Emir. J. Food Agric. 2014. 26 (1): 44-59
doi: 10.9755/ejfa.v26i1.15296
http://www.ejfa.info/

PLANT SCIENCE

Production and characterization of cellulolytic enzymes by isolated *Klebsiella* sp. PRW-1 using agricultural waste biomass

P. R. Waghmare[1], S. D. Kshirsagar[1], R. G. Saratale[3], S. P. Govindwar[1] and G. D. Saratale[1,2*]

[1]*Department of Biochemistry, Shivaji University, Kolhapur 416004, India*
[2]*Department of Environmental Biotechnology, Shivaji University, Kolhapur 416004, India*
[3]*Department of Biotechnology, Shivaji University, Kolhapur 416004, India*

Abstract

The efficient cellulolytic microorganism was isolated from soil samples collected from Shivaji University campus. Biochemical test and phylogenetic analysis of isolated culture identified as *Klebsiella* sp. PRW-1. The isolated culture could utilize pure cellulosic substrates (carboxymethylcellulose (CMC) and avicel) and different agricultural wastes like sugarcane bagasse, sugarcane barbojo, sorghum husks, grass powder, corn straw and paddy straw by producing a large amount of endoglucanase, exoglucanase, β-glucosidase, filter paperase (FPU), xylanase and glucoamylase. The reducing sugar production was found higher in the presence of grass powder and sugarcane barbojo. Effects of different physic-chemical parameters to achieve maximum cellulolytic enzymes production were systematically investigated. The effects of supplementation of different metals additives as well as the stability in the presence of higher temperature, pH and commercial detergents, on cellulolytic enzymes were also studied. The foregoing result increases the applicability of the strain for the utilization and bioconversion of lignocellulosic biomass that could be used for bioenergy production.

Key words: Cellulase, Xylanase, FPU, Commercial detergents, Sorghum husks, Sugarcane barbojo

Introduction

The beginning of the industrial revolution and the consequent tremendous usage of fossil fuels showed negative impacts on the environment mostly due to the emission of greenhouse gases (CO_2, CH_4 and CO) resulting in global warming and pollution (Saratale et al., 2008). Nevertheless, large efforts are being conducted worldwide for the development of technologies which could generate clean, sustainable energy sources particularly, lignocellulosic biomass to biofuels, to substitute fossil fuels (Ragauskas et al., 2006; Levin et al., 2006). Biofuels produced from common agricultural wastes biomass, represent CO_2 cycle, ecofriendly, cost competitive with fossil fuels, biodegradable and it also contributes to the sustainability (Saratale and Oh, 2012). The produced biofuels becomes important and promising alternative energy source for fossil fuels to protect the environment and prevents the problem of the pollution (Puppan, 2002). Some recent reports correlated the first-generation biofuels production with food security and market prices of staple food crops such as maize and rice (James et al., 2008; Keeney et al., 2009). Hence it is important to make a fast transition from the first-generation to second-generation biofuels production using the lignocellulosic biomass such as agricultural crops residues (Fischer et al., 2009).

Lignocellulosic biomass is composed of biomolecules such as cellulose (insoluble fibers of β-1, 4-glucan), hemicellulose (noncellulosic polysaccharides, including xylans, mannans, and glucans) and lignin (a complex polyphenolic structure) (Saratale and Oh, 2011). The agricultural wastes or other renewable products are, abundantly available and its production covers more than 180 million tons per year, which can accomplish about two-third of the world's energy requirement (Kim and Yun, 2006). The availability of lignocellulosic biomass is being considered as the largest renewable energy resource all over the world. Thus, it can be considered as the most promising and economically feasible carbohydrate source for

Received 18 January 2013; Revised 02 May 2013; Accepted 21 May 2013

*Corresponding Author

G. D. Saratale
Department of Biochemistry, Shivaji University, Kolhapur 416004, India

Email: gdsaratale@yahoo.co.in

producing the new generation of biofuels (Kapdan and Kargi, 2006).

The most abundant lignocellulosic agricultural wastes produced every year are corncobs, corn stover, wheat, rice, barley straw, sorghum husks, coconut husks, sugarcane bagasse, switchgrass, pineapple and banana leaves (Demain, 2005). The biodegradation of lignocellulosic materials by microorganisms is affected by some factors, namely porosity of the wastes materials and crystallinity of cellulosic fiber (Zhang et al., 2006). The molecular organization of the plant fiber cell wall, the lignocellulose complex composed of cellulose, hemicellulose, and lignin; among which lignin and hemicellulose limits the biodegradation of this complex (Lo et al., 2008). This problem can be solved by the pretreatment, which reduces crystallinity of cellulose and increases the surface area of the lignocellulosic materials and ultimately improves the biodegradation of cellulose in to fermentable reducing sugars for the production of biofuels (Kumar et al., 2008).

Klebsiella pneumoniae utilises the cellulosic biomass converting it into 2,3-butanediol, thus the conversion of major sugars present in cellulosic biomass (Yu and Saddler, 1982). *K. oxytoca* P2 can utilise the sugars glucose and arabinose to produce ethanol, which are predominant carbohydrates found in sugar beet pulp (Burchhardt and Ingram, 1992). The naturally occurring cellulolytic bacterial strain *K. oxytoca* THLC0409 can degrade micro-sized Napier grass powder producing ethanol by direct conversion (Lin et al., 2010). The genome sequence and analysis of the predicted proteomes of *K. variicola* At-22 codes for enzymes which degrades cellulose polymer is known to be involved in plant polymer degradation, including capacity in the degradation of cellulose (Suen et al., 2010).

In the present study, *Klebsiella* sp. PRW-1 was isolated from soil samples which has the ability to hydrolyse different cellulosic substrates by producing of multiple cellulolytic and hemicellulolytic enzymes under static condition. Various physico-chemical parameters were optimized to achieve maximum enzymes and reducing sugar production. To our knowledge this is the first report on *Klebsiella* sp. for the hydrolysis of different agricultural lignocellulosic substrates by producing multiple cellulolytic enzymes having stability at thermophilic, alkaline conditions and in the presence of detergents which increases the industrial applicability of this strain.

Materials and Methods

Cellulosic substrates

Cellulosic materials (pure and agricultural wastes) such as carboxymethylcellulose (CMC), avicel, paddy straw, sugarcane barbojo, sugarcane bagasse, grass powder, sorghum husks and corn straw were chosen as the carbon substrates in this study. The commercial cellulosic materials, such as CMC, birch wood xylan and avicel were obtained from Hi-Media (Mumbai, India). The agricultural wastes 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 samples were collected from the Shivaji University campus, Kolhapur (16°40'41"N 74°15'19"E / 16.67806°N 74.25528°E) and used as a screening source. The cellulose-hydrolytic bacteria were isolated by using modified Dubos salt medium with 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 30ºC for 7 days. After enrichment in CMC-amended medium for more than five times, the inoculum (0.1 ml; serially 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 evaluate 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 PRW-1, 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 gene sequencing and bacterial identification was carried out by Merck Millipore, Bangalore. Genomic DNA was extracted from the isolated cellulolytic bacteria according to the method described by Ausubel et al. (1997). The extracted DNA was then used, as a template, for PCR, to amplify the 16S rRNA gene using PCR

conditions as initial denaturation at 95º C for 5 min (1 cycle); subsequent denaturation at 95º C for 1 min, annealing at 58º C for 30 sec, extension at 72º C for 30 sec (40 cycles); final extension at 72º C for 10 min (1 cycle) and finally hold at 4º C for ∞. 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 blast with 16S rRNA sequences available in the GeneBank. Multiple alignments were performed with the program CLUSTAL X (version 1.83) (Thomson et al., 1997). Evolutionary distances were calculated using the Kimura two-parameter model. Phylogenetic tree was constructed using the neighbour-joining method with the program MEGA 4 (Kumar et al., 2004). Bootstrap values were calculated on the basis of 1000 replications.

Microorganism and culture conditions

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

Preparation of enzyme source

The isolated *Klebsiella* sp. PRW-1 was grown in the modified Dubos salt medium with 1% CMC, as the sole carbon source, at initial pH, 6.5, 30°C for 3 days, and then a subculture was used once a month and stored at 4°C. *Klebsiella* sp. PRW-1 was grown in Dubos salt medium with carbon sources including carboxymethylcellulose (CMC), avicel, paddy straw, sugarcane barbojo, sugarcane bagasse, grass powder, sorghum husks and corn straw (10 g l^{-1}), at 30°C, for 7 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 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% 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 50°C for 30 min. Exoglucanase (avicelase) activity was determined as the reaction mixture containing 1 ml of enzyme solution with 0.5 ml of 1% avicel cellulose in McIlvaine's buffer was incubated, at 50°C, for 2 h, and adding 1 ml of dinitrosalicylic acid reagent (Saratale et al., 2012). 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 50ºC temperature for 10 min. The enzyme reaction was stopped by addition of 2 ml of 1 M sodium carbonate. One unit of β-glucosidase activity is defined as that amount of enzyme which will hydrolyze 5 mM of PNPG per min (Lymar et al., 1995). Filter paper (FPase) activity was measured according to IUPAC recommendations. FPase activity was determined by measuring the reducing sugars produced from Whatman no. 1 filter paper (50 mg, 1×6 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, 2008).

Xylanase activity was determined in a reaction mixture containing 1 ml of enzyme solution diluted in McIlvaine's buffer with 1 ml of 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 aqueous suspension of 1% starch at 50°C for 10 min (Anto et al., 2006). In these enzymes test the reaction was terminated by adding 1 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 microgram of reducing sugar from the substrate per minute.

Establishment of optimum operational conditions for cellulolytic enzyme activity

The optimum temperature and pH of the cellulolytic enzymes (endoglucanase, exoglucanase, FPU, β-glucosidase, xylanase and glucoamylase) for *Klebsiella* sp. PRW-1 in the presence of agricultural wastes (10 g l^{-1}) were determined through the incubation of the enzyme and 1% (w/v) substrate at different temperature as 60, 70, 80 and 90°C, keeping constant pH 5.0 for 1 h. The effects of different pH range, from 2 to 10, at 50°C, on the cellulolytic enzymes activities were determined. The hydrolytic efficiency of *Klebsiella* sp. PRW-1 in the presence of different agricultural cellulosic substrates (paddy straw, wheat straw, sugarcane barbojo, sugarcane bagasse, grass powder, sorghum husks and corn straw, at about 10 g l^{-1}) was

determined by measuring the reducing sugar and cellulolytic enzymes production.

Effects of various metal additives on cellulolytic enzyme activity

The effect of various metal ions on the cellulolytic enzyme activity was determined, through the application 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 was incubated under optimum temperature (at 30°C and pH of 5.0), being the residual activity of each sample quantified relatively to the control containing no metal ions in the reaction mixture.

Effects of commercial detergents on cellulolytic enzyme activity

The effect of different commercial detergents on the cellulolytic enzyme activity were determined, applying Ariel (Procter & Gamble), Rin (Hindustan, Unilever), Surf excel (Hindustan, Unilever), Tide (Procter & Gamble) and Wheel (Hindustan, Unilever). To simulate washing conditions these detergents were diluted in distilled water up to a final concentration of 7.0 mg mL^{-1} and then boiled for 60 min, for the inactivation of enzymes present in detergent (De Lima et al., 2005). For the determination of the effect of detergent on the cellulolytic enzyme activity, *Klebsiella* sp. PRW-1 supernatant was mixed with the detergents solution (1:1) and incubated under optimum temperature and pH of 5.0 for 1 h. The residual activity of each sample was then quantified with control containing no detergent in the reaction mixture.

FTIR analysis

The FTIR spectra were considered to examine the functional group changes occurred due to the cellulolytic degradation of agricultural wastes material used. FTIR spectra were recorded by the FTIR spectrometer (Perkin Elmer, Spectrum one B; Shelton, WA). The FTIR analysis was performed on the microbial degraded agricultural wastes (after 7 days of incubation), being compared with control agricultural wastes material. The cellulolytic degraded grass powder, sorghum husks and sugarcane barbojo was collected and washed with distilled water for removal of bacterial cells, bound enzymes and reducing sugars. The cellulolytic degraded agricultural wastes were than dried overnight in an oven at 60ºC. The dried samples of cellulosic materials were embedded in KBr pellets with ca.1% of samples in KBr. The background FTIR spectra were obtained using a pure KBr pellet without any agricultural waste material. The FTIR spectra were recorded in the absorption band mode in the range of 4000 – 400 cm^{-1} with a resolution of 4 cm^{-1} and 32 scans.

Statistical analysis

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

Results and Discussion

Isolation and identification of cellulolytic strain

Colonies of PRW-1 strain on CMC agar plates were white colour, shiny circular colonies grows well within 3 days of incubation. Microscopic examination showed that the isolate was Gram-negative rod and non motile. The nearly full length sequence of 16S rRNA gene for isolate PRW-1, determined and based on the sequence identity of 16S rRNA gene against the Gen Bank database, indicates that the isolate was closely related to the members of the genus *Klebsiella*. The phylogenetic analysis showed about 99.4% similarity between the isolated PRW-1 strain and the *Klebsiella variicola* HUB-IV-005 strain (Figure 1). On the basis of the 16S rRNA sequence, the isolated strain was identified as *Klebsiella* sp. PRW-1. The biochemical characteristics of bacterial isolate is shown in Table 1.

Optimization of growth conditions

The *Klebsiella* sp. PRW-1, incubated in Dubos containing CMC as a sole carbon source, displayed a higher cellular growth, cellulolytic enzymes and reducing sugar production after 6 days of incubation. Better growth of *Klebsiella* sp. PRW-1 was observed under static condition, as compared with under shaking condition (120 rpm) (Figure 2). Thus, for a better cellular growth, the static condition was adopted. This condition was used for the investigation of the cellulolytic enzymes production by *Klebsiella* sp. PRW-1 in the following experiments. The better growth and cellulolytic enzymes production, was observed at 30ºC and initial Dubos medium pH of 6.5 in all cellulosic substrates used in this study.

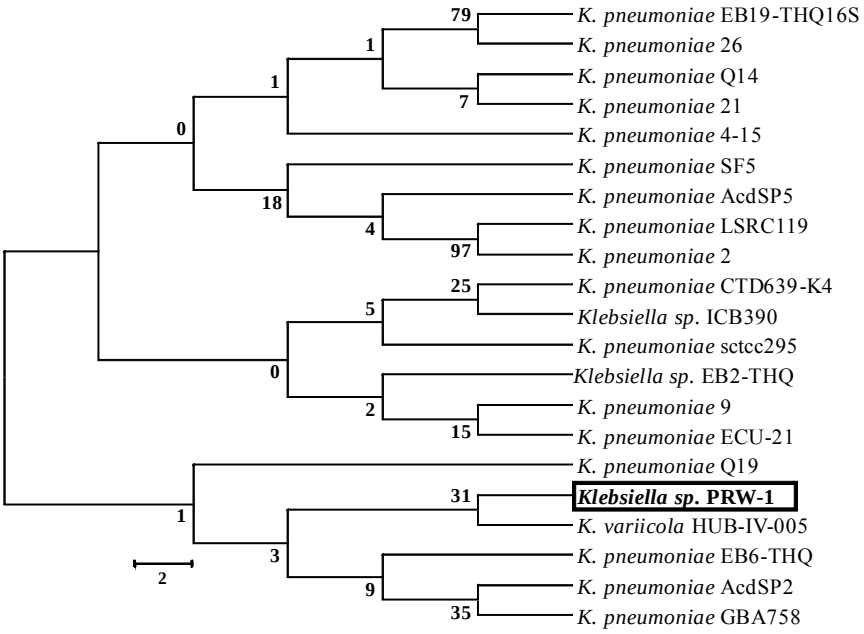

Figure 1. Neighbor-joining showing phylogenetic positions of *Klebsiella* sp. PRW-1 and *Klebsiella* species based on 16S rRNA gene sequence comparisons. *K. pneumoniae* SF5 was used as an out group Bootstrap values are indicated at nodes.

Effects of incubation time on cellulase production

Under the optimum culture conditions, the detailed growth study of *Klebsiella* sp. PRW-1 and the production of cellulolytic and hemicellulolytic enzymes, at different incubation time, showed maximum cellulolytic enzyme activities in Dubos medium containing different cellulosic substrates. The maximum enzyme activities were obtained when it entered in the late logarithmic growth phase (after 6th days of incubation) and continued to secrete enzyme also during stationary phase of growth (Figure 3). After 7 days of incubation *Klebsiella* sp. PRW-1 produced maximum reducing sugar in different cellulosic substrates. These results suggested that *Klebsiella* sp. PRW-1 produced different cellulolytic enzyme activities, which work synergistically on the hydrolysis of cellulosic substrates. The enzyme activities for endoglucanase and glucoamylase were found to be higher in sugarcane barbojo and grass powder at 6th day of incubation (Figure 3 (A) (a) and (A) (c)). The FPU activity remained higher in the grass powder and sugarcane barbojo, at the 6th day, as compare to the pure substrate CMC (Figure 3 (B) (b)).

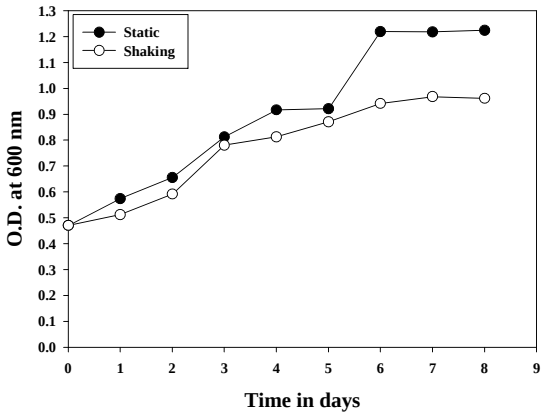

Figure 2. Growth study of *Klebsiella* sp. PRW-1 in Dubos media broth with CMC (10 g l^{-1}).

Table 1. Biochemical characteristics of isolated bacterial strain *Klebsiella* sp. PRW-1.

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

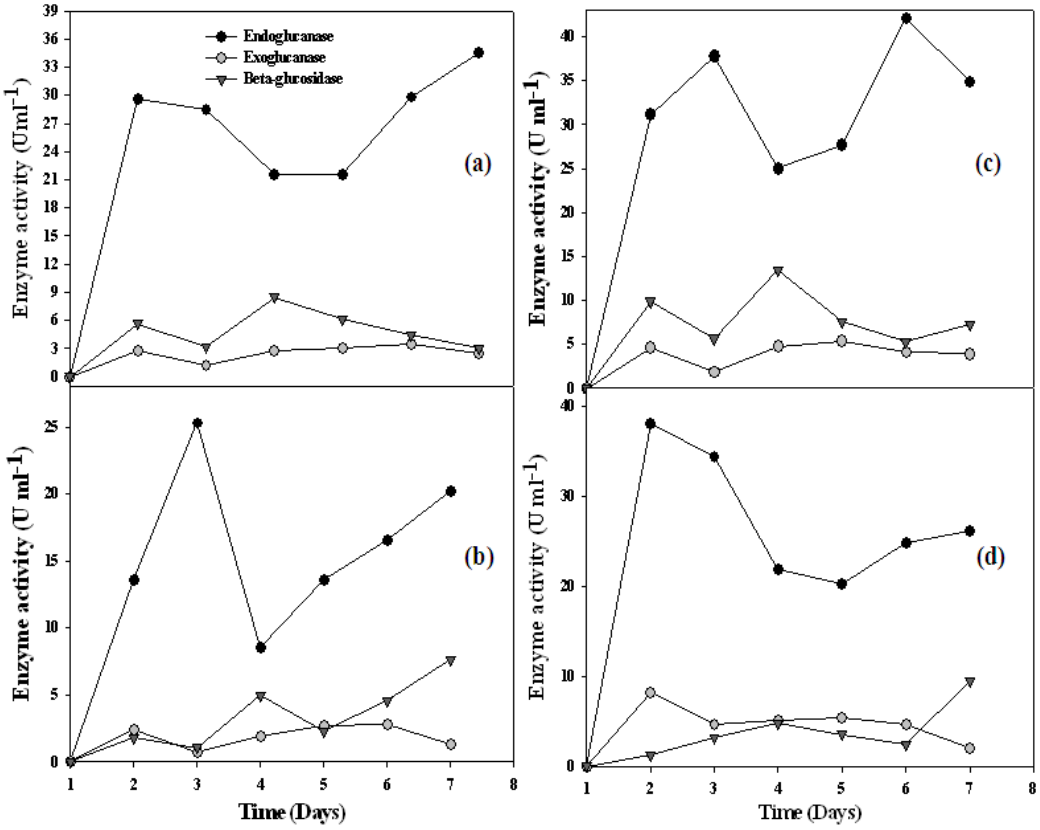

Figure 3 (A). Study of incubation time on endoglucanase, exoglucanase and β-glucosidase production by Klebsiella sp. PRW-1 (a) Sugarcane barbojo; (b) Sorghum husks; (c) Grass powder, (d) CMC.

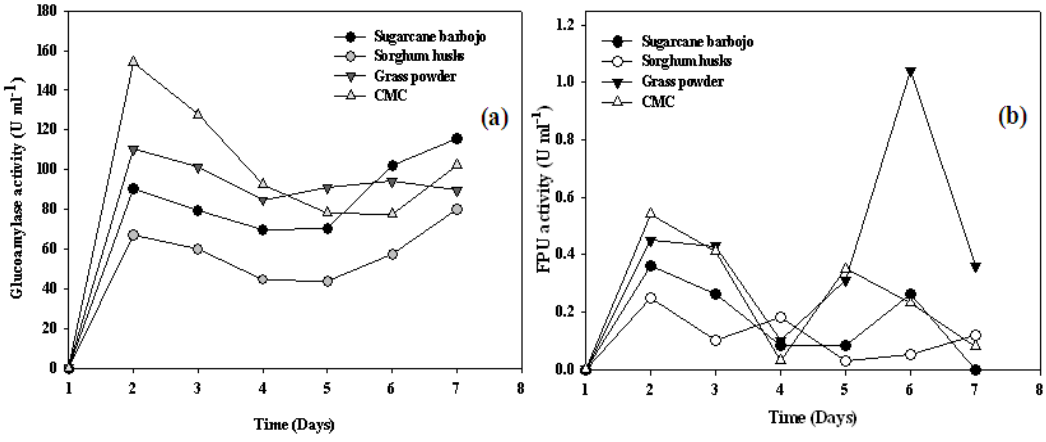

Figure 3 (B). Study of incubation time on cellulase enzyme produced by *Klebsiella* sp. PRW-1 (a) Glucoamylase (b) Filter paperase.

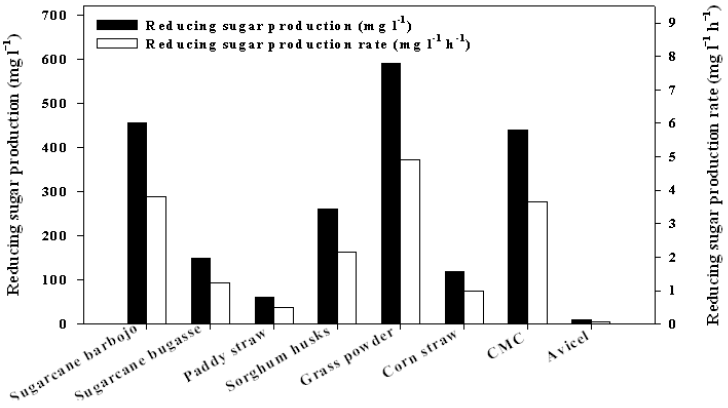

Figure 4. Effect of different cellulosic substrates on cellulose hydrolysis by *Klebsiella* sp. PRW-1 in Dubos media supplemented with 10 g l^{-1} of each cellulosic substrate at 30ºC, with initial pH 6.5 under static condition after 7 days incubation.

Effects of different cellulosic substrates on the production of reducing sugar and cellulolytic enzymes by *Klebsiella* sp. PRW-1

The isolated *Klebsiella* sp. PRW-1 could utilize different cellulosic materials (pure and agricultural wastes including; CMC, avicel, paddy straw, sugarcane barbojo, sugarcane bagasse, grass powder, sorghum husks and corn straw). The study by Zhang et al. (2006) showed a positive correlation between the production of cellulase enzyme from cellulolytic microorganisms and different composition of cellulose, hemicelluloses and lignin in different cellulosic materials. In this study we have determined the hydrolytic efficiency of *Klebsiella* sp. PRW-1 grown on the Dubos medium by taking CMC, avicel, paddy straw, sugarcane barbojo, sugarcane bagasse, grass powder, sorghum husks and corn straw 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 cellulosic substrates could be hydrolyzed by *Klebsiella* sp. PRW-1 and produces reducing sugars. Hydrolysis of sugarcane barbojo and grass powder exhibited maximum reducing sugar production rate than that of other cellulosic materials used. Among the different agricultural wastes materials, hydrolysis of grass powder gave the best hydrolysis efficiency with a maximum reducing sugar production (590 mg l^{-1}) and reducing sugar production rate (4.91 mg h^{-1} l^{-1}). After hydrolysis of other cellulosic materials such as sugarcane barbojo, CMC, sorghum husks, sugarcane bagasse, corn straw, paddy straw and avicel exhibited reducing sugar production (455, 440, 260, 150, 120, 60, 10 mg l^{-1}) and reducing sugar production rate (3.79, 3.66, 2.16, 1.25, 1.0, 0.5, 0.083 mg h^{-1} l^{-1}), respectively was observed (Figure 4).

The enzymatic hydrolysis of cellulosic feedstock has several advantages, namely mild experimental conditions, less energy consumption and avoidance of pollution over the chemical processes (Saratale et al., 2008; Zhang et al., 2006). *Klebsiella* sp. PRW-1 has ability to utilise and metabolize different cellulosic substrates for their growth and expresses multiple cellulolytic enzyme activities (e.g., endoglucanase, exoglucanase, β-glucosidase, glucoamylase and xylanase), mainly at extracellular location. In the presence of grass powder *Klebsiella* sp. PRW-1 produces the higher cellulolytic enzyme activities, while other carbon source used substrates showed moderate enzyme activities (Table 2).

Table 2. Cellulolytic and hemicellulolytic enzyme activity of *Klebseilla* Sp. PRW-1 produced extracellularly after 7 days incubation in Dubos media containing different cellulosic substrates.

Cellulosic substrate	Endoglucanase[a]	Exoglucanase[a]	Glucoamylase[a]	Xylanase[a]	FPU[a]	β-glucosidase[a]
Sugarcane barbojo	34.62 ± 0.01	2.43 ± 0.02	115.83 ± 1.18	34.24 ± 0.78	NA	3.00 ± 0.17
Sugarcane bagasse	19.44 ± 0.08	0.23 ± 0.02	62.31 ± 0.63	33.61 ± 0.78	NA	NA
Corn straw	21.04 ± 0.10	0.97 ± 0.02	45.54 ± 0.99	45.65 ± 1.03	NA	NA
Paddy straw	31.42 ± 0.16	0.73 ± 0.02	46.33 ± 0.93	38.04 ± 1.06	NA	NA
Grass powder	34.88 ± 0.04	3.96 ± 0.93	89.47 ± 0.93	51.36 ± 0.07	0.36 ± 0.01	7.24 ± 0.17
Sorghum husks	20.24 ± 0.02	1.33 ± 0.14	79.89 ± 1.04	24.73 ± 0.75	0.12 ± 0.01	7.60 ± 0.17
Carboxymethyl cellulose	26.10 ± 0.08	2.03 ± 0.10	102.25 ± 0.89	53.90 ± 0.78	0.08 ± 0.01	9.54 ± 0.14

Values are mean of three experiments, SEM (±), and by one-way ANOVA with Tukey-Kramer Multiple Comparisons Test; [a]Enzyme activity (U ml^{-1}); NA- No activity.

The maximum the cellulolytic activity of endoglucanase (34.88 U ml^{-1}), exoglucanase (3.96 U ml^{-1}) and FPU (0.36 U ml^{-1}) were observed in the presence of grass powder, as the carbon source in the Dubos media. The hemicellulolytic enzyme activity of glucoamylase (115.83 U ml^{-1}) and xylanase (51.36 U ml^{-1}) was observed when sugarcane barbojo and grass powder was used as carbon source, respectively. Similarly higher exoglucanse, β-glucosidase, xylanase and glucoamylase enzyme activities were detected in the presence of sugarcane barbojo and sorghum husks (Table 2). For the low cost and maximum production of cellulase enzyme different agricultural lignocellulosic wastes were further used as a carbon sources by *Klebsiella* sp. PRW-1. The enzymatic hydrolysis of lignocellulosic biomass produces disaccharides and oligosaccharides, which plays role as strong inducers of cellulases (Kapdan and Kargi, 2006; Saratale et al., 2012). When grass powder was use as a carbon source, the production of endoglucanase (34.88 U ml^{-1}), exoglucanase (3.96 U ml^{-1}), FPU (0.36 U ml^{-1}), β-glucosidase (7.24 U ml^{-1}), xylanase (51.36 U ml^{-1}) and glucoamylase (115.83 U ml^{-1}) was observed, which increases the applicability of this strain (Table 2). In the same contest, production of different lignocellulolytic enzymes from *Lentinus edodes* (Berk.) Sing. has been carried out by Ramkumar et al. (2011). Also Zambare (2011) with the help of response surface methodology, optimized amylase enzyme production from *Bacillus* sp. by using cellulosic material.

Effects of the temperature on the activity and stability of multiple cellulolytic enzymes produced by *Klebsiella* sp. PRW-1

The production of microbial cellulase has long been known to be influence by different factors like the strain type, reaction conditions and substrate types, since the relationship of these factors might affect the production of the cellulase enzymes (Zhang et al., 2006). The cellulases are relatively costly enzymes, and for their commercial use the production cost of cellulase should be significantly reduce for the preparation of cellulosic feedstock. The cellulolytic enzymes (exoglucanase, endoglucanase) and hemicellulolyitc enzymes (glucoamylase and xylanase) by *Klebsiella* sp. PRW-1, in the presence of different pure and agricultural cellulosic substrates, were studied at different temperatures (50–90ºC). These enzyme produced by *Klebsiella* sp. PRW-1 showed the higher performance at 50ºC, 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. The endoglucanase and glucoamylase produced in the presence of sugarcane barbojo and sorghum husks are found to be thermostable since it retains 50-60% of its activity. Whereas, in the presence of sorghum husks, enzymes which are produced maintain more than 60% initial activity at higher temperature (80ºC). The glucoamylase and xylanase enzymes produced in grass powder and CMC showed thermo stability up to 80-90% at 70ºC (Figure 5). The thermo stable nature of cellulolytic enzymes added advantages to the lignocelluloses bioconversion processes so that it

remains viable and active at higher temperature. At higher operation temperature of stability of the enzymes significantly influence the bioavailability and solubility of organic compounds. Because of these enzymes can efficiently degrade of cellulosic biomass and increased flexibility with respect to process configuration, and overall improves the economy of the process (Viikari et al., 2007). The thermo stability of this enzyme indicates industrial applicability in the food, sugar, fuel ethanol and agricultural industries where process operation applied with higher temperature (Jang and Chen, 2003).

Effects of pH on the activity and stability of multiple cellulolytic enzymes produced by *Klebsiella sp*. PRW-1

The effect of pH on endoglucanase, exoglucanase, β-glucosidase, glucoamylase and xylanase enzyme activity was studied at different pH (2–10) by maintaining similar standard assay conditions (Figure 6). The optimum pH for these enzymes produced by *Klebsiella* sp. PRW-1 was found to be 5 at temperature 50ºC and in the presence of all cellulosic substrates. The endoglucanse enzyme produced by *Klebsiella* sp. PRW-1 was found to be less stable and residual enzyme activity was only upto 20-30% than its initial activity after incubation at 50ºC for 1 h. The glucoamylase produced using the sugarcane barbojo and sorghum husks remained stable up to 70-80%, at alkaline pH 8. While the xylanase produced in the presence of grass powder showed higher alkalotolerance by retaining more than 70% initial activity of at higher pH 8. The ability of retaining the higher enzyme activity at different pH range is a potentially useful property in the processes employing alkaline delignification.

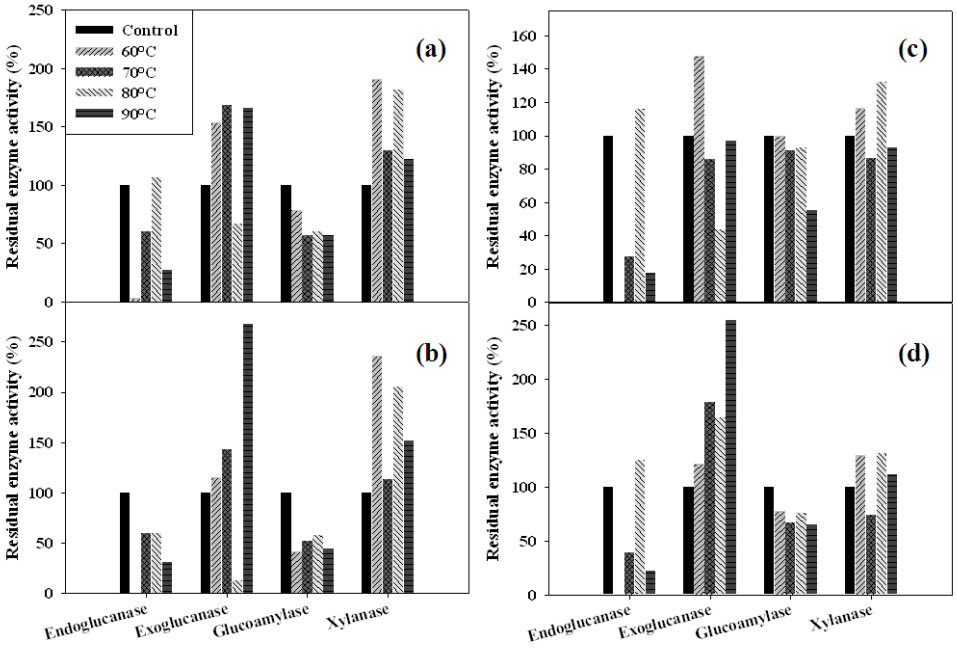

Figure 5. Thermal stability study of endoglucanase, exoglucanase, glucoamylase, xylanase and FPU produced by *Klebsiella* sp. PRW-1 using (a) Sugarcane barbojo, (b) Sorghum husks, (c) Grass powder and (d) CMC.

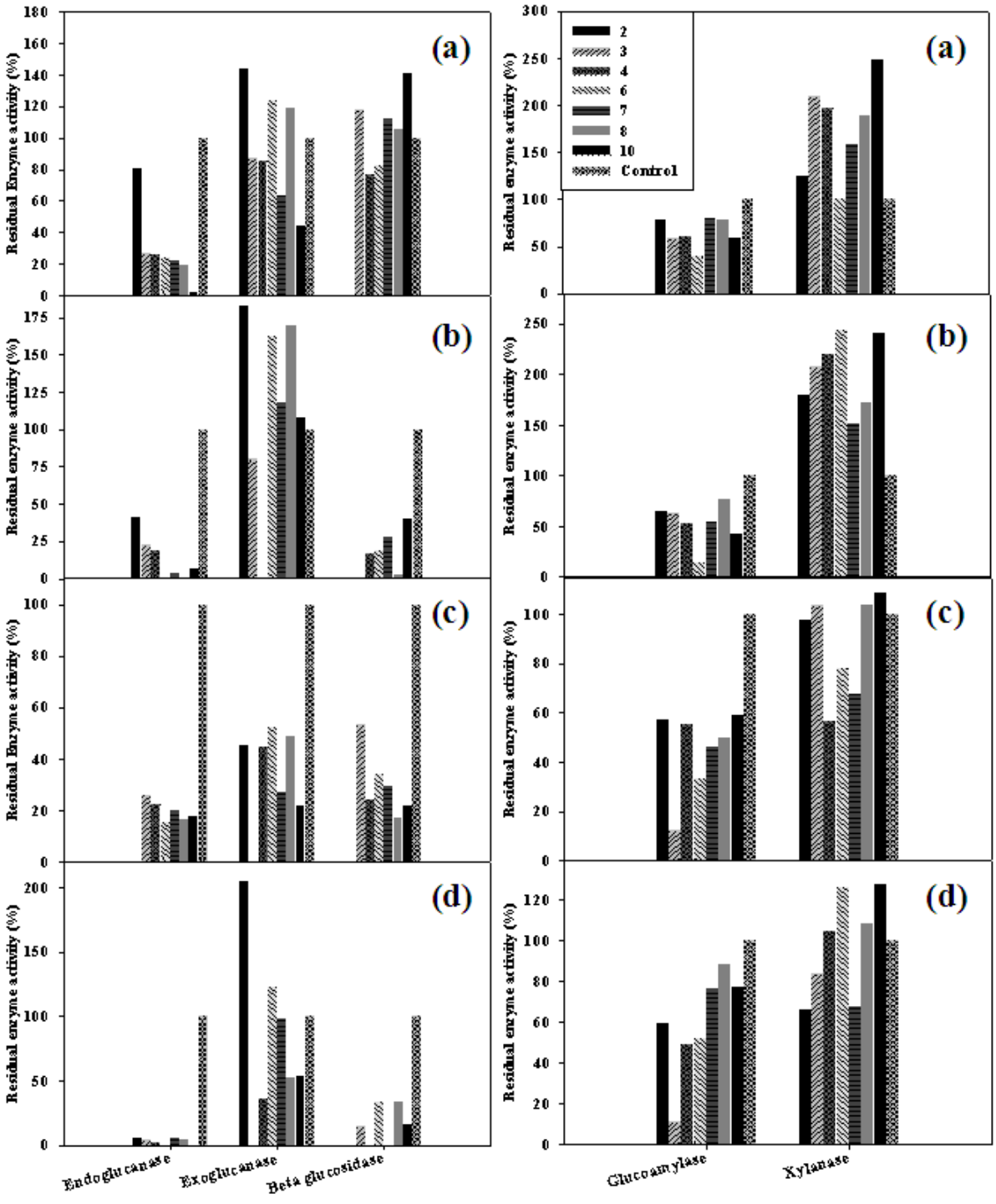

Figure 6. Study of pH stability of cellulase and hemicellulase enzymes produced by *Klebsiella* sp. PRW-1 using (a) Sugarcane barbojo; (b) Sorghum husks; (c) Grass powder and (d) CMC.

Table 3. Effects of different metal additives on endoglucanse, exoglucanse, glucoamylase, xylanase and β-glucosidase enzyme production by *Klebsiella* sp. PRW-1 in the presence of different cellulosic substrates.

Cellulosic substrate	Enzymes	Metal ion concentration (5 mM)						
		Control	$CoCl_2$	$FeCl_3$	$HgCl_2$	$CaCl_2$	$MnCl_2$	$ZnSO_4$
Sugarcane barbojo	Endoglucanase	100	696.30	244.44	NA	NA	774.07	340.74
	Exoglucanase	100	149.57	103.48	74.78	102.61	251.30	106.09
	Glucoamylase	100	277.59	368.97	NA	118.97	370.69	125.86
	Xylanase	100	150.96	75.96	NA	42.31	137.50	72.12
	β-glucosidase	100	110.34	1196.5	100.00	186.21	1041.3	172.41
Grass powder	Endoglucanase	100	204.11	228.08	NA	102.74	288.36	101.37
	Exoglucanase	100	118.32	134.35	61.83	95.42	209.92	118.32
	Glucoamylase	100	140.88	187.59	NA	77.37	229.20	76.64
	Xylanase	100	176.74	113.18	NA	38.76	198.45	113.95
	β-glucosidase	100	185.37	895.12	182.93	178.05	1292.6	214.63
Sorghum husks	Endoglucanase	100	338.24	292.65	360.29	80.88	535.29	76.47
	Exoglucanase	100	377.27	227.27	NA	68.18	854.55	377.27
	Glucoamylase	100	308.22	363.01	NA	165.75	426.03	135.62
	Xylanase	100	153.44	93.13	106.11	89.31	122.90	158.02
	β-glucosidase	100	96.30	1096.3	92.59	92.59	1355.5	166.67
Carboxymethyl cellulose	Endoglucanase	100	461.11	88.89	NA	NA	666.67	266.67
	Exoglucanase	100	238.64	172.73	14.77	159.09	322.73	167.05
	Glucoamylase	100	244.93	227.54	NA	152.17	314.49	111.59
	Xylanase	100	205.62	95.51	NA	NA	211.24	117.98

Effects of different metal additives on cellulolytic enzymes produced by *Klebsiella sp*. PRW-1

Influential studies of metal ions on enzyme are very important because it increases enzymes industrial applications. It has been previously reported that some metals acts as a cofactor of cellulase, inducing or inhibiting the amino acids of the active site of the enzymes (Haki and Rakshit, 2003). The enzyme activities of cellulase produced by *Klebsiella* sp. PRW-1 were assayed under standard optimal conditions in the presence of several metal supplements (at concentration 5 mM each) (Table 3). With the addition of 5 mM $CoCl_2$, $MnCl_2$ and $FeCl_3$ all cellulolytic enzyme activities were sharply induced in the presence of all cellulosic substrates. The enzymes produced by *Cellulomonas biazotea* NCIM-2550, *Streptomyces* sp. MDS showed the induction in $MnCl_2$, $CoCl_2$ and $FeCl_3$, respectively (Saratale et al., 2008; Saratale et al., 2012). The similar results were found to be induced due to the metal additives like $FeCl_3$, $CoCl_2$ and $MnCl_2$ in all enzyme activities in the presence of all cellulosic substrates used in this study. However addition of $HgCl_2$ showed substantial inhibitory effect on all enzyme activities in the presence of all cellulosic substrates (Table 3).

Effects of the commercial detergents on the activity and stability of multiple cellulolytic enzymes produced by *Klebsiella* sp. PRW-1

The study of commercial detergents on the cellulolytic enzyme stability increases its applicability in industry. The enzyme activity of endoglucanase produced by *Klebsiella* sp. PRW-1 using sorghum husks, sugarcane barbojo and grass powder was inhibited in the presence of all the tested detergents. While exoglucanase produced by *Klebsiella* sp. PRW-1 utilising similar substrates retained its activity and stability in the presence of all detergents (Figure 7). The exoglucanase enzyme produced using grass powder retains upto 80% than that of its initial activity. The endoglucanase enzyme found to be totally inhibited in the presence of all commercial detergents used during this study. The xylanase enzyme produced by isolated PRW-1 strain by utilising sorghum husks and CMC shown the inhibition in all detergents. Whereas this enzyme found to be stable in all detergents when enzyme was produced by utilising sugarcane barbojo and grass powder as carbon source. The inhibitory effect of commercial detergent on some enzymes mentioned in above discussion might be because of the active site modifications which alters the substrate specificities (Sukumaran et al., 2005).

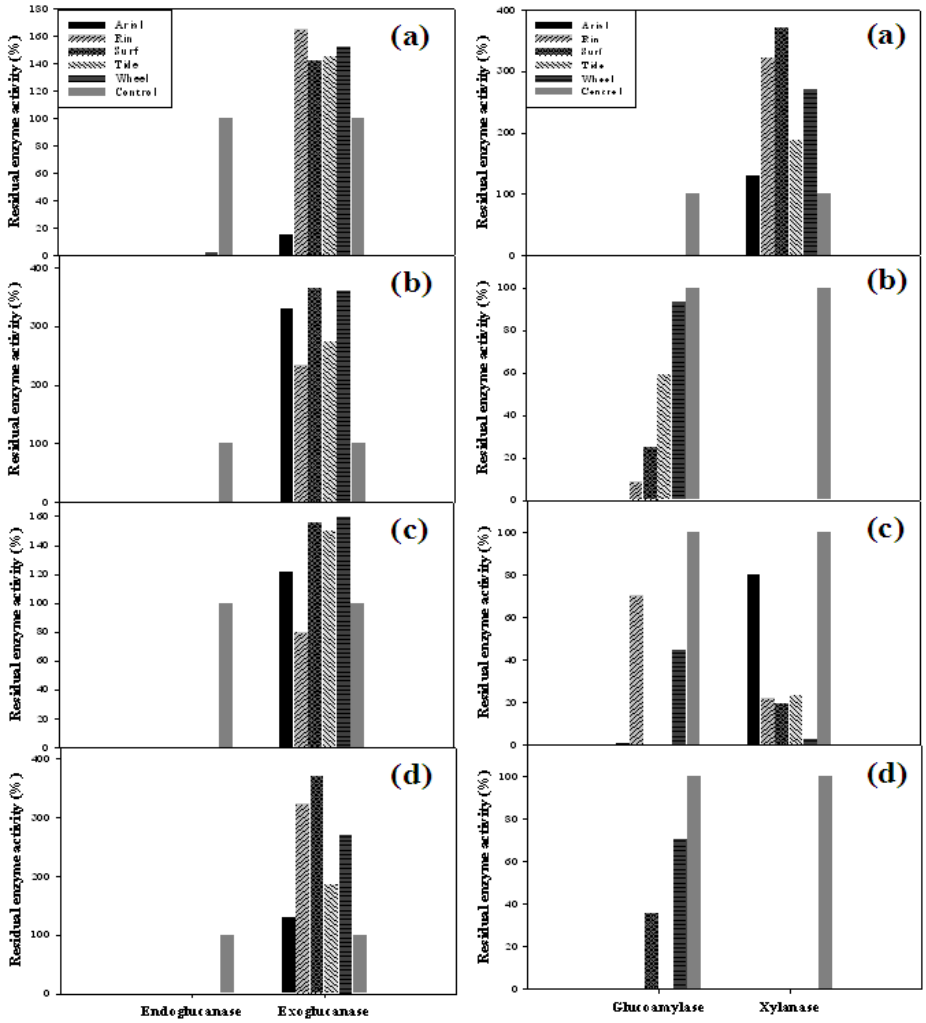

Figure 7. Detergent stability of Cellulase enzyme produced by *Klebsiella* sp. PRW-1 using (a) Sugarcane barbojo; (b) Sorghum husks; (c) Grass powder and (d) CMC.

FTIR analysis

The complex nature of agricultural lignocellulosic waste determines that most of the IR bands were observed in the fingerprint region of FTIR spectra in all substrates. For simplicity the interpretation of IR bands and reflect exact the chemical changes, the four characteristic absorption bands of lignocellulose and two characteristic absorption bands of lignin are used in this study as follows: 1736 cm^{-1} for unconjugated C-O stretch (hemicelluloses), 1372 cm^{-1} for C–H deformation (cellulose and hemicelluloses), 1160 cm^{-1} for C–O–C vibration (cellulose and hemicelluloses), 897 cm^{-1} for C–H deformation (cellulose), 1510 cm^{-1} for aromatic skeletal vibration (lignin) and 1225 cm^{-1} for C–O stretch (lignin) (Pandey and Pitman, 2003).

In the grass powder 1725 cm^{-1} for unconjugated C-O stretch (hemicelluloses), 1508 cm^{-1} for aromatic skeletal vibration (lignin) are removed, 1161 cm^{-1} for C-O-C vibration (cellulose and hemicelluloses) are removed after enzymatic degradation (Figure 8 (a)). While in sorghum husks substrate, the absorbance peak at 1736 cm^{-1} for unconjugated C-O stretch (hemicelluloses) and 1508 cm^{-1} for aromatic skeletal vibration (lignin) are removed after the enzymatic degradation (Figure 8 (b)). In case of sugarcane barbojo 1052 cm^{-1} for C-O stretch in cellulose and hemicellulose was removed while 1107 cm^{-1} for C-OH stretch for secondary alcohol was arised after the enzymatic

degradation (Figure 8 (c)). The removal of most characteristic absorption peaks of cellulose, hemicelluloses and lignin, after the incubation of 7 days showed that the *Klebsiella* sp. PRW-1 has the ability to utilize and metabolise the lignocellulosic biomass as a source of carbon for growth. This showed the potential of *Klebsiella* sp. PRW-1 for cellulolytic degradation of different cellulosic substrates by cellulolytic enzymes produced by it.

Conclusion

Isolated *Klebsiella* sp. PRW-1 strain showed its ability to grow on different cellulosic substrates (pure and agricultural wastes) by producing extracellular cellulolytic and hemicellulolytic enzymes under static submerged condition. Enhancement in the reducing sugar production was observed at the 7th day of incubation using grass powder as substrate. The FTIR analysis confirmes the utilization of cellulosic substrates as a source of carbon. Enzymes produced by *Klebsiella* sp. PRW-1 in presence of different cellulosic substrates showed higher thermal and pH stability which increases the applicability of this strain. The cellulolytic and hemicellulolytic enzymes activities were found to be induced after addition of $MnCl_2$ in the reaction mixture. The findings of this study reveals potential of *Klebsiella* sp. PRW-1 for the preparation of lignocellulosic feedstock will be useful for bioenergy production.

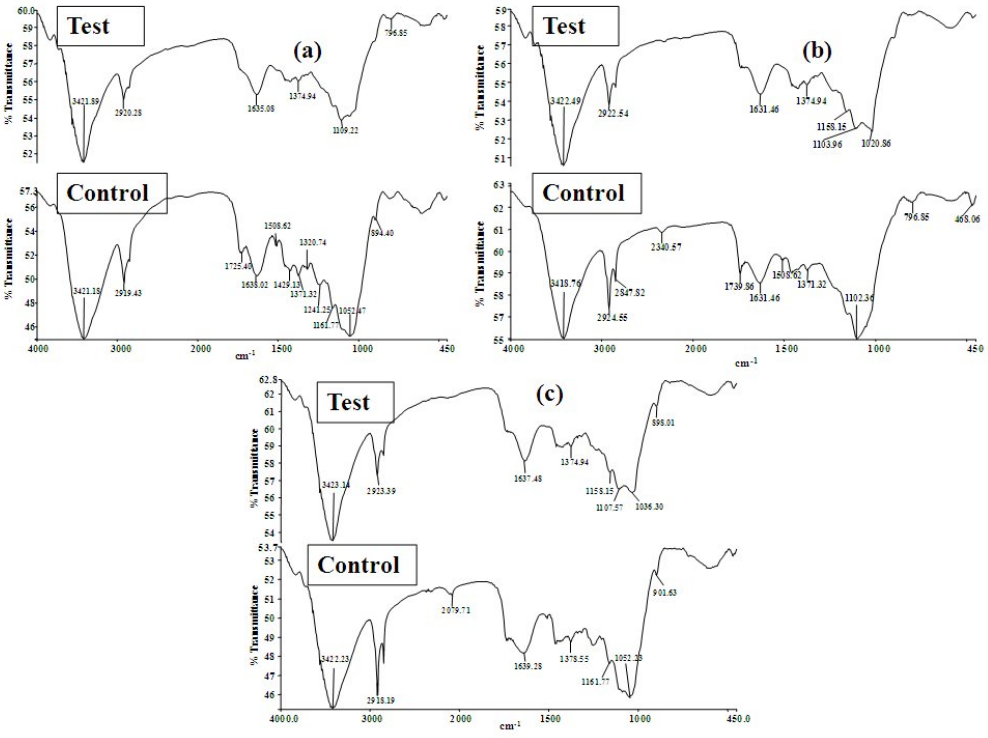

Figure 8. FTIR spectrum of (a) Grass powder; (b) Sorghum husks and (c) Sugarcane barbojo.

Acknowledgements

The author thankfully acknowledges the funding support received from University Grants Commission (41-1263/2012 SR) and Department of Science and Technology (SERC/LS-162/2011), Government of India. The authors would like to thank, Common Facility Center (CFC), Shivaji University, Kolhapur for availing FT-IR facility.

References

Adney, B. and J. Baker. 2008. Measurement of cellulase activities. Tech. report. NREL/TP-510-4262. National Renewable Energy Laboratory of the U. S. Department of Energy. Golden. Colorado. USA.

Anto, H., U. B. Trivedi and K. C. Patel. 2006. Glucoamylase production by solid-state fermentation using riceflake manufacturing waste products as substrate. Bioresour. Technol. 97:1161–1166.

Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. A. Seidman, J. G. Smith and D. J. Struhl. 1997. Current Protocols in Molecular Biology. Unit 24. John Wiley and Sons, New York.

Burchhardt, G. and L. O. Ingram. 1992. Conversion of xylan to ethanol by ethanologenic strains of *Escherichia coli* and *Klebsiella oxytoca*. Appl. Microbiol. Technol. 58:1128-1233.

De Lima, A. L. G., R. P. Nascimento, E. P. S. Bon and R. R. R. Coelho. 2005. *Streptomyces drozdowiczii* cellulase production using agroindustrial by-products and its potential use in the detergent and textile industries. Enzyme Microb. Technol. 37:272-277.

Demain, A. L., M. Newcomb and J. H. D. Wu. 2005. Cellulase, clostridia, and ethanol. Microbiol. Mol. Biol. Rev. 69:124-154.

Fischer, G., E. Hizsnyik, S. Prieler, M. Shah and H. van Velthuizen. 2009. Biofuels and food security Implications of an accelerated biofuels production. Summary of the OFID study, in OFID pamphlet series, 38, Vienna, Austria. 34.

Haki, G. D. and S. K. Rakshit. 2003. Developments in industrially important thermo stable enzymes: a review. Bioresour. Technol. 89:17-34.

James, W. E., S. Jha, L. Sumulong, H. H. Son, R. Hasan, M. E. Khan, G. Sugiyarto and F. Zhai. 2008. Food prices and inflation in developing asia: is poverty peduction coming to an end? Asian Development Bank. Manila. Philippines.

Jang, H. D. and K. S. Chen. 2003. Production and characterization of thermo stable cellulases from Streptomyces transformant T3-1. World J. Microbiol. Biotechnol. 19:263-268.

Kapdan, L. K. and F. Kargi. 2006. Biohydrogen production from waste materials. Enzyme Microbial. Technol. 38:569-582.

Keeney, R. and T. W. Hertel. 2009. The indirect land use impacts of United States biofuel policies: the importance of acreage, yield, and bilateral trade responses (2009-01). Am. J. Agric. Econ. 91(4):895-909.

Kim, J. and S. Yun. 2006. Discovery of cellulose as a smart material. Macromolecules. 39:4202-4206.

Kumar, R., S. Singh and O. V. Singh. 2008. Bioconversion of lignocellulosic biomass: Biochemical and molecular perpectives. J. Ind. Microbiol. Biotechnol. 35:377-391.

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

Levin, D. B., R. Islam, N. Cicek and R. Sparling. 2006. Hydrogen production by *Clostridium thermocellum* 27405 from cellulosic biomass substrates. Int. J. Hydrogen Energy. 31:1496-1503.

Lin, C.-W., D.-T. Tran, C.-Y. Lai, Y.-P. I and C.-H. Wu. 2010. Response surface optimization for ethanol production from *Pennisetum Alopecoider* by *Klebsiella oxytoca* THLC0409. Biomass Bioenergy. 34:1922-1929.

Lo, Y. C., M. D. Bai, W. M. Chen and J. S. Chang. 2008. Cellulosic hydrogen production with a sequencing bacterial hydrolysis and dark fermentation strategy. Bioresour. Technol. 99:8299-8303.

Lo, Y. C., G. D. Saratale, W. M. Chen, M. D, Bai and J. S. Chang. 2009. Isolation of cellulose-hydrolytic bacteria and applications of the cellulolytic enzymes for cellulosic biohydrogen production. Enzyme Microbial. Technol. 44:417-425.

Lymar, E. S., B. Li and V. Renganathan. 1995. Purification and Characterization of a Cellulose-Binding β-Glucosidase from Cellulose-Degrading Cultures of *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. 61(8):2976-2980.

Pandey, K. K., A. J. Pitman. 2003. FTIR studies of the changes in wood chemistry following decay by brown-rot and white-rot fungi. Int. Biodeterior. Biodegrad. 52:151-160.

Puppan, D. 2002. Environmental evaluation of biofuels. Period. Polytech. Ser. Soc. Man. Sci. 10:95-116.

Ragauskas, A. J., C. K. Williams, B. H. Davison, G. Britovsek, J. Cairney, C. A. Eckert, W. J. Jr. Frederick, J. P. Hallett, D. J. Leak, C. L. Liotta, J. R. Mielenz, R. Murphy, R. Templer and T. Tschaplinski. 2006. The path forward for biofuels and biomaterials. Science 311:484-489.

Ramkumar, L., T. Ramanathan and T. Nedumaran. 2011. *In vitro* effect of organic and inorganic additives from the production of radial mycelial growth and lignocellulolytic enzyme in *Lentinus edodes* (Berk.) Sing. Emir. J. Food Agric. 23(1):71-79.

Saratale, G. D. and S. E. Oh. 2012. Lignocellulosics to ethanol: the future of the chemical and energy industry. Afr. J. Biotechnol. 11:1002-1013.

Saratale, G. D. and S. E. Oh. 2011. Production of thermotolerant and alkalotolerant cellulolytic enzymes by isolated *Nocardiopsis* sp. KNU. Biodegradation. 22:905-919.

Saratale, G. D., S.-D. Chen, Y.-C. Lo, R. G. Saratale, J.-S. Chang. 2008. Outlook of biohydrogen production from Lignocellulosic feedstock using dark fermentation - a review. J. Sci. Ind. Res. 67:962-679.

Saratale, G. D., R. G. Saratale, Y. C. Lo and J. S. Chang. 2010. Multicomponent cellulase production by *Cellulomonas biazotea* NCIM-2550 and their applications for cellulosic biohydrogen production. Biotechnol. Prog. 26:406-416.

Saratale, G. D., R. G. Saratale and S. E. Oh. 2012. Production and characterization of multiple cellulolytic enzymes by isolated *Streptomyces* sp. MDS. Biomass Bioen. 47:302-315.

Suen, G., J. J. Scott, F. O. Aylward, S. M. Adams, S. G. Tringe, A. A. Pinto-Tomás, C. E. Foster, M. Pauly, P. J. Weimer, K. W. Barry, L. A. Goodwin, P. Bouffard, L. Li, J. Osterberger, T. T. Harkins, S. C. Slater, T. J. Donohue and C. R. Currie. 2010. An insect herbivore microbiome with high plant biomass-degrading capacity. PLoS Genet. 6(8):e1001129.

Sukumaran, R. K., R. R. Singhania and A. Pandey. 2005. Microbial cellulase - production, application and challenges. J. Sci. Ind. Res. 64:832-844.

Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin and D. G. Higgins. 1997. The CLUSTAL X windows interface: exible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25:4876-4882.

Viikari, L., M. Alapuranen, T. Puranen, J. Vehmaanpera and M. Siika-Aho. 2007. Thermostable enzymes in lignocelluloses hydrolysis. Adv. Biochem. Eng. Biotechnol. 108:121-145.

Yu, E. K. C. and J. N. Saddler. 1982. Power solvent production by *Klebsiella pneumoniae* grown on sugars present in wood hemicellulose. Biotechnol. Lett. 4:121-126.

Zambare, V. P. 2011. Optimization of amylase production from *Bacillus* sp. using statistics based experimental design. Emir. J. Food Agric. 23(1):37-47.

Zhang, Y. H. P., M. E. Himmel and J. R. Mielenz. 2006. Outlook for cellulase improvement: screening and selection strategies. Biotechnol. Adv. 24:452-482