time

Enterobacter sp. SUK-Bio grown in Dubos medium containing different cellulosic substrates showed maximum cellulolytic and hemicellulolytic enzyme activities in the late logarithmic growth phase (after day 8 of incubation) and continued to secrete enzyme.

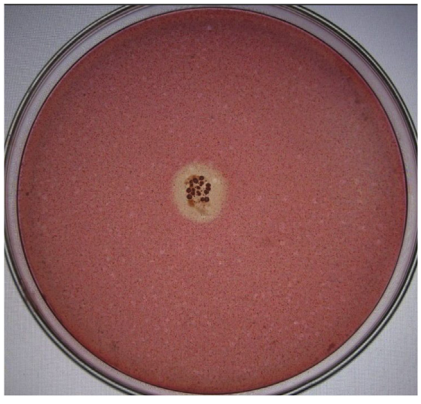

Fig. 1. Screening of isolated bacteria *Enterobacter* sp. SUK-Bio for cellulolytic activity using Congo red staining method showing zone of clearance.

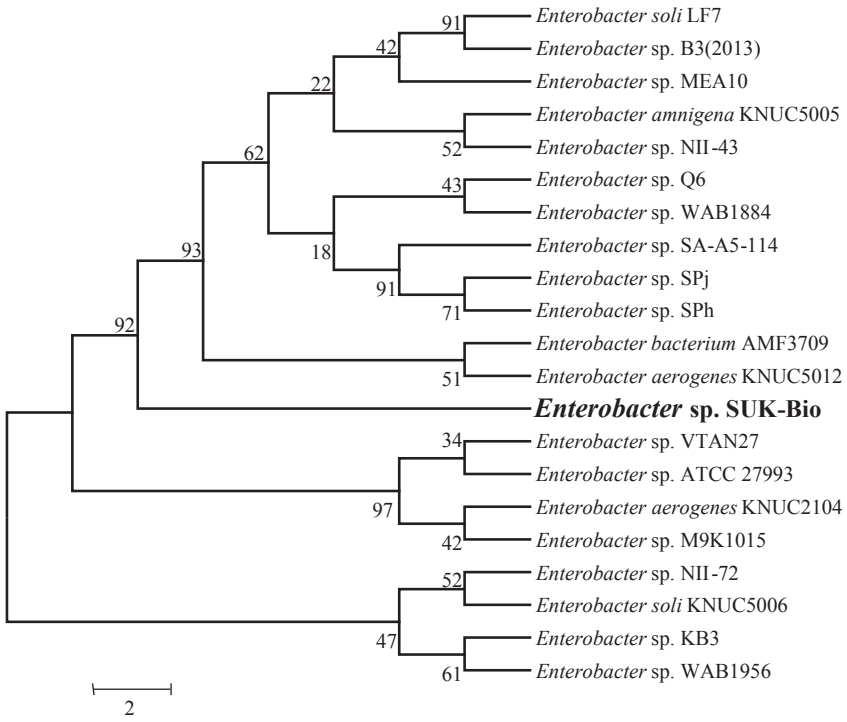

Fig. 2. Phylogenetic position of the isolated strain, *Enterobacter* sp. SUK-Bio (accession number KC825345.1), shown using neighbor joining tree based on the 16S rRNA sequences of the genus *Enterobacter*, where levels of bootstrap support percentage are indicated by the numbers at the nodes based on the neighbor joining of 1000 replicates.

Table 1
Biochemical characteristics of isolated bacterial strain *Enterobacter* sp. SUK-Bio.

Enterobacter sp. SUK-Bio	
Characters	
Gram staining	Gram negative
Mobility	Non-motile
Biochemical test	
Lactose	+
Xylose	+
Maltose	+
Fructose	−
Dextrose	−
Galactose	+
Raffinose	+
Trehalose	+
Melibiose	+
Sucrose	+
L-Arabinose	+
Mannose	+
Inulin	−
Sodium gluconate	−
Glycerol	+
Salicin	+
Dulcitol	−
Inositol	−
Sorbitol	+
Mannitol	+
Adonitol	+
Arabitol	+
Erythritol	−
α-Methyl-D-glucoside	+
Rhamnose	+
Cellobiose	+
Melezitose	+
α-Methyl-D-mannoside	−
Xylitol	+
ONPG	+
Esculin hydrolysis	+
D-Arabinose	+
Citrate utilization	+
Malonate utilization	+
Sorbose	+

On day 8 of incubation, *Enterobacter* sp. SUK-Bio produced maximum reducing sugar in different agricultural wastes used as carbon source. The production of cellulolytic (endoglucanase, exoglucanase, β-glucosidase, FPU) and hemicellulolytic (glucoamylase, xylanase) enzymes by *Enterobacter* sp. SUK-Bio was observed on day 8 of incubation using sorghum husk as a substrate (Fig. 3).

Effects of different cellulosic substrates

All cellulosic substrates used in this study were hydrolyzed by *Enterobacter* sp. SUK-Bio and produced reducing sugars. Among the different agricultural wastes materials, sorghum husk showed efficient hydrolysis with the maximum reducing sugar production (554 mg/L) and reducing sugar production rate (3.84 mg/h/L). The other cellulosic materials: sugarcane trash, wheat straw, grass powder, CMC and water hyacinth exhibited reducing sugar production amounts of 253 mg/L, 255 mg/L, 322 mg/L, 366 mg/L and 245 mg/L, respectively, with reducing sugar production rates of 1.75 mg/h/L, 1.77 mg/h/L, 2.23 mg/h/L, 2.54 mg/h/L and 1.70 mg/h/L, respectively (Fig. 4).

Enterobacter sp. SUK-Bio could utilize and metabolize different cellulosic substrates for their growth and expresses multiple cellulolytic enzyme activities (endoglucanase, exoglucanase, β-glucosidase, glucoamylase, xylanase) mainly at an extracellular location. In the presence of grass powder *Enterobacter* sp. SUK-Bio produced higher cellulolytic enzyme activity, while other carbon source substrates showed moderate enzyme activities (Table 2).

Various agricultural lignocellulosic wastes were used for screening low cost and maximum production of cellulase enzyme as carbon sources by *Enterobacter* sp. SUK-Bio. The maximum cellulolytic activity of endoglucanase (12.24 U/mL), exoglucanase (2.52 U/mL), FPU (0.15 U/mL) and the hemicellulolytic activity of glucoamylase (33.26 U/mL) and xylanase (26.26 U/mL) were observed in the presence of sorghum husk as the sole carbon source in the Dubos media. The maximum β-glucosidase activity (37.10 U/

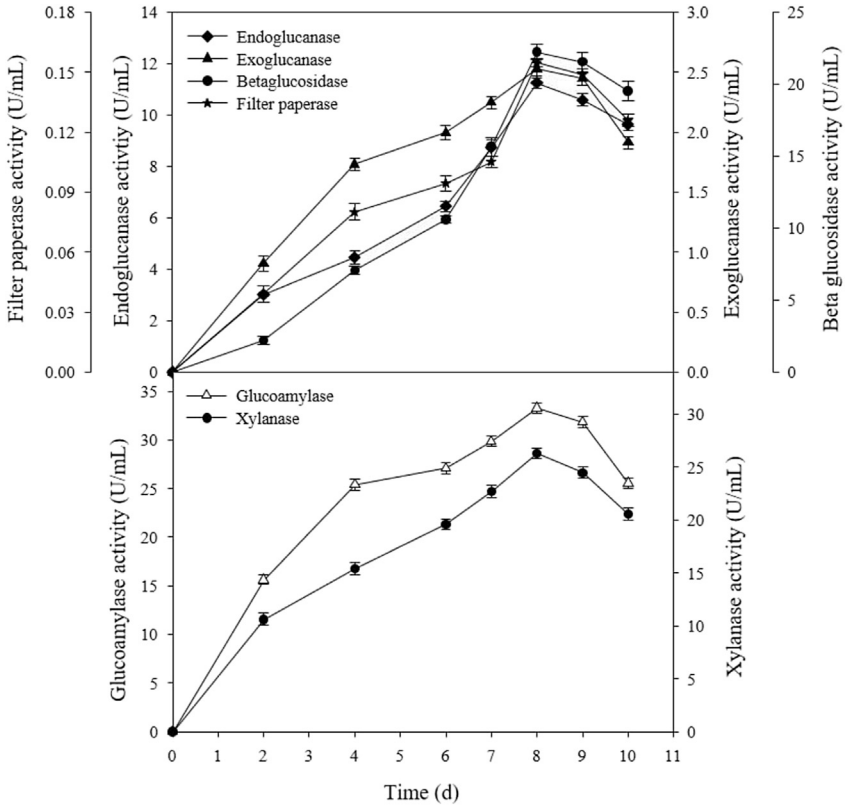

Fig. 3. Study of incubation time on endoglucanase, exoglucanase, beta-glucosidase, filter paperase, glucoamylase and xylanase production by *Enterobacter* sp. SUK-Bio using sorghum husk.

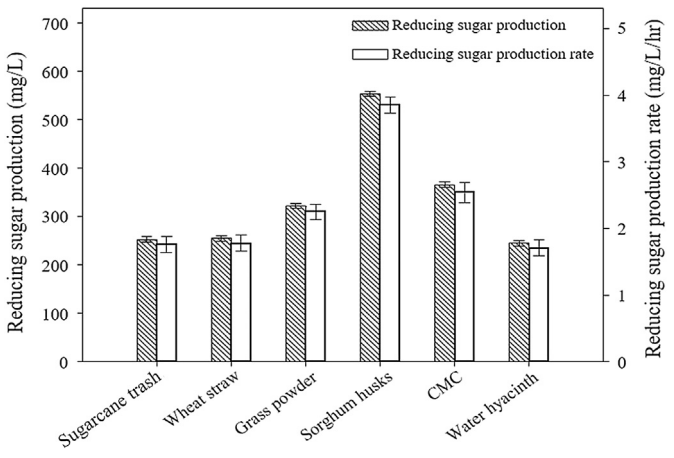

Fig. 4. Effect of different cellulosic substrates on reducing sugar production and reducing sugar production rate on 8 d incubation, where error bars show ± SD ($n = 3$).

mL) was observed when grass powder was used as a carbon source by *Enterobacter* sp. SUK-Bio (Table 2).

Effects of the temperature and pH

The cellulolytic and hemicellulolytic enzymes produced by *Enterobacter* sp. SUK-Bio in the presence of sorghum husk were studied at different temperatures (50–90 °C) and over a pH range (4–8). The optimum production of all enzymes by *Enterobacter* sp. SUK-Bio was at pH 5 and 50 °C in the presence of sorghum husk. The thermostability of all enzymes was assessed by incubating the enzymes at different temperatures for 1 h. Enzymes produced in the presence of sorghum husk could maintain more than 70% of initial activity at the higher temperature (80 °C).

Effects of different metal additives

Enzyme activities of cellulase produced by *Enterobacter* sp. SUK-Bio were assayed under standard optimal conditions with supplementing metal ions (Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{3+}, Hg^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+} each at a concentration of 5 mM). Supplementation of Co^{2+}, Fe^{3+} and Mn^{2+} showed sharp induction in enzyme activities produced by *Enterobacter* sp. SUK-Bio using agricultural wastes. All activities of the enzymes produced on the cellulosic substrates were inhibited by Hg^{2+} metal ions (Table 3).

Determination of sugar production using high-performance thin-layer chromatography

The reducing sugars produced during microbial degradation of sorghum husk by *Enterobacter* sp. SUK-Bio strain were qualitatively determined using HPTLC. The HPTLC analysis confirmed the production of hexose and pentose sugars like glucose and xylose, respectively, after microbial degradation of sorghum husk (Fig. 5).

Fourier-transform infrared spectroscopy analysis

The most representative peaks observed from FTIR analysis were the broad absorption at 3300 and 2900 cm^{-1} corresponding to the stretching of H-bonded OH groups and C–H groups, respectively (Blackwell et al., 1970; Wang et al., 2007). Peaks at 1736 and 1630 cm^{-1} corresponded to carbonyl stretching (C=O) for

Table 2
Cellulolytic and hemicellulolytic enzyme activity (U/mL) of *Enterobacter* sp. SUK-Bio produced extracellularly at day 8 incubation in Dubos media containing different cellulosic substrates.

Cellulosic substrate	Endoglucanase	Exoglucanase	Glucoamylase	Xylanase	FPU	β-glucosidase
Sugarcane trash	5.44 ± 0.02[a]	0.85 ± 0.01	13.46 ± 0.05	9.6 ± 0.61	0.04 ± 0.021	15.90 ± 0.65
Wheat straw	3.59 ± 0.08	0.82 ± 0.08	11.34 ± 0.04	9.2 ± 0.26	0.04 ± 0.028	21.20 ± 0.71
Grass powder	4.61 ± 0.10	1.16 ± 0.02	15.38 ± 0.55	9.6 ± 0.56	0.05 ± 0.04	37.10 ± 0.92
Sorghum husks	12.24 ± 0.20	2.52 ± 0.02	33.26 ± 0.35	26.26 ± 0.22	0.15 ± 0.02	22.22 ± 0.54
Carboxymethyl cellulose	1.06 ± 0.07	1.72 ± 0.02	16.15 ± 0.10	15.46 ± 0.75	0.08 ± 0.01	NA
Water hyacinth	1.92 ± 0.05	0.54 ± 0.01	10.19 ± 0.11	6.0 ± 0.12	0.01 ± 0.01	4.41 ± 0.21

NA = no activity.
[a] Values are mean of three experiments, SEM (±) and no significant differences using one-way analysis of variance with Tukey-Kramer multiple comparisons test.

Table 3
Effects of different metal additives on endoglucanase, exoglucanase, glucoamylase, xylanase and filter paperase enzyme production by *Enterobacter* sp. SUK-Bio in the presence of different cellulosic substrates.

Cellulosic substrate	Enzyme	Metal ion concentration (5 mM)								
		Control	$CaCl_2$	$CoCl_2$	$CuSO_4$	$FeCl_3$	$HgCl_2$	$MgCl_2$	$MnCl_2$	$ZnSO_4$
Sorghum husks	Endoglucanase	100	62.0	288.0	72.0	117.0	NA	60.0	345.0	86.0
	Exoglucanase	100	68.8	189.6	91.2	110.4	41.6	76.0	226.4	94.4
	Glucoamylase	100	73.3	223.8	3.0	131.7	NA	50.5	175.2	90.1
	Xylanase	100	98.2	293.0	50.9	145.6	NA	140.4	324.6	110.5
	Filter paperase	100	73.0	197.3	94.6	102.7	10.8	62.2	389.2	75.7
Grass powder	Endoglucanase	100	35.2	188.3	38.9	75.9	NA	22.2	205.6	50.6
	Exoglucanase	100	89.5	147.4	94.7	109.6	2.5	83.3	201.8	107.9
	Glucoamylase	100	67.9	221.0	49.4	112.3	NA	79.0	212.3	88.9
	Xylanase	100	96.4	171.4	92.9	94.6	1.1	71.4	171.4	80.4
	Filter paperase	100	78.4	186.5	81.1	94.6	4.9	67.6	224.3	83.8
Water hyacinth	Endoglucanase	100	61.3	253.2	75.8	111.3	NA	56.5	277.4	102.4
	Exoglucanase	100	52.2	115.6	75.6	84.4	35.6	58.9	197.8	82.2
	Glucoamylase	100	87.3	191.5	105.6	115.5	NA	71.8	269.0	104.2
	Xylanase	100	69.6	173.9	102.2	102.2	4.8	78.3	184.8	84.4
	Filter paperase	100	28.0	156.0	NA	40.0	NA	NA	256.0	20.0
Wheat straw	Endoglucanase	100	19.5	343.9	84.1	132.9	NA	36.6	379.3	95.1
	Exoglucanase	100	56.5	138.9	91.1	85.6	3.6	64.4	183.3	90.0
	Glucoamylase	100	92.6	321.4	17.1	167.1	NA	54.3	211.4	98.6
	Xylanase	100	59.2	212.2	128.6	100.0	4.1	69.4	206.1	67.3
	Filter paperase	100	105.9	282.4	135.3	117.6	7.6	70.6	588.2	129.4

NA = no activity.

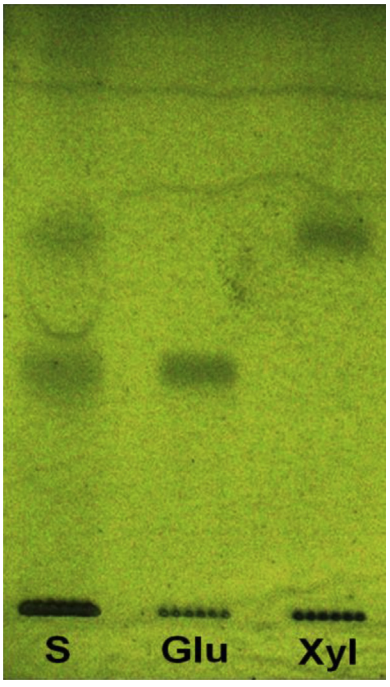

Fig. 5. High-performance thin-layer chromatography analysis for detection of sugars produced after 8 d incubation of *Enterobacter* sp. SUK-Bio grown on sorghum husk, where S = standard sugars (glucose and xylose); Glu = glucose; Xyl = xylose.

acetyl groups in hemicelluloses and for the aldehyde group present in lignin, respectively (Ramle et al., 2012). The peak at 1510 cm^{-1} showed C=C stretching of the aromatic ring of lignin (Lionetto et al., 2012). The peaks at 1430 cm^{-1} revealed the symmetric CH_2 bending and wagging (Cao and Tan, 2004), while the peak at 1375 cm^{-1} showed C–H bending (Colom and Carrillo, 2002). The peak at 1225 cm^{-1} was the bending of O–H (Oh et al., 2005). C–O–C stretching at the β (1–4) glycoside linkages gave two peaks at 1160 cm^{-1} (Oh et al., 2005). The peak at 1020 cm^{-1} was indicative of C–O stretching at C-6. The peaks between 1000 cm^{-1} and 1200 cm^{-1} depicted C–O–C stretching, C–O covalent bonds and O–H linkages that are prevalent in cellulose, hemicellulose and lignin (Kazeem et al., 2017). The FTIR spectrum profiles for native sorghum husk and after microbial degradation of sorghum husk were different (Fig. 6). Major changes occurred in FTIR spectrum with the elimination of bands such as 1736 cm^{-1}, 1510 cm^{-1}, 1224 cm^{-1} and 1160 cm^{-1} while the band of 1020 cm^{-1} arose after the microbial degradation of sorghum husk.

The TCI was determined from the infrared ratio A_{1375}/A_{2900} between different peak heights 1375–2900 cm^{-1} for native sorghum husk and sorghum husk after microbial degradation. The TCI of microbially degraded sorghum husk (0.98) had decreased compared to native sorghum husk (1.02), which might have been due to the removal of amorphous cellulose from sorghum husk during microbial degradation.

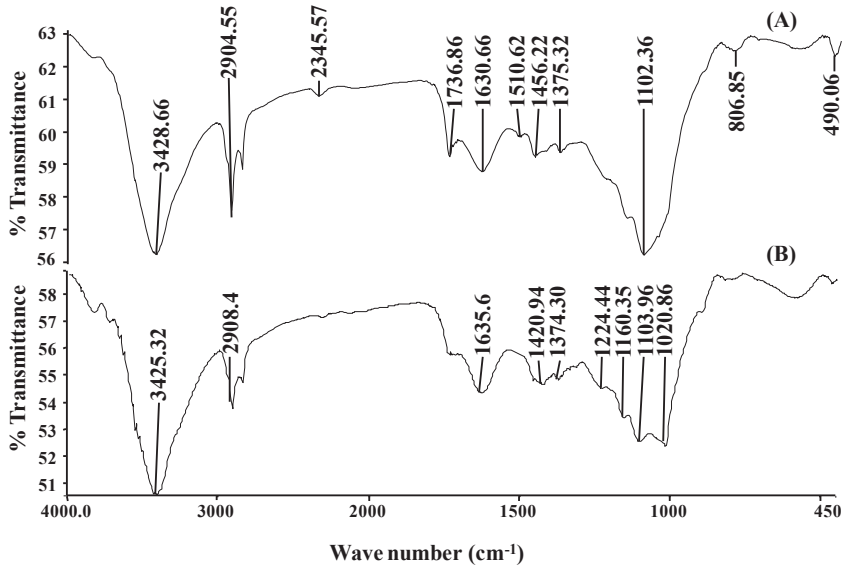

Fig. 6. Fourier-transform infrared spectroscopy spectrum: (A) sorghum husk; (B) sorghum husk after day 8 incubation with *Enterobacter* sp. SUK-Bio.

Discussion

Primary screening of isolates using the Congo red CMC agar method confirmed the secretion of cellulose-degrading enzymes resulting in a zone of clearance which was formed due to the degradation of CMC by the cellulase enzyme produced by *Enterobacter* sp. SUK-Bio. Further biochemical characterization showed that SUK-Bio isolate could utilize different sugars. The *Enterobacter* sp. SUK-Bio isolate also could utilize different cellulosic materials like CMC, sugarcane trash, grass powder, sorghum husk, wheat straw and in particular, water hyacinth, a low cost and complex agricultural lignocellulosic biomass. Zhang et al. (2006) correlated the production of cellulase enzyme from cellulolytic microorganisms and different composition of cellulose, hemicellulose and lignin in different cellulosic materials. The current study determined the hydrolytic efficiency of *Enterobacter* sp. SUK-Bio grown on Dubos medium utilizing CMC, sugarcane trash, grass powder, sorghum husk, wheat straw and water hyacinth as the carbon source to explore the effect of carbon sources on cellulose hydrolysis. The enzymatic hydrolysis of cellulosic feedstock has several advantages, namely mild experimental conditions, less energy consumption and avoidance of pollution from the associated chemical processes (Zhang et al., 2006; Saratale et al., 2008). Disaccharides and oligosaccharides produced due to enzymatic hydrolysis of lignocellulosic biomass play a role as strong inducers of cellulases (Kapdan and Kargi, 2006).

The temperature and pH are considered important factors for the production of microbial cellulase along with the strain type, reaction conditions and substrate type, since the action of these factors might affect the production of the cellulase enzymes (Zhang et al., 2006). Thermostable enzymes retain their stability and activity even at higher temperatures than the optimum growth conditions of the microorganisms (Haki and Rakshit, 2003). A thermostable nature is advantageous for the lignocellulosic bioconversion process as enzymes remain active at the higher temperature. Thus these enzymes can efficiently degrade the lignocellulosic biomass used in this study and finally produce reducing sugars, with an overall improvement in the economy of the process (Viikari et al., 2007). The thermostability of these enzymes indicates their industrial applicability in the food, sugar, fuel ethanol and agricultural industries where processing is at higher temperature (Jang and Chen, 2003).

Metal ions play a very important role in enzyme activity. Their influence on enzyme activity increases the industrial applicability of enzymes. Haki and Rakshit (2003) reported some metal ions which act as a cofactor for active sites cellulase. Chivero et al. (2001) also reported the inhibition effect of Hg^{2+} ions on enzyme activity; this might be due to their interaction with sulfhydryl groups, suggesting that there is an important cysteine residue in or close to the active site of the enzyme.

The HPTLC profile of the separated sugars was comparable with the hydrolysate supernatant obtained after microbial degradation of sorghum husk. Thus, this mixture of hexose and pentose sugars produced in hydrolysate can be further used for bioethanol production using a co-fermentation approach (Binod et al., 2012; Waghmare et al., 2014a).

FTIR analysis is widely used for the determination of structural changes in the complex organic material (Casas et al., 2012). The lignocellulosic biomass is also the most complex structure as it is composed of alkanes, alcohols, aromatics, esters, and ketones with the different oxygen-containing functional groups (Adel et al., 2010). In the present study, the FTIR spectra revealed the structural changes occurred in sorghum husk after microbial degradation by *Enterobacter* sp. SUK-Bio. The changes between the FTIR spectrum profiles for native sorghum husk and after microbial degradation of sorghum husk might have been due to functional group modifications. Major changes occurred in the FTIR spectrum with the elimination of bands such as 1736 cm^{-1}, 1510 cm^{-1}, 1224 cm^{-1} and 1160 cm^{-1} while the band of 1020 cm^{-1} arose after the microbial degradation of sorghum husk. The elimination of the lignin band in the FTIR spectrum indicated the removal of lignin from the sorghum husk after microbial degradation that enhanced reducing sugar production by eliminating lignin inhibition.

In conclusion, the isolated *Enterobacter* sp. SUK-Bio strain could utilize different cellulosic substrates (pure and agricultural wastes) and produce extracellular cellulolytic and hemicellulolytic enzymes. The enhancement of reducing sugar production and reducing sugar production rate was observed at day 8 of incubation using sorghum husk as the substrate. Enzymes produced by *Enterobacter* sp. SUK-Bio in the presence of agricultural waste biomass showed higher thermal and pH stability which increases the applicability of the isolated bacterial strain. The cellulolytic and hemicellulolytic enzymes activities increased after the addition of

$MnCl_2$ and $CoCl_2$ in the reaction mixture, while all enzymes were inhibited by the addition of $HgCl_2$. Changes in the FTIR pattern of degraded sorghum husk, the decrease in the total crystallinity index and production of glucose and xylose confirmed the utilization of sorghum husk as a carbon source. Thus, the present study revealed the potential of *Enterobacter* sp. SUK-Bio for bioenergy production by utilizing agricultural waste biomass.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgements

The first author acknowledges the UGC (University Grants Commission), New Delhi for providing financial assistance through a UGC-NET-JRF fellowship during this research work.

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