Presentations and Publications

List of Presentations:

1. **Thite V. S.**, Patel K. M. and Nerurkar A. S. (2016). Submerged fermentation of crude agrowastes substrates for xylanopectinollytic enzyme(s) production from isolated *Bacillus* spp. Conference paper presented at 57th Annual conference of Association of Microbiologists of India (AMI-2016), Guwahati University, Guwahati, Assam, India.
2. **Thite V. S.** and Nerurkar A. S. (2016). Xylano-pectinolytic accessory enzymes of *Bacillus* spp. from ruminant dung for enzymatic agro-waste biomass saccharification. Conference paper presented at 38th Symposium on Biotechnology for Fuels and Chemicals, organized by Society for Industrial Microbiology and Biotechnology, at Baltimore, Maryland, USA.
3. **Thite V. S.,** Bhaadwaj P. H. Ruchi A. and Nerurkar A. S. (2015). Production of xylano-pectinolytic enzymes from *Bacillus* spp., their substrate specificity and potential for agro-waste saccharification. Conference paper presented at 56th Annual conference of Association of Microbiologists of India (AMI-2015), JNU, New Delhi, India.
4. **Thite V. S.**, Ruchi A. and Nerurkar A. S. (2015). Potential of cellulase free xylano-pectinolytic enzymes from *Bacillus* spp. For agro-waste saccharification", Conference paper presented at International conference on New Horizons in Biotechnology (NHBT-2015) jointly organized by the Biotech Research Society, India, and CSIR-National Institute for Interdisciplinary Science and Technology, (November 2015), Thiruvananthapuram, Kerala, India.
5. **Thite V. S.** and Nerurkar A. S. (2015). Pectinases of *Bacillus* spp., isolated from ruminant dung as potential accessory enzymes for agro-waste saccharification. Conference paper presented at Researcher's Ferret Confabulation (REFECO'15), IIT-Gn. Gujarat, India.
6. **Thite V. S.**, Pandit A. S. and Nerurkar A. S. (2014). Potential of polysaccharide hydrolases of xylano-pectinolytic *Bacillus* spp. isolated from ruminant dung in improvement of enzymatic agro-waste saccharification. Conference paper presented at the 55th Annual Conference of Association of Microbiologists of India (AMI-2014), TNAU, Tamilnadu, India.
7. **Thite V. S.**, Mangalvedhekar M. and Nerurkar A. S. (2013). Quantitative Screening and Identification of Cellulase free Xylanase and Pectinase Producing Bacteria

from Different Ruminant Dung Samples. Conference paper presented at the 54th Annual Conference of Association of Microbiologists of India (AMI-2013), MDU, Rohtak, Haryana, India.

8. **Thite V. S.**, Bhosale M. A. and Nerurkar A. S. (2012). Isolation and Qualitative Screening of Multiple Polysaccharide Hydrolase Producing Bacteria. Conference paper presented at the 53rd Annual Conference of Association of Microbiologists of India (AMI-2012), KIIT, Bhubaneswar, Odisha, India.

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1. **Thite, V. S.**, & Nerurkar, A. S. (2015). Xylanases of *Bacillus* spp. isolated from ruminant dung as potential accessory enzymes for agro-waste saccharification. Letters in applied microbiology, 60(5), 456-466.
2. **Thite, V. S.**, & Nerurkar, A. S. (2018). Physicochemical characterization of pectinase activity from *Bacillus* spp. and their accessory role in synergism with crude xylanase and commercial cellulase in enzyme cocktail mediated saccharification of agrowaste biomass. Journal of applied microbiology, 124(5), 1147-1163.

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ORIGINAL ARTICLE

Xylanases of *Bacillus* spp. isolated from ruminant dung as potential accessory enzymes for agro-waste saccharification

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Significance and Impact of the Study: The uncontrolled use of fossil fuels and concerns about its future availability, have invoked interest over unconventional alternative energy sources like solar, hydro-power, geothermal, nuclear and biomass. Plants, being largest renewable biomass on earth, have received consideration as a source of biofuels. Ruminant dung isolates M35, R31 and J208 belonging to *Bacillus* sp. produces majorly endo-xylanase when induced with wheat bran. Such plant cell wall degrading endo-xylanases with broad pH optima and mesophilic nature can act as accessory enzymes with cellulases to enhance the saccharification of plant biomass in biofuel industries.

Keywords
accessory xylanase, agro-wastes, *Bacillus altitudinis*, *Bacillus safensis*, enzyme cocktail, saccharification.

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Abstract

Polysaccharide hydrolase producing bacteria were isolated for biomass saccharification step in two-step bioethanol production. Xylanolytic bacteria were found to be common in ruminant dung. Seven *Bacillus* dung isolates exhibited high xylanolytic activity; three of which were identified as *Bacillus safensis* and four as *Bacillus altitudinis*, based on 16S rDNA and *gyrB* gene sequencing. Interestingly, comparison of activity profiles for *B. safensis* M35 and *B. altitudinis* R31 and J208 crude xylanases showed activity in similar temperature and pH ranges of 40–60 °C and 6·0–9·0, respectively, even though they were isolated from three different dung sources. Furthermore, 22–28% viscosity reduction of beechwood xylan substrate by all the three xylanases points towards their endo-acting nature. Endo-acting xylanases are envisaged as accessory enzymes which help expose the cellulose fibres for the subsequent action of the core enzyme cellulases. In this study, when supplemented to the commercial cellulase as a cocktail, the accessory role of the crude xylanases from the selected *Bacillus* strains was established as 1·3, 2·33 and 1·9 fold increase in saccharification of barley husk, sugarcane bagasse and wheat husk was achieved, respectively.

Introduction

The term 'Lignocellulosic Biomass' is coined for plant biomass containing complex polysaccharides such as cellulose, a major component followed by hemicellulose and pectin, recalcitrant lignin and some amount of proteins, allowing its worldwide use as largest renewable energy source since centuries (Mckendry 2002). These natural, potentially cheap and abundant polysaccharides are found as agricultural waste (husks and straws of grain plants, corn stalks, etc.), industrial waste (pulp and paper, sugarcane bagasse (SCB), oilseed cakes, etc.), forestry residues, municipal solid waste, etc. (Ramachandran *et al.* 2007; Cardona *et al.* 2010).

Polysaccharides accounts for about ≥50% of the biomass and can be utilized in two steps to produce the alternative fuel, i.e. ethanol. (i) Enzyme catalyzed hydrolysis of lignocellulosic polysaccharides to reducing sugars, and (ii) Fermentation of these sugars to ethanol by yeasts or bacteria. Structural porosity, cellulose fibre crystallinity, lignin and hemicellulose content etc., factors have been identified to affect the cellulose hydrolysis. The noncovalent association of hemicellulose-cellulose microfibrils results in a complex network, where cellulose fibrils are

embedded in lignified hemicellulose network. This hemicellulose covering limits cellulose accessibility to hydrolases (Berlin *et al.* 2007). Lignin and hemicellulose removal, reduction of cellulose crystallinity and increase of porosity by pretreatment processes can significantly improve the hydrolysis (Yoshida *et al.* 2008). Enzymatic digestion of lignocellulosic materials needs synergistic action of a group of different functional enzymes. 'Core enzymes' such as endoglucanases and cellobiohydrolases break down majorly cellulose. 'Accessory enzymes' such as variety of hemicellulases cleave physical shields of hemicelluloses that cover cellulose (Banerjee *et al.* 2010). It has been reported that Celluclast, a commercial cellulase from Novozymes, Denmark, was not sufficient to remove all of the xylan, when used on pretreated lignocellulose substrates. Such deficiencies of cellulases can be compensated with supplementation of noncellulosic accessory hydrolases having appropriate activities with core cellulase mixtures (Qing and Wyman 2011). Therefore, the improved accessibility of cellulose surface to cellulases by the efficacy of these accessory enzymes has been identified as one of the important factors for increasing hydrolytic saccharification.

In this study, isolation of xylanolytic bacteria from lignocellulose digesting ruminant dung or decaying niches was carried out. The best xylanase producers selected were identified as *Bacillus* spp. using molecular techniques and their crude xylanases were studied for various physical parameters. Potential of these xylanases as accessory enzyme to commercial cellulase mediated saccharification of agro-wastes was evaluated.

Results and discussion

Isolation of xylanolytic bacteria

Ruminant dung and manure are reported to harbour a large number of xylanolytic, pectinolytic and cellulolytic micro-organisms (Wei *et al.* 2009; El-shishtawy *et al.* 2014). Accordingly manure samples from farm yard and fresh dung samples of four different ruminants viz., camel, buffalo, cow and bull, fed agro-wastes were collected and subjected to enrichment under selection pressure of polysaccharides like cellulose, xylan and pectin in presence of glucose to obtain polysaccharide hydrolase like xylanase, pectinase and cellulase producing isolates free of catabolite repression as reported in many bacterial system (Beg *et al.* 2000). When nutrient richness and glucose were tapered in subsequent steps of enrichment culture technique, presence of polysaccharides as sole carbon source facilitated the enrichment of polysaccharide hydrolase producing cultures free of catabolite repression. After enrichment, 249 of total bacterial isolates were obtained: 35 from bull dung, 78 from cow dung, 35 from buffalo dung, 36 from camel dung and 65 from manure. Qualitative screening of xylanolytic bacteria was performed on dye tagged pure xylan and crude xylan source wheat bran (WB). As shown in Fig. 1a, hydrolysis of dye tagged xylan particles released the bound dye, covalently linked with xylan giving a clear zone of hydrolysis (Ten *et al.* 2004), while in the case of WB substrate (Fig. 1b) breakdown of -1,4-β D xylosidic bond from xylan decreases the intensity of Congo red in the zone of hydrolysis which when flooded with 1 N HCl gives blue zone of hydrolysis in purple background confirming the xylanase activity (Teather and Wood 1982). Qualitative screening revealed xylanolytic nature of 65 isolates, which were further analyzed based on the clearance zone to colony size ratio (Cz/Cs), and quantified for xylanase activity after induction with pure and crude inducers. Use of different xylan substrates with different types and degrees of substitution offers poor comparability of reported xylanase activities. The methods used for the assay of endoxylanase activity are in most cases reported as based on the release of reducing sugars from partially soluble xylan substrate, hence realistic comparison between the methods in practice is extremely difficult (Bailey *et al.* 1992). Considering that clearance zone is due to both endo- and exo- xylanases, the high Cz/Cs ratio (data not shown) and high enzyme activity when induced by WB and Xylan, were the basis of selection of the seven isolates, M18, M33, M35, R30, R31, J208 and J216 (Fig. 1c) for further studies.

Phenotypic and molecular characterization and identification

Gram-positive nature, endospore formation, rod shape, aerobic and catalase positive tests categorized the seven chosen isolates as *Bacillus* sp. Species level identification was carried out with the help of 16S rRNA gene and *gyrB* gene sequencing analysis. 16S rRNA gene sequence of isolates revealed ≥98% identity with three reference type strains in BLAST analysis, i.e. *Bacillus safensis* FO-36b^T, *Bacillus altitudinis* 41KF2b^T and *Bacillus pumilus* ATCC 7061^T. Since several reports have been published stating that strains with >99% 16S rRNA gene sequence similarity may not belong to the same species (La Duc *et al.* 2004), comparative *gyrB* gene sequence analyses was carried out. The results of phylogenetic trees constructed individually for seven selected strains associated with other reference sequence of type strains retrieved from GenBank, based on 16S rRNA gene sequences (Fig. 1d) and *gyrB* gene sequence (Fig. 1e) showed similar cluster pattern in both cases. Camel dung isolates M18, M33 and M35 clustered with *B. safensis* FO-36b^T whereas buffalo dung isolates J208, J216; and bull dung isolates R30, R31 with *B. altitudinis* 41KF2b^T. *B. pumilus* ATCC 7061^T was

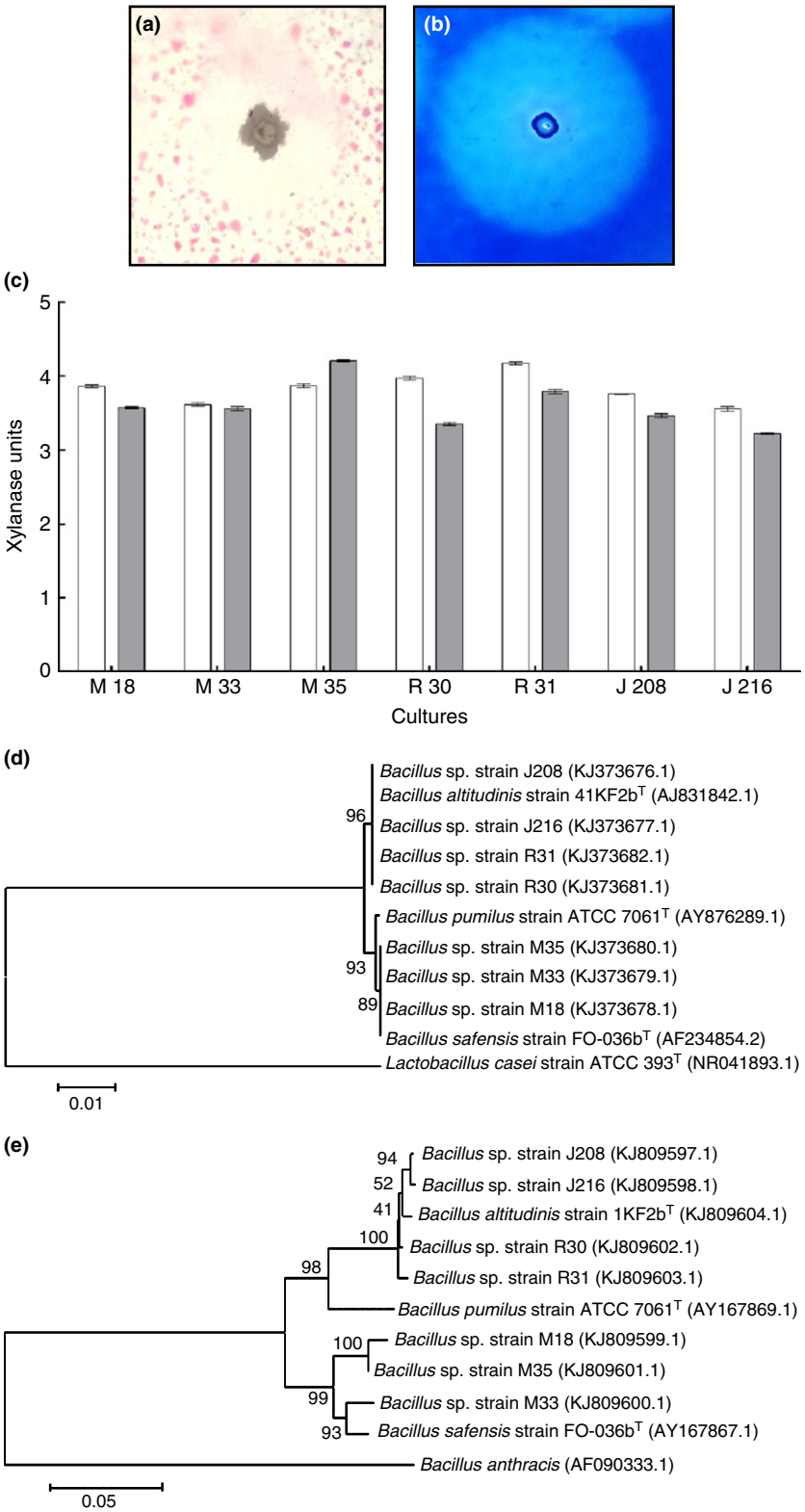

Figure 1 Screening of xylanolytic bacteria and their phylogenetic analysis. Xylanolytic activity by the isolates on (a) Dye tagged xylan medium and (b) Wheat Bran (WB) medium. (c) Crude xylanase activity of the selected cultures after induction by xylan (white bar) and WB (grey bar) (n=3). Phylogenetic tree of isolates and reference type strains based on (d) 16S rRNA gene sequences with *Lactobacillus casei* strain ATCC 393 as an outgroup and (e) *gyrB* gene sequences with *Bacillus anthracis* as an outgroup. Scale bar represents genetic distance in terms of 0·01 substitutions per nucleotide position for (d) and of 0·05 substitutions per nucleotide position for (e). Numbers at nodes indicate percentages of occurrence in 1000 bootstrapped trees. Accession numbers are given in parenthesis.

sorted on a separate clade between two clusters, having a close proximity with *B. safensis* group with respect to 16S rRNA gene analysis whereas with *B. altitudinis* group with respect to *gyrB* gene analysis. Similar kind of results in case of *B. safensis* and in case of *B. altitudinis* have been reported (Satomi *et al.* 2006; Shivaji *et al.* 2006). 16S

rRNA gene sequences and *gyrB* gene sequences for seven isolates and later one for *B. altitudinis* 41KF2b^T were submitted to NCBI GenBank database under the Accession No. KJ373676.1–KJ373682.1 and KJ809597.1–KJ809604.1 respectively.

Effect of pH and temperature on xylanase activity

In preliminary analysis isolates M35, R31 and J208 out of selected seven isolates exhibited xylanase activity over 5·0–9·0 pH range at 40°C and over 30–70°C temperature range at pH 7·0. Xylanases from mesophilic *Bacillus* sp. MX47 has demonstrated the pH and temperature optima of 8·0 and 40°C (Chi *et al.* 2012). Interestingly, the three xylanolytic isolates M35, R31 and J208 earmarked for further studies also possessed appreciable pectinase activity in same range of pH and temperature (data not shown) therefore they were selected for further analysis.

The broad activity range of pH and temperature under study is a useful attribute for agro-waste saccharification. The activity of the crude xylanase from all three cultures was good within 45–60°C temp range and within the pH range 5·0–9·0 when combine effects of pH and Temperature were studied (Fig. 2a–c). Cloned xylanase KRICT PX1 from *Paenibacillus* sp. HPL-001 has shown maximum activity at pH 5·5 and 9·5 at 50 and 45°C, respectively (Hwang *et al.* 2010).

Effect of temperature on xylanase stability

Relative residual xylanase activity with increase in preincubation time and temperature assayed to study the thermal effect on enzyme stability demonstrated that at 60°C, $t_{1/2}$ in case of M35 xylanase was observed to be ~30 min (Fig. 2d), whereas it was ~18 min for R30 and J208. At 50°C (Fig. 2e) the $t_{1/2}$ for J208 xylanase was ~58 min whereas in case of R31 and M35 xylanases it increased gradually. At 40°C, (Fig. 2f) more than 90% M35 xylanase and more than 80% R31 and J208 xylanase relative residual activity was retained up to 150 min. In spite of 45–60°C being the range of maximum activity, extended exposure of the enzymes at more than 50°C resulted in decline in residual activity. Hence, the temperature of 40°C can be preferred for long-term incubation. Notably, similar results have been observed with xylanase from *Bacillus* sp. SV-34S within 15 min of preincubation at different temperatures showed the relative residual activity of about 80% till 60°C (Mittal *et al.* 2013).

Effect of pH on xylanase stability

Relative residual xylanase activity with increase in preincubation time up to 24 h with various pH buffers at 40°C was assayed to study the pH effect on enzyme stability. M35 xylanase (Fig. 2g) was found to be most stable at pH 6–9 and also in native form. More than 70% activity was retained after 18 h and ~50% of its residual activity after 24 h. R31 xylanase was found to be least stable. As shown in Fig. 2g–i pH 8·0 and pH 7·0 were found to be most stabilizing for xylanases from all three sources. In another similar findings, recombinant xylanase XynE2 was shown to be stable over a broad pH range, retaining more than 80% of the initial activity after incubation in buffers of pH 4·6–pH 12·0 at 37°C for initial 1 h (Wang *et al.* 2010).

Time course studies of production of xylanase

Xylanase production was observed within 12 h of induction and attaining maximum level of activities in all three cultures at 24 h, thereafter there was gradual decrease in production which is least in case of M35 (Fig. 3a). Such reduction in xylanase yield could be due to nutrients depletion or the proteolysis activity as all three strains are protease positive. In a study with *B. altitudinis* DHN8, highest xylanase production was obtained at 42 h of incubation and then it declining gradually thereafter (Adhyaru *et al.* 2014).

Viscosity measurement

If an enzyme has a purely exo-activity, unless it is present in high amount it cannot lead to the sudden decrease in viscosity within short time period. The increase in reducing sugar by all the three xylanases from 0·4 to 2·0 μmol of xylose equivalent per ml in initial 10 min time corresponds to an average 25–28% decrease in viscosity (Fig. 3b). M35 xylanase decreased viscosity faster as well as released more of reducing sugar than the other two enzymes. Similar result was observed in case of thermostable endo-xylanase from *Bacillus stearothermophilus* T-6 (Khasin *et al.* 1993). Cleavage of polymer at endo- position only can lead to sudden decrease in viscosity. Both, rate of increase in released reducing sugar (enzyme activity) and rate of decrease in viscosity, tend to decrease with gradual increase in time because, breakdown of polymer length significantly decreases available endo-action sites for enzymes establishing endo-acting nature of all three xylanases.

Influence of crude xylanase supplementation in commercial cellulase-mediated saccharification of lignocellulosic substrate

Proximate chemical composition of three different agro-wastes without any pretreatment in terms of % Cellulose,

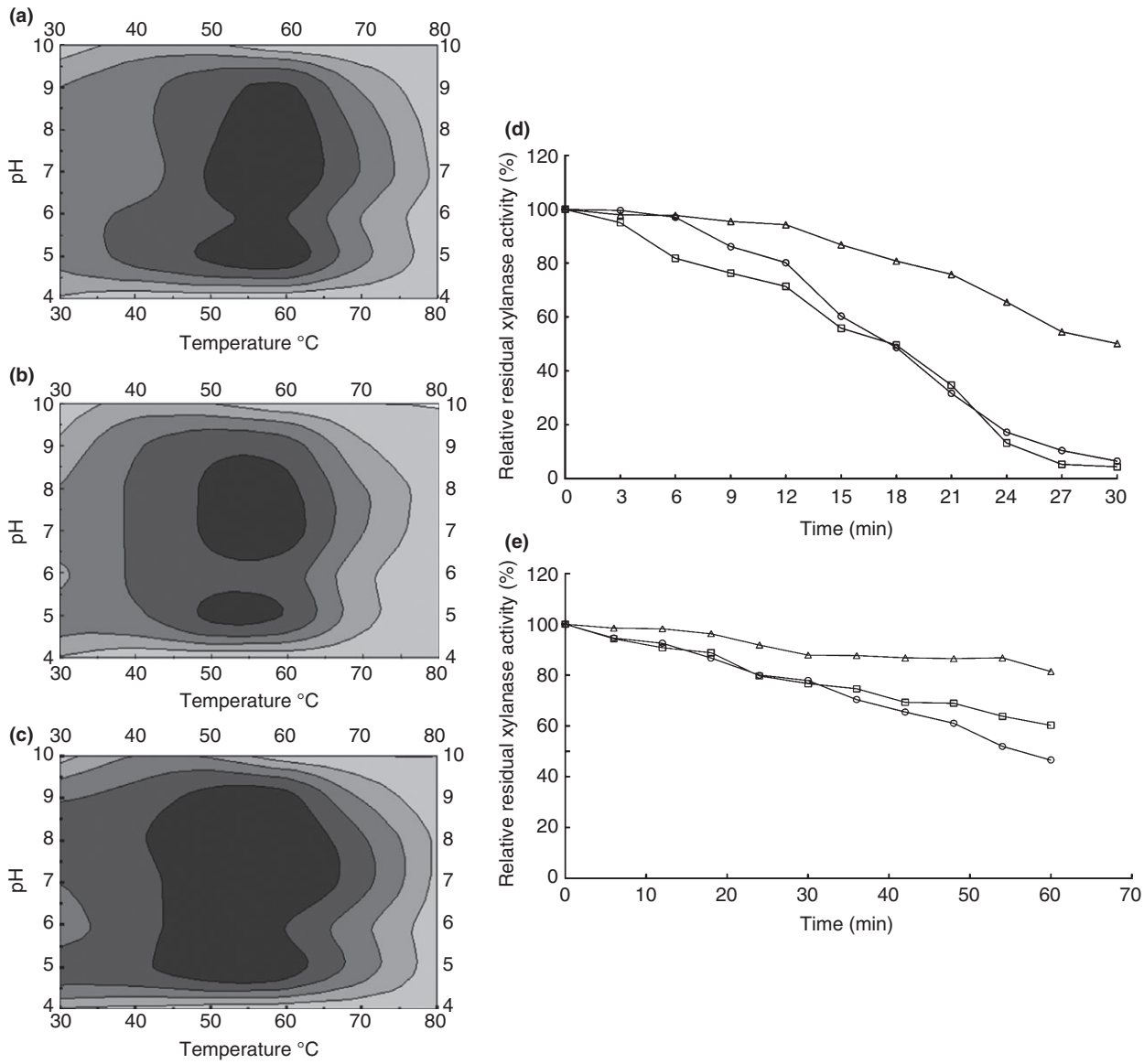

Figure 2 Effect of temperature and pH on enzyme activity (n=3): combined effect of temperature (*X*-axis) and pH (*Y*-axis) on xylanase activity (*Z*-axis) of (a) M35, (b) R31 and (c) J208 represented in terms of units (as six shades of gray, in increasing order with units <0, 0–1, 1–2, 2–3, 3–4, >4). Effect of temperature (d) 60°C, (e) 50°C and (f) 40°C on stability of xylanases from M35(open triangle), R31(open square) and J208 (open circle); Effect of pH 4·0 (close circle), 5·0 (open square), 6·0 (close square), 7·0 (open triangle), 8·0 (close triangle), 9·0 (open diamond), 10·0 (close diamond), on stability of xylanases from (g) M35, (h) R31 and (i) J208, in comparison with un-buffered (open circle) xylanase.

Hemicellulose and Lignin are as follows: barley husk (BH) 39, 12 and 22 (Bledzki *et al.* 2010a); SCB 43, 31 and 12 (Martin *et al.* 2007); wheat husk (WH) 36, 18 and 16 (Bledzki *et al.* 2010b). Because of such diverse compositions of agro-waste residues, the effectiveness of each enzyme on crude plant material was studied. Broad pH optima, endo-acting and mesophilic thermal nature of enzyme, would be considered as attributes of an accessory enzyme in biofuel industry. Activity of cell-free supernatant from individual cultures on beechwood xylan and carboxymethyl cellulose sodium (CMC-Na) salt indicated the cellulase free nature of xylanase from all three cultures whereas Primafast® 200 showed both cellulase and xylanase activities (Fig. 4a). Saccharification treatment of these agro-wastes individually with Primafast® 200 cellulose (C) and three different M35, R31 and J208 xylanases (X) revealed synergism. Synergism by xylanase can be established only when the product released by the enzyme cocktail ((C + X)) exceeds the sum of the product released by the individual enzyme when used separately

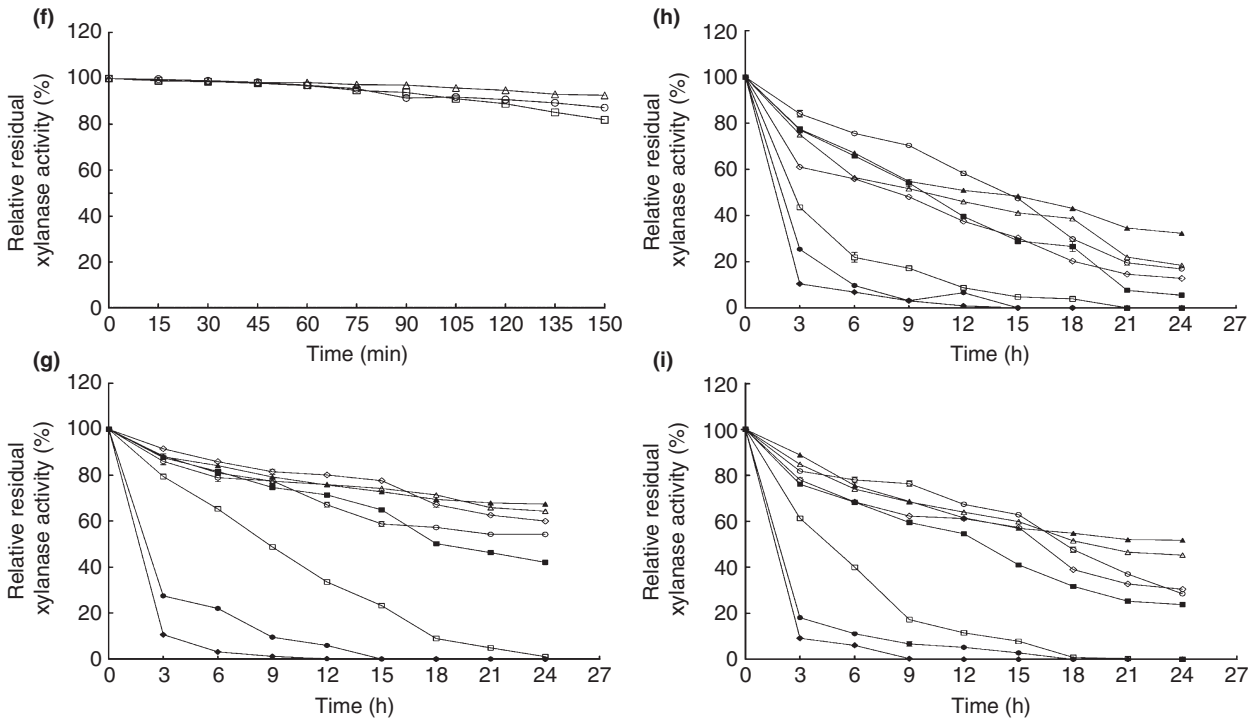

Figure 2 *(continued)*

in the same amount as in the cocktail in unit time, moreover the ratio of the two (degree of synergism) should exceed 1·0. Crude xylanase when applied in supplementation with cellulase for 18 h, aided effective saccharification of agro-wastes, represented as saccharification in terms of 'reducing sugar μg ml^{-1}' (Fig. 4b–d). Enhanced % saccharification was observed at every 6 h of intervals (Table 1). The Degree of synergism decreased with the increased hydrolysis time in major cases suggesting that the hydrolysis time is an important parameter (Li *et al.* 2014). Maximum of 1·3, 2·33 and 1·9 fold increase in % saccharification was observed in case of BH, SCB and WH, respectively, in first 6 h of time. Although Primafast® 200 has xylanase, supplementation in case of two different concentrations of all three xylanases yielded ratio larger than 1 as fold increase in % saccharification up to 18 h, suggesting synergism and therefore establishing its accessory role. Such results have been reported for multicomponent enzyme cocktail preparations (Banerjee *et al.* 2010; Li *et al.* 2014).

Ruminant dung can be an excellent source of xylanolytic bacteria as was observed in this study. The strains M18, M33, M35 identified to be *B. safensis* and are reported for the first time to occur from dung samples. Whereas, strains R30, R31, J208, J216 identified to be *B. altitudinis* have been shown to be present in ruminant dung and soil. Remarkably, the enzymes from *B. safensis* M35, *B. altitudinis* R31 and J208 were found to share similar properties although isolated from three varied sources that is camel, bull and buffalo dung samples, respectively. This can be attributed to enrichment strategy employed for the samples. All three crude xylanases were found suitable for an 'accessory role' in cellulose mediated saccharification of agro-wastes for which they were isolated. Such cellulase free endo-acting xylanase with broad pH spectrum can be considered as important features for application in many other industrial processes.

Materials and methods

Isolation of xylanolytic bacteria

Enrichment culture technique was used to obtain polysaccharide hydrolase producing bacteria, free of glucose catabolite repression, from yard manure and dung samples of four different ruminant mammals, i.e. cow, bull, buffalo and camel. Enrichment was initiated by inoculating 0·5 g of sample in 0·65% nutrient broth (NB), amended with glucose (HiMedia, Mumbai, India, GRM077) and 0·5% each polysaccharides like beechwood xylan (Sigma-Aldrich, Missouri, USA, X-4252), pectin (HiMedia, RN396) and cellulose (HiMedia, GRM126). After incubating for 24 h at 30°C at 180 rev min^{-1}, serial transfers were made twice in media containing 0·327%

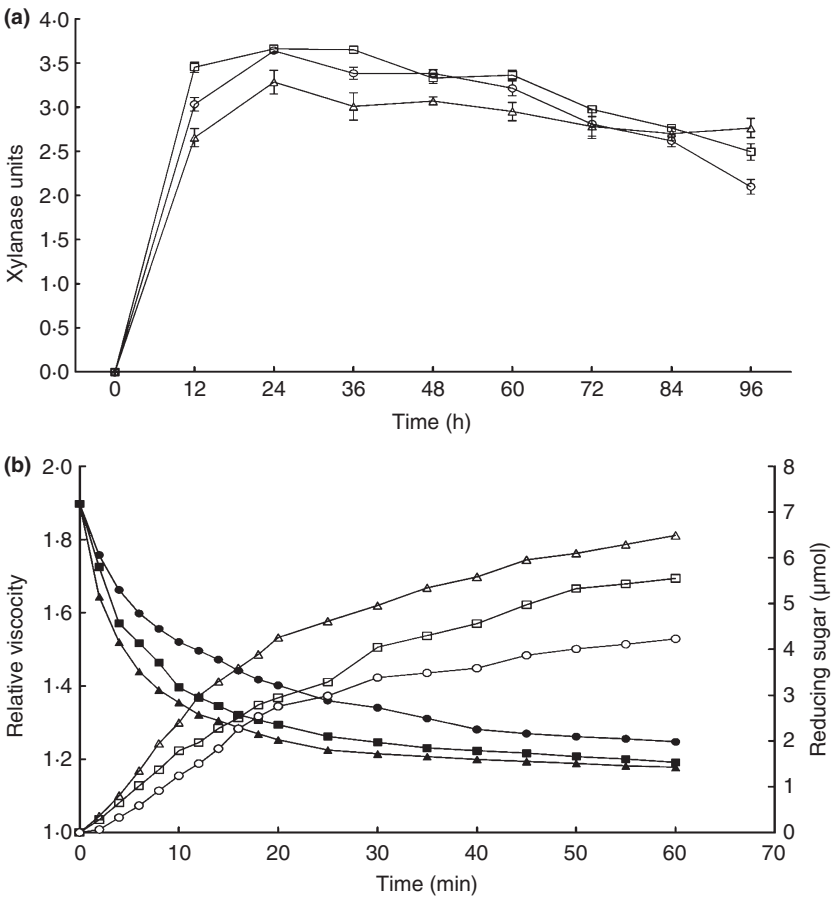

Figure 3 (a) Time course xylanase production by isolates M35 (open triangle), R31 (open square) and J208 (open circle) on Wheat Bran. (b) Drop in viscosity (close symbols) and formation of reducing sugar (open symbols) during the degradation of xylan by xylanase of isolate M35 (triangle); isolate R31(square) and isolate J208 (circle) (n=3).

Bushnell Haas Medium (BHM; HiMedia, M350), 0·5% polysaccharides with and without glucose sequentially.

Selected isolates were screened for xylanolytic activity on two different media. First, medium containing pure substrate, i.e. 0·5% NB amended with chromogenic beechwood xylan prepared by covalent coupling of chromogenic dye Cibacron Brilliant Red 3B-A (Sigma-Aldrich, 228451) to beechwood xylan as per reported procedure (Ten *et al.* 2004). Disappearance of coloured particles in clear zone indicated xylanolytic activity. Second, medium containing 0·5% NB amended with crude xylan substrate, i.e. 0·5% WB flooded with 0·5% Congo red and washed with 5% NaCl where zone of clearance indicated the xylanolytic activity which was intensified to blue colour by 1 N HCl (Teather and Wood 1982) in purple background.

Xylanase assay

About, 0·5% inoculum of 0·4 $OD_{600\ nm}$ overnight grown cultures in NB was inoculated in induction medium containing 0·327% BHM, 0·025% Yeast Extract, 0·075% Peptone and 0·5% either WB or beechwood xylan and incubated for 96 h at 37°C at 180 rev min^{-1}. Xylanase assay system consisted of 0·5% beechwood xylan in 50 mmol l^{-1} phosphate buffer pH 7·0 and appropriately diluted cell free supernatant as crude enzyme solutions. After 10 min incubation at 40°C, reducing sugar released in reaction mixture was measured by di-nitro salicylic acid (DNS) method (Miller 1959; Ghose and Bisaria 1987). One unit of xylanase activity was defined as the amount of enzyme releasing one μmol of xylose from xylan under optimum conditions per minute.

Phenotypic and molecular identification of selected strains

Morphology and various biochemical traits were analyzed for selected isolates. 16S rRNA gene partial sequence and *gyrB* gene sequence alignment analysis was conducted for molecular characterization of the isolates. 16S rRNA gene fragment was amplified by PCR using 27F, 1541R primers (Löffler *et al.* 2000). *gyrB* gene fragment was amplified by PCR using universal degenerate primers UP-1, UP-2r (Yamamoto and Harayama 1995). Amplified PCR products were sequenced at Xcelris laboratory, Ahmedabad, India. The sequences were then retrieved and aligned with the nearest neighbour sequences in the GenBank database. Neighbour-joining phylogenetic analysis was performed

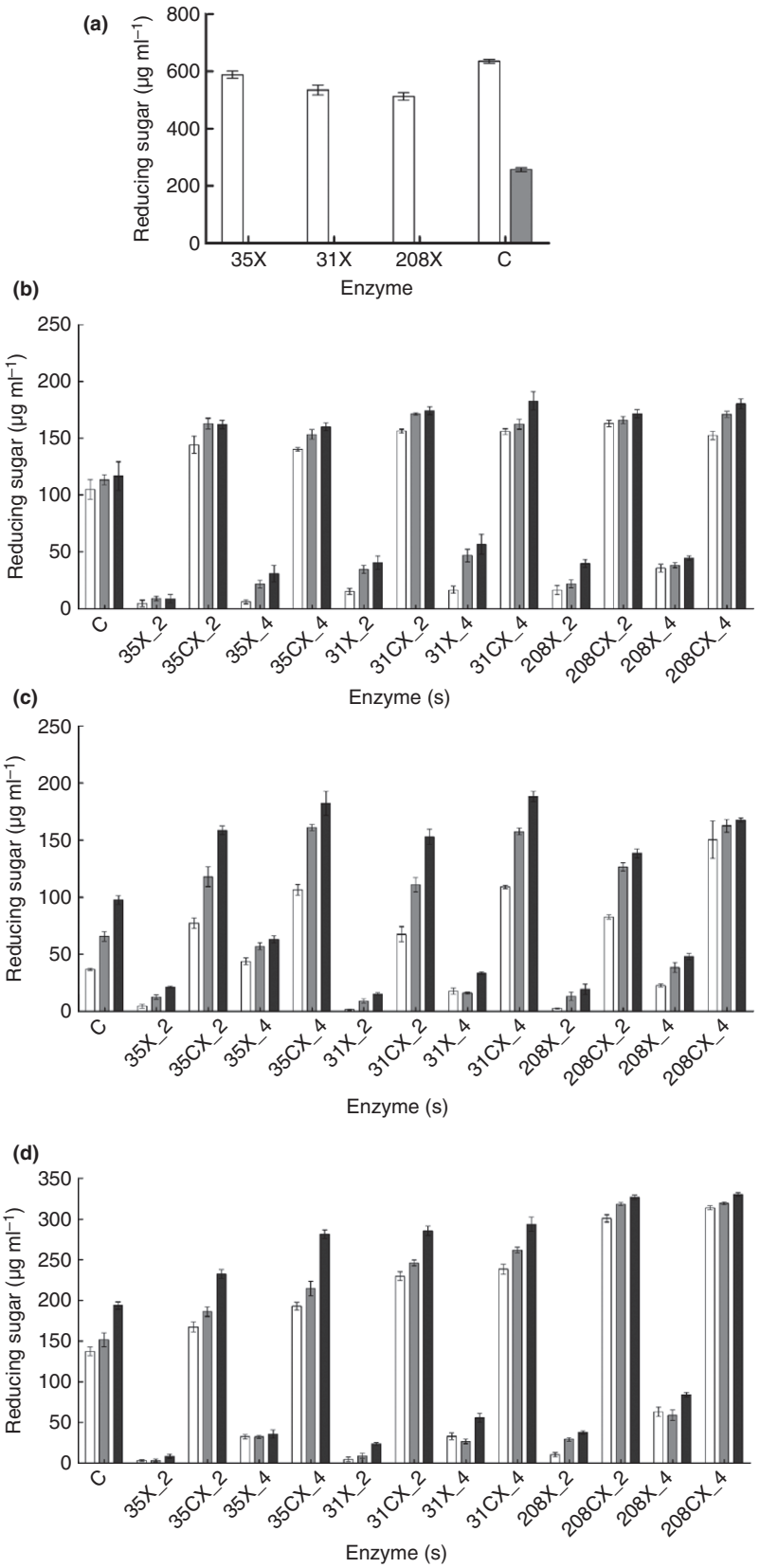

Figure 4 (a) Release of reducing sugars by crude xylanase (X) and commercial cellulase Primafast® 200 (C) from Beechwood Xylan (white bar) and carboxymethyl cellulose sodium salt (grey bar). (b–d) Increase in saccharification of Barley husk (BH) (b), Sugarcane bagasse (SCB) (c) and Wheat Husk (WH) (d). Letters on *X* axis indicates the application of enzymes. 200 and 400 μg of xylanase (as X_2 and X_4), cellulase (C) and cocktail (CX_2 and CX_4) for different time period of 6 h (white bar), 12 h (grey bar) and 18 h (black bar) (n=3).

Table 1 % Saccharification and fold increase in % Saccharification (degree of synergism)

Substrate	Enzyme Time	Sum of % saccharification by individual enzymes (C and X_2 or X_4)			% Saccharification by enzyme mixture (CX_2 or CX_4 Cocktail)			Fold increase in % saccharification (degree of synergism)		
		6 h	12 h	18 h	6 h	12 h	18 h	6 h	12 h	18 h
Barley husk (BH)	M35X_2	0·58 ± 0·04	0·64 ± 0·02	0·66 ± 0·06	0·74 ± 0·05	0·83 ± 0·03	0·83 ± 0·03	1·28	1·30	1·26
	M35X_4	0·59 ± 0·04	0·71 ± 0·03	0·77 ± 0·07	0·72 ± 0·01	0·78 ± 0·03	0·82 ± 0·03	1·22	1·10	1·06
	R31X_2	0·63 ± 0·04	0·77 ± 0·03	0·82 ± 0·07	0·80 ± 0·01	0·87 ± 0·02	0·89 ± 0·03	1·27	1·13	1·09
	R31X_4	0·64 ± 0·04	0·83 ± 0·04	0·90 ± 0·08	0·80 ± 0·02	0·87 ± 0·01	0·93 ± 0·06	1·25	1·05	1·03
	J208X_2	0·64 ± 0·05	0·71 ± 0·03	0·81 ± 0·06	0·83 ± 0·02	0·85 ± 0·02	0·87 ± 0·03	1·30	1·20	1·07
	J208X_4	0·74 ± 0·04	0·79 ± 0·02	0·84 ± 0·051	0·78 ± 0·02	0·87 ± 0·02	0·92 ± 0·03	1·05	1·10	1·10
Sugarcane bagasse (SCB)	M35X_2	0·24 ± 0·01	0·42 ± 0·02	0·63 ± 0·02	0·40 ± 0·03	0·61 ± 0·06	0·81 ± 0·03	1·67	1·45	1·29
	M35X_4	0·43 ± 0·02	0·64 ± 0·03	0·84 ± 0·02	0·55 ± 0·03	0·82 ± 0·02	0·93 ± 0·07	1·28	1·28	1·11
	R31X_2	0·22 ± 0·01	0·40 ± 0·02	0·60 ± 0·02	0·35 ± 0·05	0·57 ± 0·05	0·78 ± 0·02	1·59	1·43	1·30
	R31X_4	0·30 ± 0·01	0·44 ± 0·02	0·69 ± 0·09	0·56 ± 0·01	0·80 ± 0·02	0·96 ± 0·03	1·87	1·82	1·39
	J208X_2	0·23 ± 0·01	0·42 ± 0·03	0·62 ± 0·03	0·43 ± 0·01	0·65 ± 0·03	0·71 ± 0·03	1·87	1·55	1·15
	J208X_4	0·33 ± 0·01	0·55 ± 0·03	0·76 ± 0·02	0·77 ± 0·12	0·83 ± 0·04	0·85 ± 0·01	2·33	1·51	1·12
Wheat husk (WH)	M35X_2	0·73 ± 0·02	0·81 ± 0·04	1·04 ± 0·024	0·85 ± 0·04	0·95 ± 0·04	1·18 ± 0·04	1·16	1·17	1·13
	M35X_4	0·88 ± 0·03	0·95 ± 0·04	1·18 ± 0·033	0·98 ± 0·03	1·10 ± 0·06	1·42 ± 0·04	1·11	1·16	1·20
	R31X_2	0·74 ± 0·03	0·84 ± 0·04	1·12 ± 0·022	1·17 ± 0·04	1·25 ± 0·03	1·44 ± 0·04	1·58	1·49	1·29
	R31X_4	0·88 ± 0·03	0·92 ± 0·04	1·28 ± 0·034	1·21 ± 0·04	1·33 ± 0·03	1·48 ± 0·06	1·38	1·45	1·16
	J208X_2	0·77 ± 0·03	0·94 ± 0·04	1·19 ± 0·023	1·52 ± 0·03	1·61 ± 0·01	1·65 ± 0·02	1·97	1·71	1·39
	J208X_4	1·03 ± 0·04	1·09 ± 0·05	1·04 ± 0·031	1·59 ± 0·02	1·61 ± 0·01	1·67 ± 0·01	1·54	1·48	1·61

based on maximum composite likelihood method with support of Kimura-2-parameter model and 1000 bootstrap values with MEGA 6.0 program developed by Tokyo Metropolitan University, Hachioji, Tokyo, Japan.

Effect of pH and temperature on xylanase activity

Xylanase activity at various pH and temperature was assayed using cell free supernatant of WB induced culture as crude enzyme source. Assay mixture containing 0·5% xylan was prepared in different assay buffers (50 mmol l^{-1}); pH 4·0, 5·0 and 6·0 Citrate-Phosphate buffer; pH 7·0, 8·0 and 9·0 Tris–Cl buffer; pH 10·0 Carbonate-Bicarbonate buffer. Crude xylanase was incubated with a set of buffered substrate at a range of temperature starting with 30–80°C with the increase of 10°C per set. Contour plot was prepared for xylanase units with respect to pH and temperature conditions using MINITAB 14 developed by Minitab, Ltd., Coventry, UK.

Effect of temperature on xylanase stability

Effect of temperature on stability of crude xylanase was determined by preincubating enzyme in pH 8·0 buffer at 40, 50 and 60°C for 120, 60 and 30 min respectively. The residual xylanolytic activity at various incubation time was assayed at 40°C for 10 min incubation using the DNS method (Miller 1959; Ghose and Bisaria 1987).

Effect of pH on xylanase stability

Effect of pH on stability of crude xylanase was determined by preincubating enzyme in various pH buffers mentioned above (50 mmol l^{-1}) at 40°C for 24 h. After every three hours, residual xylanolytic activity was assayed at 40°C for 10 min incubation using the DNS method (Miller 1959; Ghose and Bisaria 1987).

Time course studies of xylanase production

Induction of xylanase was carried out in 50 ml 0·5% WB induction medium in 250 ml Erlenmeyer flask at 37°C, 180 rev min^{-1} for 96 h. During the course of incubation, cell free culture supernatant was harvested as crude enzyme source after interval of 12 h and assayed for xylanase activity on 50 mmol l^{-1} pH 8·0 buffered 0·5% xylan as above.

Viscosity measurement

To study the endo- or exo- acting nature of xylanase from selected *Bacillus* sp. viscosity decrease and reducing sugar release from xylan were determined simultaneously. The reaction was performed with 50 mmol l^{-1} Tris-Cl buffer (pH 8·0) containing 3·0% xylan in an Ostwald Capillary Viscometer for viscosity measurement and same in a culture tube for reducing sugar analysis.

Influence of crude xylanase supplementation in commercial cellulase mediated saccharification of lignocellulosic substrate

Crude xylanase from individual cultures and commercial cellulase Primafast® 200 (Dupont, Jiangsu, China) were studied for cellulase and xylanase activities on 0·5 ml of 0·5% of either beechwood xylan or CMC-Na salt (Sigma Aldrich, 21902) substrate, prepared in 50 mmol l^{-1} Tris-Cl Buffer pH 7·0. One hundred microlitre of cell free culture supernatant as an enzyme source was added and system was incubated for 10 min at 40°C. End products were detected using DNS method (Miller 1959; Ghose 1987; Ghose and Bisaria 1987). Saccharification of three different agro waste biomass, BH, SCB and WH was carried out individually at substrate loading of 2% (w/v) in 25 ml of 50 mmol l^{-1} of Tris-Cl buffer pH 7·0 containing Tetracycline 100 μg ml^{-1} to prevent microbial contamination. The reaction mixture was incubated on orbital shaker incubator at 160 rev min^{-1} and 40°C up to 18 h. 0·2 mg (X_2) and 0·4 mg (X_4) of all three xylanase per 0·5 mg of dry substrate were loaded individually with 0·8 mg cellulase (C) per 0·5 g of dry substrate in two separate experiments. Sample was collected after every 6 h and analyzed for release of reducing sugar by DNS method as mentioned earlier. % Saccharification and degree of synergism were calculated by following calculations.

$$\%\,\text{Saccharification} = \frac{\text{reducing sugar released (mg ml}^{-1})}{\text{Substrate used (mg ml}^{-1})}$$

$$\text{Degree of synergisms} = \frac{\%\,\text{saccharification by enzyme cocktail}}{\text{sum of }\%\,\text{saccharification by individual enzyme}}$$

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Conflict of Interest

No conflict of interests declared.

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ORIGINAL ARTICLE

Physicochemical characterization of pectinase activity from *Bacillus* spp. and their accessory role in synergism with crude xylanase and commercial cellulase in enzyme cocktail mediated saccharification of agrowaste biomass

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Keywords
agrowaste biomass, *Bacillus altitudinis*, *Bacillus safensis*, enzyme cocktail, pectinase activity, saccharification, sugarcane bagasse, synergism.

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Abstract

Aim: The aim of this study was to evaluate the physicochemical properties of the crude pectinase activity from three *Bacillus* isolates of ruminant dung origin and study their synergism with crude xylanases from the same *Bacillus* spp. and a commercial cellulase to evaluate their accessory role in improved biomass saccharification.

Methods and Results: Pectinolytic crude culture filtrate obtained from three ruminant dung isolates, *Bacillus safensis* M35, *Bacillus altitudinis* R31 and *Bacillus altitudinis* J208, on crude pectin containing medium possessed polygalacturonate hydrolase, pectate lyase and pectin lyase activities. Studies regarding their stability under various temperature and pH conditions revealed their mild acidic to alkaline and mesophilic nature with enzyme activity falling within the pH range 6·0–9·0 and temperature range 30–60°C. The pectinase activity was categorized as endolytic as it brought about ~50% reduction in relative viscosity of pectic polymer within initial 10 min of incubation. Synergism of pectinase activity with crude xylanase activities and/or commercial cellulase was clearly demonstrated as ~1·6 to ~1·9-fold increase in agrowaste biomass saccharification was obtained confirming the role of pectinases as accessory enzymes.

Conclusion: Synergism of the broad-spectrum endopectinase activity obtained from three *Bacillus* isolates with accessory crude xylanases from the same isolates and commercial cellulase enhanced the agrowaste saccharification and confirmed the accessory role of crude pectinase as they formed an efficient enzyme cocktail functioning in a contributive manner for improvement of agrowaste biomass saccharification.

Significance and Impact of the study: Mesophilic crude endopectinases obtained from *Bacillus* spp. isolated from ruminant dung possessed activity in broad pH and temperature ranges as well as broad substrate specificity. Moreover, their synergism with crude xylanase and Primfast®200 cellulase demonstrated the potential to form efficient enzyme cocktail for application in plant biomass saccharification process.

Introduction

Diverse interactions between semi-crystalline cellulose fibres and amorphous hemicellulosic glucans as well as polyphenolic lignins, small polypeptides and small organic-inorganic constituents are observed in primary and secondary plant cell wall (Hamelinck *et al.* 2005; Hendriks and Zeeman 2009; Albersheim *et al.* 2011).

Several covalent (e.g. ester bonds at feruloyl acid ester) and noncovalent (e.g. hydrogen bonds) interactions between lignin and hemicelluloses result in a complex matrix, called 'lignocellulose' which embed cellulose fibrils in lignified hemicellulosic network. Middle lamella is a cementing layer between the wall of two plant cells and is mainly composed of calcium pectate, a calcium salt of pectic acid. Besides this, the pectic polymers like rhamnogalacturonans I and II react with other hemicellulose in the complex matrix and such cross-linking secures cellulose fibres against hydrolysis by making them inaccessible to hydrolases (Berlin *et al.* 2007; Achyuthan *et al.* 2010). Due to their recalcitrant properties the natural process of breakdown and mineralization of cellulose, hemicelluloses and lignin complex is slow and incomplete. Reports suggested that the diverse community of bacterial, archaeal and eukaryotic gut symbionts ease the digestion process of less lignified cellulosic forage in ruminant cattle (Dehority 2002) and recalcitrant woody biomass in termites (Brune 2014). Removal of lignin and hemicellulose, decrease in the crystalline structure of cellulose and increase in porosity of cell wall structure by pretreatments, etc., are the methods which can significantly improve the saccharification of cellulose, a first step in biofuel production process (Yoshida *et al.* 2008). Thus, agricultural waste materials of lignocellulosic nature and low animal fodder value can be used for production of bioethanol.

In India, sugarcane bagasse (SCB) is a major plant waste product of the sugar factories (Pandey *et al.* 2000). Conversion of SCB, barley husk (BH), corn stover (CS), and other plant residues into fuel ethanol and other organic chemicals have provided attractive opportunities for more sustainable development of agricultural resources (Qureshi *et al.* 2014). Enzymatic hydrolysis of cellulose contained in such plant residues is the first step that leads to sugar release in fuel ethanol production. The exo- and endo-cellulases belonging to the group of core enzymes alone are not sufficient and the assistance of some accessory or auxiliary group of enzymes such as xylanases and pectinases is necessary for increased saccharification (Hu *et al.* 2011; Delabona *et al.* 2013; Li *et al.* 2014). The accessory enzymes like xylanases and pectinases unmask the cellulose present in the plant biomass by hydrolysing the hemicellulosic and pectic shield facilitating its access to cellulolytic enzymes consequently enhancing the sugar release.

Synergism among saccharifying enzymes has been studied between the different enzymes of core cellulase group or between that of core cellulase and accessory group comprising of hemicellulases and pectinases through either additive or substitutive method (Kumar and Wyman 2009; Hu *et al.* 2011; Kostylev and Wilson 2012; Zhang *et al.* 2013). Commercial cellulases after supplementation with Multifect Pectinase have been reported to show enhancement in glucan conversion of the dilute acid pretreated CS (Berlin *et al.* 2007) and ammonia fibre expansion treated rice straw (Zhong *et al.* 2009). Improved saccharification in case of wheat bran and SCB has been demonstrated by the enzyme cocktail containing cellulase, pectinase and xylanase enzymes produced by fungus *Chrysoporthe cubensis* in comparison with commercial enzymes (Maitan-Alfenas *et al.* 2015).

The agrowaste saccharification by plant cell wall degrading commercial cellulases and accessory xylanase and pectinase enzymes can be improved due to their concerted synergistic actions, provided the enzymes can breakdown diverse range of complex plant polysaccharides in broad range of pH and temperature. Agrowaste from different sources possesses variations in composition of cellulose, hemicellulose, pectin, lignin and hence the complexities observed in structural polysaccharides also varies (Krawczyk *et al.* 2008; Bledzki *et al.* 2010). Furthermore, crude pectinases produced by diverse *Bacillus* spp. include hydrolase and lyase activities, cleaving the pectic substrates by hydrolysis or by β-elimination (Kashyap *et al.* 2000; Yadav *et al.* 2009; Zhou *et al.* 2017). In our earlier work, the crude xylanases produced by xylano-pectinolytic *Bacillus safensis* M35, *Bacillus altitudinis* R31 and *B. altitudinis* J208 isolated from ruminant cattle dung were shown to act as accessory enzymes in a cocktail with commercial cellulase Primafast®200 (Thite and Nerurkar 2015). Interestingly, these three *Bacillus* isolates also showed pectinase activity. As per our knowledge, studies regarding the characterization of pectinase enzymes from *B. safensis* and *B. altitudinis* strains and their application in agrowaste plant biomass saccharification have not been reported.

In this context, the aim of the present investigation was to characterize the crude pectinase activity from *B. safensis* M35, *B. altitudinis* R31 and *B. altitudinis* J208 with respect to their physicochemical parameters and to study the synergism between the crude pectinases and crude xylanases as well as commercial cellulase Primfast200 to establish the role of pectinases as accessory enzymes.

Materials and methods

Materials

Pectin (polygalacturonic acid methyl ester, GRM 396), polygalacturonic acid (PGA, RM4779), bushnell haas medium (BHM, M350), nutrient broth (M002), peptone

(bacteriological, RM001), yeast extract (YE, RM027), agar agar type I (GRM666), 2-thiobarbituric acid (TBA, RM1594) and 3,5-dinitrosalicylic acid (DNS, GRM1582) were purchased from HiMedia (Mumbai, Maharashtra, India). All other chemicals for assay were laboratory grade and purchased from Merck (Mumbai, Maharashtra, India). Citrus peel (CP), BH, SCB and wheat husk (WH) were procured locally. All % unit values for chemicals and media components are in % w/v unless mentioned. Primafast200 was procured from Dupont (Genencor, Shanghai, China) and is referred to as commercial cellulase in present studies.

Micro-organisms and enzyme preparations

Three *Bacillus* isolates *B. safensis* M35 (MCC 3305), *B. altitudinis* R31 (MCC 3308) and *B. altitudinis* J208 (MCC 3307) obtained from fresh dung samples of camel, bull and buffalo, respectively, were used as source of crude pectinolytic enzymes (Thite and Nerurkar 2015). The isolates were maintained at 6–8°C on nutrient agar plates. Crude pectinases were produced by growing three *Bacillus* isolates individually in medium containing 0·327% w/v BHM with 0·025% w/v YE and 0·075% w/v peptone, amended with 0·5% w/v CP in 250 ml Erlenmeyer flask and incubated at 37°C, 180 rev min^{-1} for 96 h. At the end of incubation, culture broth was centrifuged at 19 230 ***g*** for 10 min to obtain cell free supernatant as crude pectinase enzyme which was used for physicochemical characterization of pectinase activities. Commercially prepared core cellulase along with accessory enzymes such as crude pectinases prepared as mentioned above and crude xylanases prepared as described in our earlier saccharification studies from same *Bacillus* isolates were used (Thite and Nerurkar 2015).

Pectin lyase (PNL) and polygalacturonate lyase (or pectate lyase, PL) assay

Pectin lyase (PNL) and polygalacturonate or pectate lyase (PL) activities were determined in crude pectinases by measuring unsaturated oligogalacturonides released as a result of the enzymatic cleavage of pectin or PGA respectively using TBA reagent (adapted and modified as per, Nedjma *et al.* 2001). A 250 μl of 0·5% w/v substrate (pectin or PGA) in 50 mmol l^{-1} Tris-Cl pH 8·0 was incubated with 50 μl of crude enzyme at 50°C for 30 min. Reaction was stopped by adding 0·5 ml of 1 mol l^{-1} NaOH and incubating at 76°C for 10 min. Addition of 0·6 ml of 1 mol l^{-1} HCl followed by 0·5 ml of 40 mmol l^{-1} TBA and incubation at 76°C for 10 min developed a pink colour, which was measured as $A_{550\ nm}$. One unit of PNL or PL activity was defined as the amount of enzyme that caused a change in the absorbance of 0·01 per hour at optimum condition.

Polygalacturonase (or polygalacturonate hydrolase, PG), xylanase and cellulase assay

Polygalacturonase, xylanase and cellulase activities were determined by measuring reducing sugar released as a result of hydrolysis of the polymer substrate (PGA for polygalacturonase, xylan for xylanase and CMC-Na salt for cellulase) using DNS reagent (adapted and modified, Miller 1959; Ghose and Bisaria 1987). A 250 μl of 0·5% w/v substrate in 50 mmol l^{-1} Tris-Cl pH 8·0 was incubated with 50 μl crude enzyme at 40°C for 10 min. Reaction was stopped by adding 300 μl of DNS reagent and boiled for 10 min. After cooling, volume was made up to 1·5 ml and $A_{540\ nm}$ was measured. The reducing sugar formed was quantified using D-galacturonic acid or D-xylose or D-glucose as a standard, respectively. One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol of respective reducing sugar per min at optimum conditions.

Substrate specificity of crude pectinase activity

Crude pectinase activity from all three *Bacillus* isolates was analysed individually with each of the four substrates having different degrees of esterification (DE) as follows: PGA (0% DE; HiMedia), low methoxylated pectin (35% DE, Genu® pectin LM12-1 CG; CPKelco, Lille Skensved, Denmark), High methoxylated pectin (70% DE, Genu pectin 150 USA-SAG; CPKelco) and pectin (65–70% DE; HiMedia), hereafter referred as DE0, DE35, DE65, DE70. Activity on PGA (DE0) was considered as 100% and relative activity (RA) for other substrates having DE35, DE65 and DE70 was calculated. The substrate specificity was determined based on lyase activity for PNL and PL assays and hydrolase activity for PG assay.

Zymogram analysis of crude pectinase activity

SDS-PAGE was performed using a 10% polyacrylamide gel (adapted and modified, Laemmli 1970) incorporated with PGA (DE0) and pectin (DE70) substrates individually for activity staining (zymogram analysis). Protein molecular weight marker (PMWM) containing mixture of five different proteins named carbonic anhydrase (29 kDa), ovalbumin (43 kDa), bovine serum albumin (BSA) (66 kDa), phosphorylase b (97·4 kDa) and myosin from rabbit muscle (205 kDa) was run alongside the crude protein samples from individual isolate. Following the completion of electrophoresis at 80V for 180 min,

each gel was cut in two parts. Part of the gel containing PMWM was stained with silver salts (Sambrook *et al.* 2001). The other part of gel containing the substrate was renatured and activity staining was performed. For renaturation, gel was washed three times with 2% v/v Triton X-100 in 50 mmol l^{-1} Tris-Cl pH 8·0 for 1 h each, followed by incubation of 24 h in 50 mmol l^{-1} Tris-Cl pH 8·0, and then, zymogram was developed using 0·1% w/v toluidine blue-O in 50 mmol l^{-1} Tris-Cl pH 8·0 (Maisuria and Nerurkar 2012). Molecular weight of the protein bands showing pectinase activities from each *Bacillus* isolates was determined with reference PMWM using ALPHA EASE software ver. 2.0.

Effect of pH and temperature on crude pectin lyase activity

A 0·5% w/v pectin was prepared in different assay buffers (50 mmol l^{-1}) of pH 4·0, 5·0 and 6·0 (citrate–phosphate buffer); pH 7·0, 8·0 and 9·0 (Tris-Cl buffer); and pH 10·0 and 11·0 (carbonate–bicarbonate buffer). Crude pectinases were incubated with each of the buffered substrates at 30, 40, 50, 60, 70 and 80°C temperature individually. Pectin lyase activity was measured as described above. Contour plot for PNL activity units from individual culture with respect to pH and temperature was drawn using Akima's polynomial method for interpolation with MINITAB 17.

Effect of temperature on stability of crude pectin lyase activity

Effect of temperature on stability of crude PNL activity was determined by pre-incubating the crude enzyme in pH 8·0 buffer at 40, 50, 60 and 70°C for 360, 240, 120 and 60 min, respectively, and residual PNL activity was assayed as mentioned above.

Effect of pH on stability of crude pectin lyase activity

Effect of pH on stability of crude PNL activity was determined by pre-incubating the crude enzyme in 50 mmol l^{-1} buffers of pH 4·0, 5·0, 6·0, 7·0, 8·0, 9·0, 10·0 and 11·0 (as described above) at 40°C for 24 h. After every 6 h, residual PNL activity was assayed as mentioned above.

Effect of crude pectinase on relative flow rate and viscosity of pectin

The crude pectinases were incubated individually with 20 ml of 50 mmol l^{-1} Tris-Cl buffer (pH 8·0) containing 3·0% w/v pectin, and the relative flow rate of the assay mixture was measured with an Ostwald capillary viscometer. The end products released were detected using TBA method for PNL activity. One unit of endopectinases was defined as an amount of the enzyme required to obtain ~30–50% decrease in relative viscosity of pectin by pectinases after a definite time (1, 10, 20, 60 or 120 min) under optimum incubation conditions (Angelova 2008). Flow time of the buffered distilled water was taken as control, and relative flow time of the pectin substrate was calculated using the following formula:

$$\text{Relative flow time or relative viscosity} = \frac{T_t}{T_s} \quad (1)$$

where T_t = flow time at incubation of t min; T_s = flow time of solvent.

HPLC analysis for enzymatic degradation products of pectin

Pectin lyase assay was performed for individual crude pectinases and 20 μl of aliquot was withdrawn after 10 h and loaded on HPLC (SHIMADZU). Using Agilent Hi-Plex-H column containing strong cation-exchange resin made up of sulfonated, cross-linked styrene-divinylbenzene copolymer in hydrogen form as a stationary phase and deionized water as mobile phase, separation of released products under isocratic conditions was performed, and chromatograms were compared with 5 mmol l^{-1} D-galacturonic acid monomer, buffered pectin substrate and crude cell free supernatant enzyme as controls to detect change in peak of substrate and products.

Effect of crude pectinase supplementation to xylanase and/or commercial cellulase cocktail mediated plant biomass saccharification

Three different agrowaste biomasses BH, SCB and WH were dried, partially ground and stored in airtight container. 2·0% w/v of each agrowaste biomass substrate was suspended in 10 ml of 50 mmol l^{-1} Tris-Cl buffer pH 7·0. The assay mixture contained sodium azide ($Na\text{-}N_3$; Sigma-Aldrich, Steinheim, Germany 71289) with ampicillin, kanamycin, and streptomycin each 100 μg ml^{-1} to prevent microbial contamination during assay. Protein concentrations from crude pectinases, xylanases from individual *Bacillus* isolates and commercial cellulase were estimated with Bradford method using BSA (MB083 HiMedia) as a standard (Kruger 1996). In two separate experiments, the assay system contained either 0·2 or 0·4 mg protein individually for both xylanase (X_2 or X_4) and/or pectinase (P_2 or P_4)

Table 1 Substrate specificity of pectinases for hydrolase and lyase activities

Activity	Culture	PGA (DE0)	Pectin (DE35)	Pectin (DE65)	Pectin (DE70)
Hydrolase	*Bacillus safensis* M35	100 ± 4·36	89·59 ± 1·28	82·37 ± 1·02	80·78 ± 2·66
	Bacillus altitudinis R31	100 ± 5·30	85·07 ± 1·45	66·97 ± 1·12	66·09 ± 0·72
	B. altitudinis J208	100 ± 5·10	97·80 ± 2·31	81·27 ± 3·42	77·09 ± 0·89
Lyase	*B. safensis* M35	100 ± 1·90	81·47 ± 1·35	52·64 ± 1·70	21·47 ± 0·20
	B. altitudinis R31	100 ± 3·30	95·32 ± 1·38	49·38 ± 2·20	15·38 ± 1·22
	B. altitudinis J208	100 ± 1·02	93·22 ± 0·65	75·54 ± 2·86	37·47 ± 2·35

Values presented are mean ± SEM.

per 200 mg of dry agrowaste biomass. Each of the set was amended with or without 0·8 mg protein for cellulase (C) per 500 mg of dry agrowaste biomass and incubated in shaking condition at 160 rev min^{-1}, 40°C up to 60 h. Enzyme assays with individual enzymes (C, P and X), combination of two enzymes (CP, CX and PX), and all three enzymes together (CPX) were performed separately for each agrowaste BH, SCB and WH. After 12 and 60 h of incubation, samples were analysed for released reducing sugar by DNS method, and results were compared with chemical saccharification of selected biomass with 1 mol l^{-1} HCl.

Estimation of reducing sugars after plant biomass saccharification

Reducing sugars were estimated using DNS reagent method (adapted and modified as per Miller 1959); 300 μl of DNS solution was added to 300 μl of sampled plant hydrolysate. The mixture was boiled for 10 min. After cooling, the volume was made up to 1·5 ml and $A_{540\ nm}$ was measured. Reducing sugar released after hydrolysis was quantified using D-glucose as standard and was calculated as mg of reducing sugar released per substrate loading.

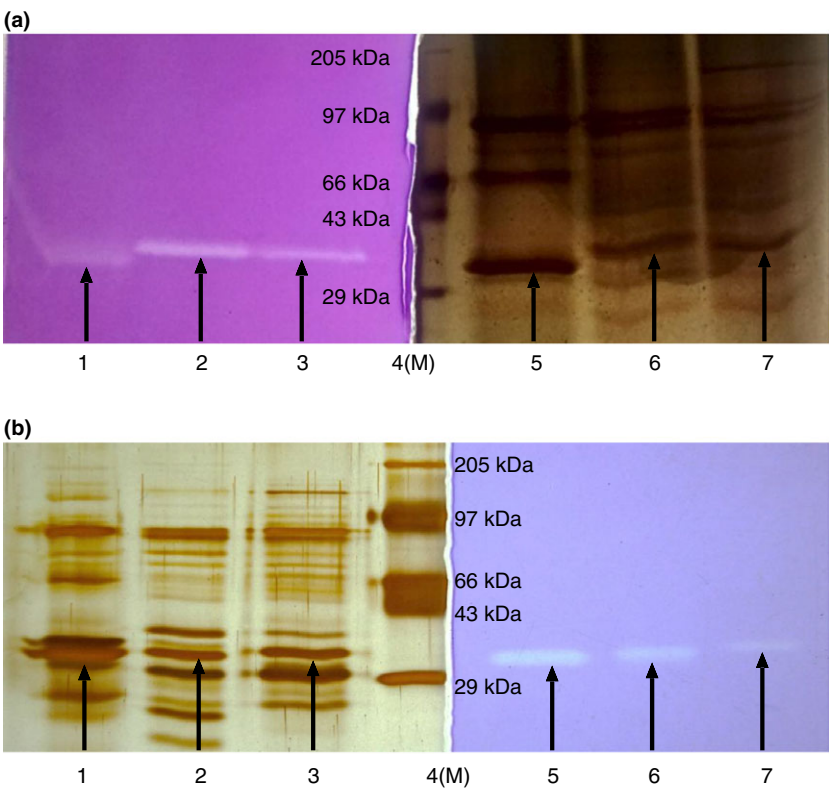

Figure 1 Zymogram and SDS-PAGE analysis of crude pectinase enzymes from *Bacillus safensis* M35, *B. altitudinis* R31 and *B. altitudinis* J208: Zymogram and silver stain of (a) pectin and (b) PGA incorporated in 10% acrylamide gel; lanes 1 and 5: *B. safensis* M35, lanes 2 and 6: *B. altitudinis* R31 and lanes 3 and 7: *B. altitudinis* J208, lane 4(M): protein marker. [Colour figure can be viewed at wileyonlinelibrary.com]

Calculation of % saccharification and degree of synergism (DS)

% Saccharification was calculated as the amount of reducing sugar in % released after hydrolysis of total substrate provided.

$$\%\,\text{Saccharification} = \frac{\text{reducing sugar released}\,(\text{mg ml}^{-1})}{\text{Substrate used}\,(\text{mg ml}^{-1})} \times 100 \quad (2)$$

Degree of synergism was calculated from the ratio of % saccharification by enzyme cocktail and sum of % saccharification by individual enzymes.

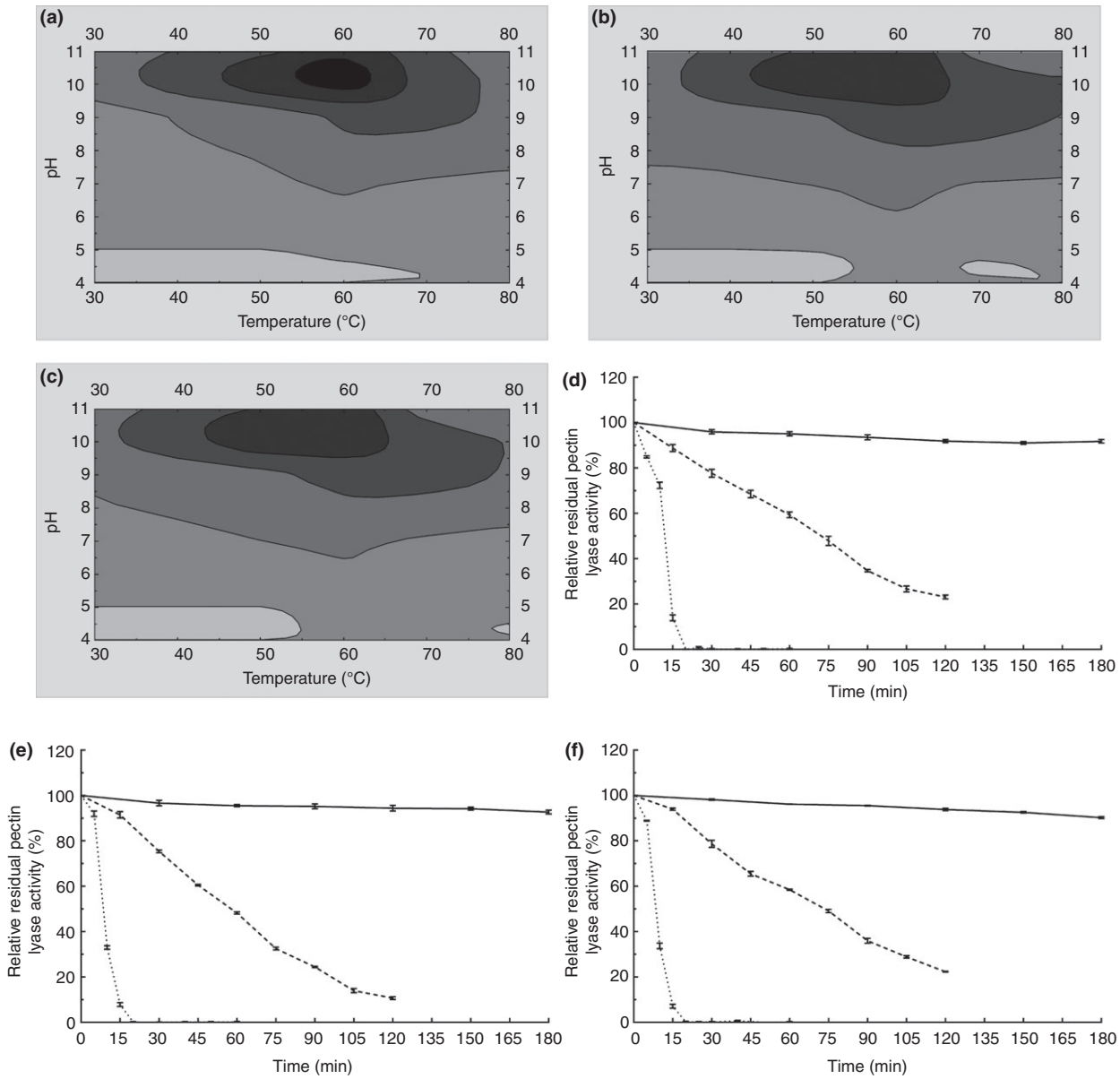

Figure 2 Effect of temperature and pH on activity and stability of crude pectin lyase from *Bacillus safensis* M35, *Bacillus altitudinis* R31 and *B. altitudinis* J208: (a–c) Combined effect of temperature (*x*-axis) and pH (*y*-axis) on pectin lyase activity (*z*-axis) of M35, R31 and J208, respectively, as grouped in units <0, 0–100, 100–200, 200–300, 300–400, >400 each represented by the shade of grey with increasing intensity with unit numbers; (d–f) effect of temperature 50°C (——), 60°C (– –) and 70°C (– ·) on stability of pectin lyase from M35, R31 and J208, respectively. (g–i) Effect of pH on stability of pectin lyase from M35, R31 and J208, respectively, pH 4·0, 5·0, 6·0, 7·0, 8·0, 9·0, 10·0, 11·0 separated by (– – –) and in comparison with unbuffered pectinase; Error bars represented as standard error of the mean (SEM) for $n = 3$.

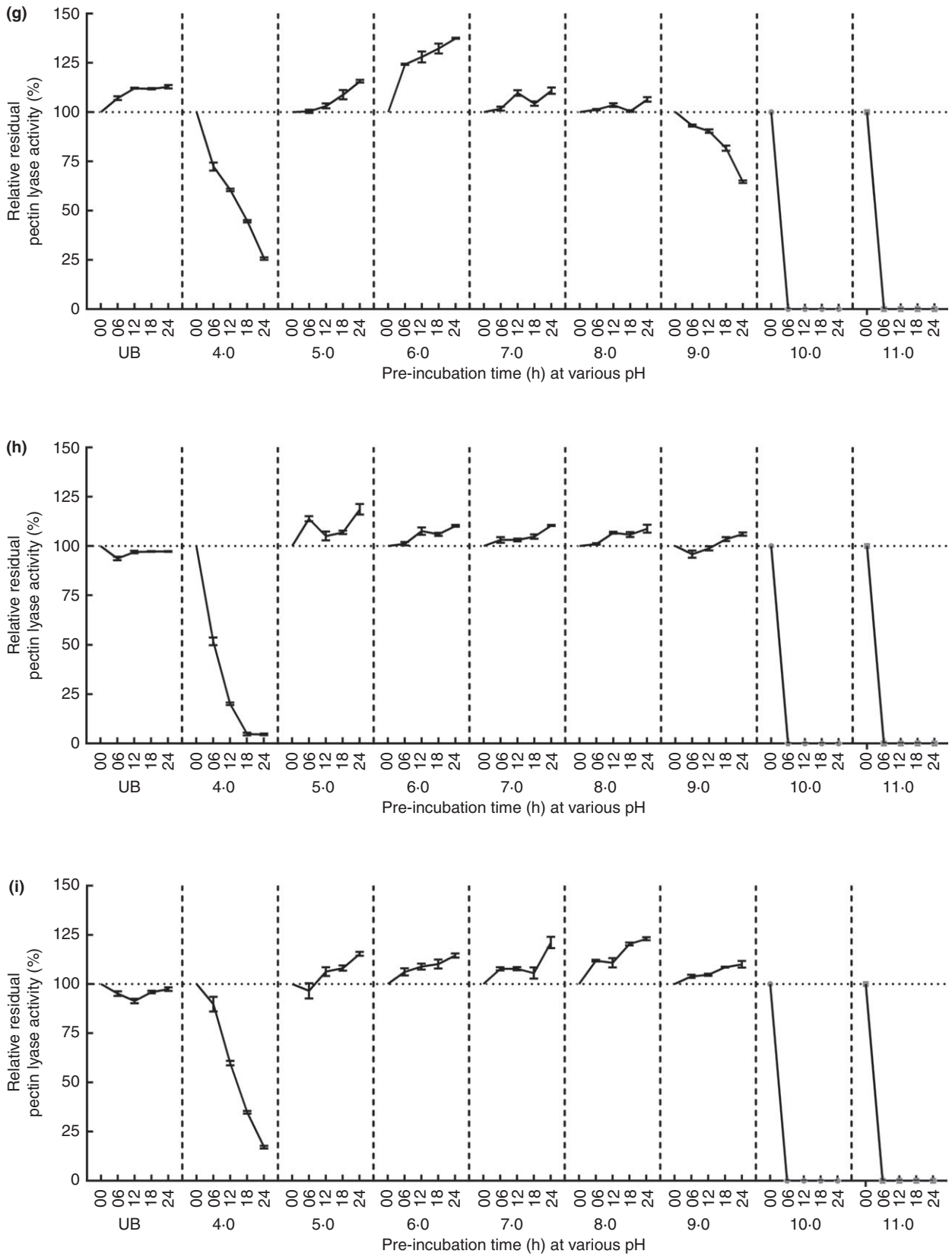

Figure 2 Continued.

$$\text{Degree of synergism} = \frac{\%\,\text{saccharification by enzyme cocktail}}{\text{sum of \% saccharification by individual enzyme}} \quad (3)$$

Results

Pectinases activity and substrate specificity

Crude pectinases produced by *B. safensis* M35, *B. altitudinis* R31 and *B. altitudinis* J208 exhibited all three PG, PL and PNL activities. Polygalacturonate hydrolase activity on DE35 pectin and DE65-DE70 pectin were found to be in range of 85–97% and 66–80%, respectively, relative to 100% on PGA (Table 1). While pectin and PL activities on DE35 pectin and DE65-DE70 pectin were found to be in range of 81–93% and 15–38%, respectively, relative to 100% on PGA. It was observed that as the DE of the substrates increased, both the hydrolase and lyase activities decreased for all the pectinases, viz., PG, PL and PNL.

Zymogram of crude pectinase activity

Approximate molecular weights of the pectinase activity bands were observed to be ~33 kDa for *B. safensis* M35 and ~35 kDa for *B. altitudinis* R31 and *B. altitudinis* J208 as calculated from the zymogram of both the pectin and PGA incorporated gels developed with toluidine blue-O dye for the activity bands and the silver stained gels (Fig. 1a,b). Notably a single activity band with same molecular weight was observed on both the pectin and PGA zymograms from all three *Bacillus* isolates.

Effect of temperature and pH on crude pectin lyase activity

Maximum PNL activity of 422 and 377 and 360 units was obtained at pH 10·0 and 60°C, respectively, from *B. safensis* M35, *B. altitudinis* R31 and *B. altitudinis* J208. Crude pectinases from the three *Bacillus* isolates remained active over a broad range of temperature 50–60°C and pH 6·0–11·0, and the activity gradually increased from pH 6·0 up to pH 10·0 with temperature range of 40–60°C, suggesting mesophilic alkaline nature of pectinases. A bell-shaped curve was obtained for activities at pH 11·0 for all temperatures under study as well as at 70 and 80°C for all pH values under study (Fig. 2a–c). Both *B. altitudinis* R31 and J208 showed more than 70–80 units activity at pH 7·0, whereas at pH 6·0, they exhibited more than 55 units PNL activity in 40–70°C temperature range. Thus, even though the optimum pH for the enzymes was alkaline, they were able to work at mild acidic pH. While ~65 and ~90 units PL activity was observed at pH 7·0 for 40 and 70, respectively, ~210 and ~235 units activity was observed at pH 11·0 for 40 and 70°C, respectively, by *B. safensis* M35. PL activities from both *B. altitudinis* R31 and J208 were detected to be ~230 units at pH 11·0, 40°C and ~160 units at pH 11·0, 70°C.

Effect of temperature on stability of crude pectin lyase activity

Relative residual pectinase activity assayed to study the thermal effect on enzyme stability demonstrated that crude pectinases from all three *Bacillus* isolates maintained more than 90% activity up to 8 h at 40°C (data not shown) and 4 h at 50°C. While at 60°C, $t_{1/2}$ was observed to be approximately 80 min for *B. safensis* M35 and 60 min for *B. altitudinis* R31 and J208. At 70°C, $t_{1/2}$ decreased to less than 15 min (Fig. 2d–f).

Effect of pH on stability of crude pectin lyase activity

Relative residual pectinase activity in different pH buffers at 40°C revealed that *B. safensis* M35 pectinase was stable over pH range 6–9 retaining ~95–100% activity in all cases., whereas *B. altitudinis* R31 and *B. altitudinis* J208 pectinases were most stable over the pH range 5–9 showing more than 100% RA (Fig. 2g,h). Also, it was observed that the buffered enzyme (pH range 5·0–9·0) was more active as compared with unbuffered condition for all three *Bacillus* isolates.

Effect of crude pectinase on relative flow rate and viscosity of pectin

The crude pectinases from *B. safensis* M35, *B. altitudinis* R31 and *B. altitudinis* J208 showed a rapid relative decrease of 47·50, 73·83 and 60·83% in viscosity, respectively, within initial 10 min of incubation with gradual

Figure 3 Mode of action of crude pectin lyase from *Bacillus safensis* M35, *Bacillus altitudinis* R31 and *B. altitudinis* J208: (a) Drop in viscosity (close symbols, dark) and accumulation of unsaturated oligosaccharides (open symbols, white) during the breakdown of pectin substrate by pectin lyase of *B. safensis* M35 (▲, △), *B. altitudinis* R31 (■, □) and *B. altitudinis* J208 (●, ○); error bars represented as standard errors of the mean (SEM) for $n = 3$. (b–f) HPLC chromatogram of (b) pectin as substrate control; (c) D-galacturonic acid as monomer control; (d–f) pectin after 10 h of incubation with individual pectinases from (d) M35, (e) R31 and (f) J208; dashed line (-----) at *x*-axis indicates the time span of 5 min on chromatogram. [Colour figure can be viewed at wileyonlinelibrary.com]

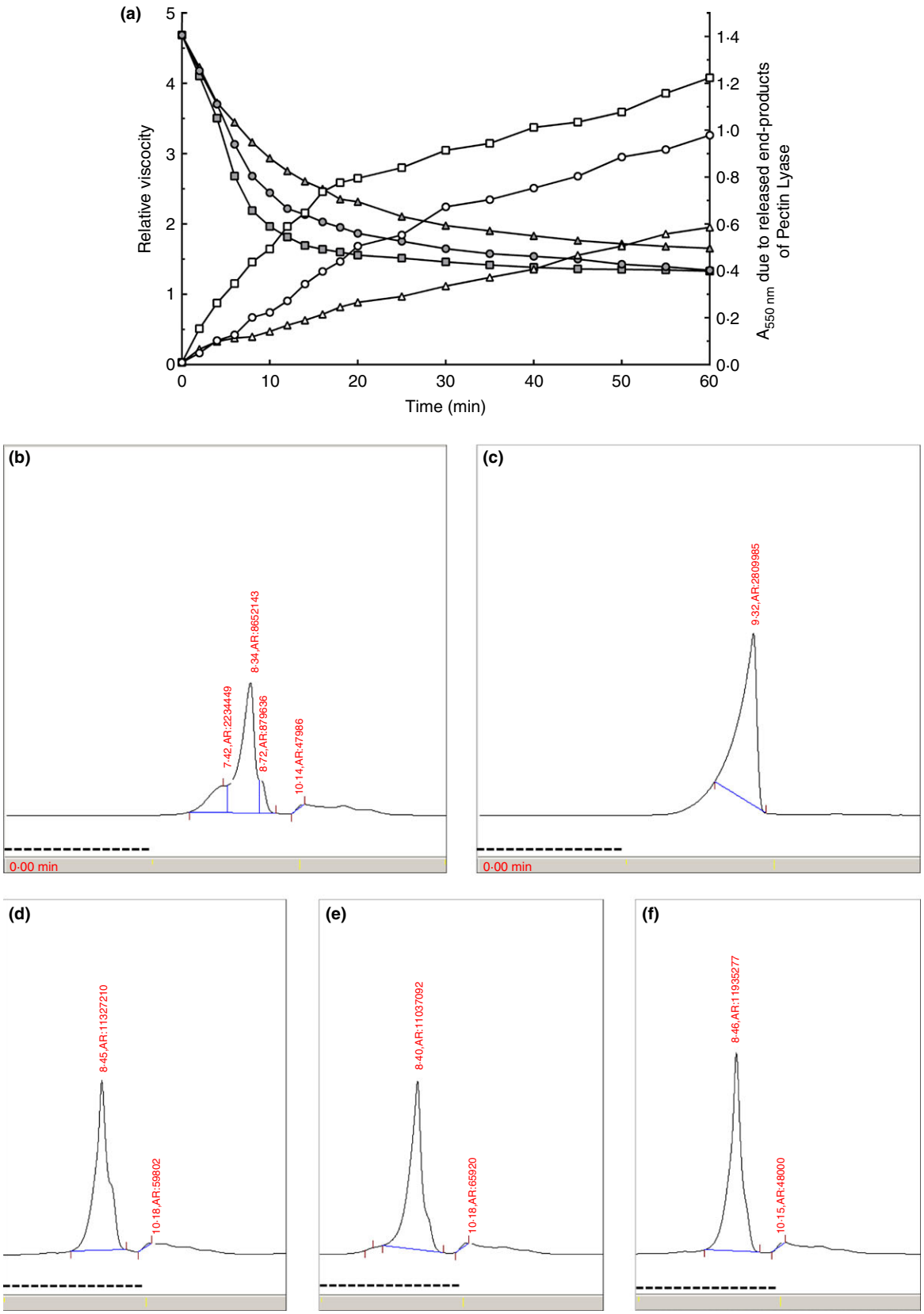
(a)
Relative viscocity
Time (min)
$A_{550\ nm}$ due to released end-products of Pectin Lyase
(b)
7·42,AR:2234449
8·34,AR:8652143
8·72,AR:879636
10·14,AR:47986
0·00 min
(c)
9·32,AR:2809985
0·00 min
(d)
8·45,AR:11327210
10·18,AR:59802
(e)
8·40,AR:11037092
10·18,AR:65920
(f)
8·46,AR:11935277
10·15,AR:48000

Table 2 Protein content and enzyme activities for various enzyme preparations

Enzyme and preparation	Protein (μg ml^{-1})	PNL (U ml^{-1})	PL (U ml^{-1})	PG (U ml^{-1})	Xylanase (U ml^{-1})	FPA (FPU per ml)
Pectinase						
Bacillus safensis M35	52·1 ± 2·7	9461·3 ± 106·2	10 425·6 ± 160·4	11·3 ± 0·7	0·5 ± 0·1	0·0
Bacillus altitudinis R31	67·4 ± 5·6	9625·0 ± 399·2	12 996·8 ± 98·3	14·6 ± 0·5	0·3 ± 0·0	0·0
B. altitudinis J208	65·5 ± 3·9	14 077·3 ± 124·1	14 221·2 ± 84·5	14·8 ± 0·2	0·7 ± 0·1	0·0
Xylanase						
B. safensis M35	66·3 ± 5·2	578·9 ± 35·2	701·8 ± 54·9	4·8 ± 0·1	4·8 ± 0·0	0·0
B. altitudinis R31	36·7 ± 3·3	1872·5 ± 120·3	2677·3 ± 178·3	4·1 ± 0·0	5·0 ± 0·1	0·0
B. altitudinis J208	39·4 ± 2·2	2261·3 ± 280·5	2848·0 ± 90·2	4·9 ± 0·1	5·5 ± 0·1	0·0
Commercial cellulase	529·2 ± 14·1	122·6 ± 19·2	170·1 ± 12·2	4·6 ± 0·1	4·8 ± 0·2	4·0 ± 0·2

Values presented are mean ± SEM.

increase in released end products (Fig. 3a). Both the decrease in viscosity and end product release were rapid in the initial duration of 0–10 min, which thereafter gradually slowed down. In HPLC analysis, the pectin substrate control demonstrated three individual peaks very close to each other at retention time of 7·4, 8·3 and 8·7 min (Fig. 3b). As the enzyme reaction progressed up to 10 h, the former peak at 7·4 min disappeared and the latter two peaks at 8·3 and 8·7 min merged together at 8·4 min with increase in peak area as can be seen from Fig. 3d–f. Moreover, even after incubation of 10 h, the enzyme failed to release the monomeric end products as no peak corresponding to D-galacturonic acid (at 9·3 min) was observed (Fig. 3c).

Effect of crude pectinase supplementation to xylanase and/or commercial cellulase cocktail mediated plant biomass saccharification

Wheat bran induced crude enzymes from individual *Bacillus* cultures possessed xylanolytic activity on beechwood xylan, and CP induced crude enzymes from the same *Bacillus* possessed pectinolytic activity on pectin as well as on PGA. None of the cultures exhibited cellulolytic activity on filter paper. However, in addition to cellulolytic activity, commercial cellulase possessed xylanase activity equivalent to the xylanolytic enzymes of the *Bacillus* isolates and PG, PL and PNL activities less than the pectinolytic enzymes of the *Bacillus* isolates. Table 2 represents xylanase, PG, PL, PNL and cellulase on various substrates by all the enzymes used in the study.

In general, the saccharification treatment of the agrowastes BH, SCB and WH with pectinase and xylanase from individual cultures and commercial cellulase (C) separately released more reducing sugar as the reaction progressed from 12 to 60 h. Xylanases from individual cultures released more while the pectinases released less reducing sugar than cellulase from all three agrowastes tested (Fig. 4a–c). Accordingly, 4 U of commercial cellulase released approximately 4·6, 5·0 and 9·6 mg of reducing sugars from BH, SCB and WH, respectively, which is similar to the amount of sugar released by M35 xylanase (4·1, 5·3 and 9·1 mg) and less when compared with R31 xylanase (7·5, 7·2 and 9·7 mg) and J208 xylanase (8·7, 9·3 and 14·0 mg) as can be seen from Fig. 4a–c.

Crude pectinase when applied in supplementation with xylanase and/or cellulase, incubation up to 60 h aided effective agrowaste saccharification. Enhanced % saccharification was observed at 60 h when compared with 12 h. Comparison of saccharification fold increase between different cocktail groups (PX, CP and CPX) revealed effective increase in fold saccharification for higher enzyme concentration (Supporting information, Tables S1–S3).

When crude xylanase and crude pectinase were applied as (PX) cocktail, maximum of 9·88% saccharification was observed as compared with 8·38%, which is the cumulative value of individual enzymes after 60 h on WH. 9·37 and 6·69 were % saccharification values obtained by the PX cocktail as compared with cumulative values of 5·69 and 5·67 for BH and SCB, respectively, at 60 h (Fig. 5a–c and Table S1). Similarly, during application of cellulase-pectinase (CP), cocktail observed value of % saccharification was 11·72 against cumulative value of 6·20 at 60 h on WH. 6·45 and 4·82 were % saccharification values obtained by the CP cocktail against cumulative values of 3·63 and 3·51 for BH and SCB, respectively (Fig. 5d–f and Table S2).

The CPX cocktail, CP_4X_4 provided maximum values of 10·58, 9·68 and 16·14 for % saccharification against cumulative % saccharification values of 7·99, 8·20 and 13·21 at 60 h on BH, SCB and WH with fold increase of 1·32, 1·18 and 1·22. With supplementation of higher dosage, that is, 0·4 mg when compared with 0·2 mg of enzymes with fixed amount of cellulase, the saccharification yield of agrowastes increased gradually with time and resulted in higher % saccharification and eventually positive fold increase (Fig. 6a–c and Table S1). When the

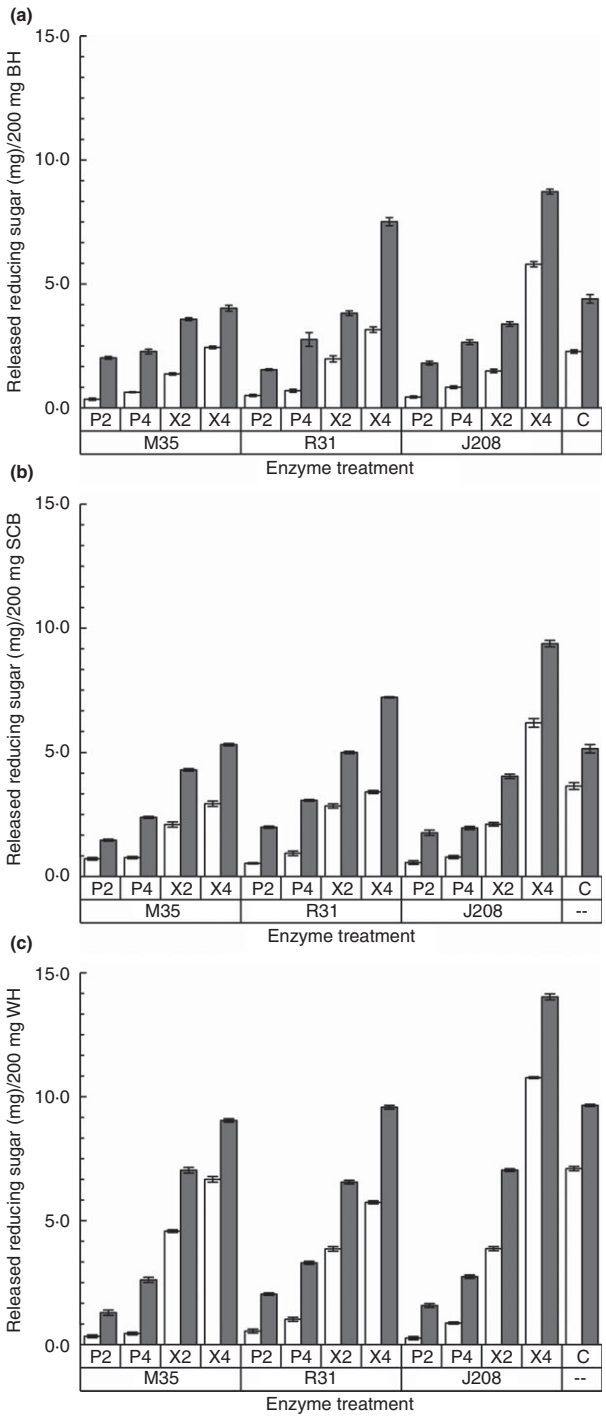

Figure 4 Hydrolysis of barley husk (BH), sugarcane bagasse (SCB) and wheat husk (WH) by pectinase, xylanase and commercial cellulase individually: Effect of 0·2 and 0·4 mg of individual pectinase (P_2 and P_4, respectively) and xylanase (X_2 and X_4, respectively) and commercial cellulase (C) on (a) BH, (b) SCB and (c) WH hydrolysis. 0·2 or 0·4 mg of enzyme from individual culture is separately represented in group of 12 h (white bar, □) and 60 h (grey bar, ▩); error bars represented as standard error of the mean (SEM), for $n = 3$.

CPX cocktail efficacy was compared with chemical method of acid hydrolysis by HCl (Fig. 6d), enhanced % saccharification of SCB and WH was observed.

Discussion

Bioethanol production from plant biomass is a two-step process which consists of saccharification of plant biomass to fermentable sugars and subsequent fermentation of sugars to alcohol. Saccharification is carried out by microbial enzyme cocktails of plant polysaccharide degrading enzymes that are compatible with each other. Such application necessitates that the enzyme should possess several qualities like inexpensive and rapid production on raw material, broad substrate specificity with broad temperature and pH optima and endo-acting mode of action.

Wheat bran has moderate while CP has high fraction of pectic polymer content. Therefore, they were amended separately as an enzyme inducer in the medium for production of pectinase. Increased production of PG, PL and PNL was observed for all three *Bacillus* isolates in wheat bran or CP containing production media after supplementation with YE and peptone (Fig. S1). Kashyap *et al.* (2000) has also reported increased pectinase production in medium supplemented with YE and peptone by *Bacillus* sp. DT7.

The ratio of esterified galacturonic acid groups to total galacturonic acid groups in the homogalacturonan backbone of a pectic substrate is termed as degree of methyl esterification (DE). Pectin is made up of homogalacturonan with high DE, whereas pectic acid or PGA is composed of homogalacturonan without any esterification. As shown in Table 1, increase in DE gradually decreased the amenability of pectin to the enzyme reaction, and thus, breakdown of pectic substrates with high DE is comparatively more difficult than breakdown of PGA by both the hydrolase and lyase enzymes. All three pectinase enzymes PG, PL and PNL were able to hydrolyse simple to highly esterified complex pectic polymers due to their broad-spectrum substrate specificities. Such broad substrate preference has been reported in case of *Erwinia carotovora* subsp. *carotovora* (Ecc) BR1 towards moderate and high methyl esterified pectin for hydrolase activity (Maisuria *et al.* 2010) and for lyase activity (Maisuria and Nerurkar 2012), respectively.

When NCBI database of shotgun sequencing genome assemblies for type strains *B. safensis* Fo36b^T (ASJD01000001.1–ASJD01000038.1) and *B. altitudinis* 41Kf2b^T (ASJC01000001.1–ASJC01000039.1) were analysed for presence of pectinase(s), annotations revealed the presence of genes only for PL and pectin methyl esterase (PME) enzymes in both the organisms (protein IDs for PL: KDE28588.1 and for PME: KDE29732.1 from B. *safensis*

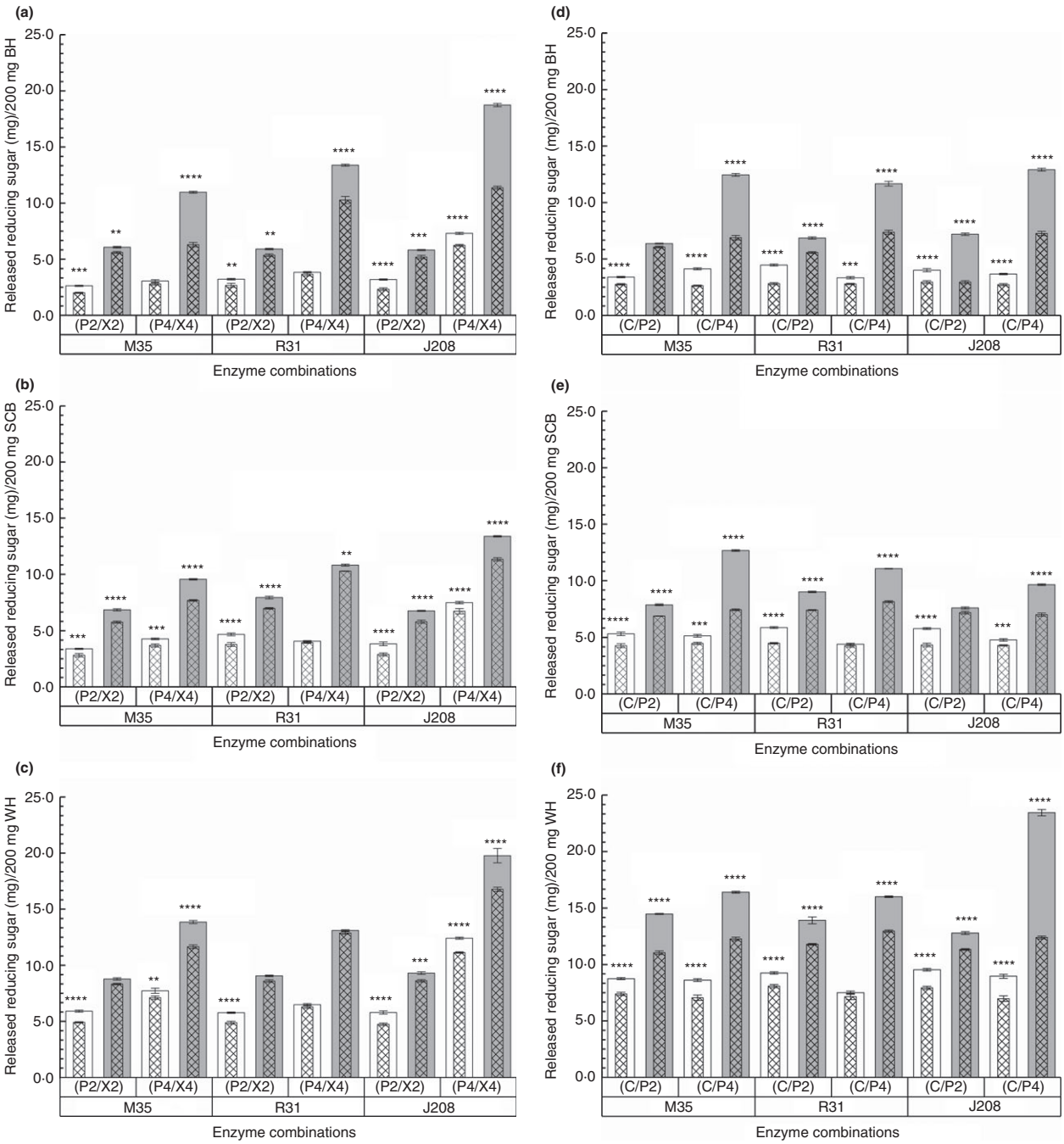

Figure 5 Hydrolysis of barley husk (BH), sugarcane bagasse (SCB) and wheat husk (WH) by cocktail of two enzymes: (a–c) Synergistic effect of pectinase (P) and xylanase (X) in combination on (a) BH, (b) SCB and (c) WH hydrolysis; (d–f) Synergistic effect of pectinase (P) and cellulase (C) in combination on (d) BH, (e) SCB and (f) WH hydrolysis; yield of enzyme activity is represented on left *y*-axis as groups of 0·2 and 0·4 mg enzyme load for each culture and in turn overlapped for sum of yield of individual enzyme (crossed pattern, 12 h white ⊠ and 60 h grey ⊠) with yield of enzyme cocktail (clear pattern, 12 h white □ and 60 h grey ▣); error bars represented as standard error of the mean (SEM), and asterisk marks (*) above each group of two bars represent significant differences ($P < 0{\cdot}01$) for $n = 3$.

Fo36b^T; for PL: KDE31317.1 and for PME: KDE30325.1 from *B. altitudinis* 41Kf2bT.). PME catalyses the de-esterification of methyl ester linkages from galacturonan backbone of pectic substrates and releases pectic acids and methanol. This acidic pectin is the substrate for polygalacturonate hydrolase and/or lyase enzymes.

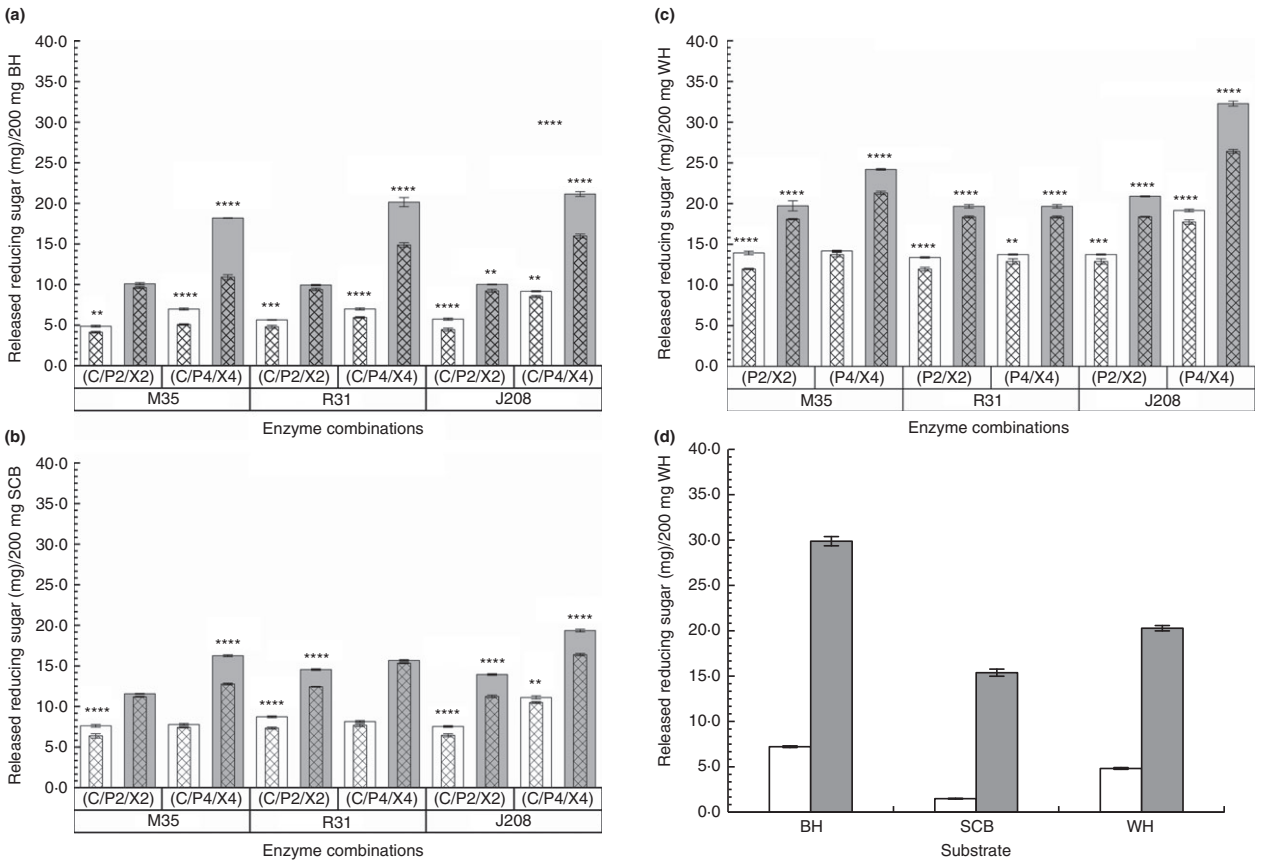

Figure 6 Hydrolysis of barley husk (BH), sugarcane bagasse (SCB) and wheat husk (WH) by three enzyme cocktail and acid: (a–c) Synergistic effect of pectinase (P) and xylanase (X) and cellulase (C) in combination on (a) BH, (b) SCB and (c) WH hydrolysis; (d) chemical hydrolysis of agro-waste by HCl; yield of enzyme activity is represented on left *y*-axis as groups of 0·2 and 0·4 mg enzyme load for each culture and in turn over-lapped for sum of yield of individual enzyme (crossed pattern, 12 h white ⊠ and 60 h grey ⊠) with yield of enzyme cocktail (clear pattern, 12 h white □; and 60 h grey ■). Error bars represented as standard error of the mean (SEM), and asterisk marks (*) above each group of two bars represent significant differences ($P < 0{\cdot}01$) for $n = 3$.

Since, PME activity was not present and lyase and hydrolase activities were observed in the crude pectinase preparation of all three *Bacillus* isolates, there was a possibility that the activity band observed on pectin and PGA zymogram might be due to PL. Notably only one single band from each isolate on silver stained gel was found to correspond with the activity band obtained on developed zymogram of both pectin and PGA substrates (Fig. 1a,b). The SDS gel analysis results along with genome database search also suggest that there must be only single lyase enzyme produced by the three *Bacillus* strains, which acts on both PGA and pectin substrates. In literature too genus *Bacillus* has been reported to produce alkaline PL predominantly (Zhou *et al.* 2017). Our results suggest that pectinases from all three *Bacillus* isolates were broad-spectrum lyases as they were able to hydrolyse pectic substrates with varying DE. Such pectinases can be of particular interest because they break pectin polymers causing increase in structural porosity and also open up the pockets in structural complex matrix of cell wall to expose cellulose for cellulase activity (Yadav *et al.* 2009).

When there are several enzymatic processes to be carried out in a single system (such as saccharification of diverse polysaccharides by enzyme cocktail), one of the major limiting factor is different optimal conditions (e.g. temperature and pH) for individual reaction. The core enzyme cellulase has been reported to be active in 4·0–7·0 pH and 40–65°C temperature range (Cardona and Sanchez 2007). In this context, the broad activity range of pH and temperature is a beneficial attribute of the enzymes for their industrial applications. Pectinolytic enzymes from the three *Bacillus* isolates were mesophilic as they demonstrated activity over the 40–60°C range of temperature and 6·0–10·0 range of pH (Fig. 2a–c). The results also revealed that the pectinases of all three

isolates *B. safensis* M35, *B. altitudinis* R31 and *B. altitudinis* J208 were active in neutral to moderate alkaline (pH 8·0–10·0) conditions. Additionally, pectinases of *B. altitudinis* R31 and J208 were also active in mild acidic pH conditions. Together the pH range of the activity of the three pectinases in a cocktail would be extended from 6·0 to 10·0 at around 40°C temperature. During long duration of incubation, the enzymes showed stability at 40 and 50°C, which is the range suitable for application in biomass saccharification. As shown in Fig. 2d–f with extended exposure of the enzyme at higher temperature of 60 and 70°C, destabilizing the enzyme results in the reduction of the residual activity. Pectin lyase from *Brevibacillus borstelensis* (P35) possessed optimum temperature of 60°C at pH 8·0 (Demir *et al.* 2014). In comparison to fungal pectinases which are more stable at low pH, pectinases from all three *Bacillus* isolates were active at mild acidic to alkaline range from pH 6·0 to 10·0 (Fig. 2g–i). Similar kind of alkali stable bacterial pectinase has been reported from *Bacillus* sp. DT7 for a range of pH 6·0–9·0 (Kashyap *et al.* 2000). While applications of the acidic pectinases and alkaline pectinases are limited in food and textile industries, respectively, the stability of pectinases from the three *Bacillus* isolates in mild acidic to moderate alkaline pH would be an asset to varied applications considering their mesophilic nature. Xylanolytic activity suitable for agrowaste saccharification from these isolates *B. safensis* M35, *B. altitudinis* R31 and *B. altitudinis* J208 has been reported earlier in the temperature range of 30–60°C and pH range of 5·0–9·0 (Thite and Nerurkar 2015).

Viscosity is a characteristic of the fluid that opposes relative motion between two surfaces of the fluid which are moving at different velocities. Positive correlations between the molecular weight or length of the polymers or branching pattern of the polymer with viscosity has been shown by Mortimer *et al.* (1964). Breakdown of the polymer eases the branching and reduces the length and molecular weight of the polymer, which consequently reduces the force required for movement of two fluid layers. This results in increased motion of the layers and reduced flow time and viscosity. Breakdown of the α (1→4) linkage between the D-galacturonic acid residues in backbone of PGA chain is responsible for the drop in viscosity of pectin solution. If this cleavage is of the terminal bonds of the polysaccharide backbone, drop in the viscosity will be slow with rapid increase in end products, that is, monomer accumulation, while only the cleavage of α (1→4) linkages at positions away from the terminus of the polysaccharide (endo cleavage) can drop the viscosity faster with gradual increase in the end products of unsaturated oligogalacturonides. As observed in Fig. 3a, rapid decrease in viscosity by pectinases of three *Bacillus* spp. within initial 10 min suggests their endo-acting nature. *Aspergillus niger* endopectinase has been shown to reduce 32·7% viscosity after 30 min and 50·0% after 60 min in the studies by Maiorano and Ogaki (1995).

HPLC analysis of the reaction product after incubation period of 10 h showed that the pectin substrate peak obtained at RT 7·4 min out of three peaks at RT: 7·4, 8·3 and 8·7 min disappeared, while the one at RT 8·4 min increased, suggesting accumulation of breakdown products of the pectic substrate (Fig. 3d–f). The peak corresponding to D-galacturonic acid monomer (RT: 9·3 min) was absent. The above results clearly pointed that the end products are oligomers and not monomers, which in turn confirmed the endo-acting mode of the three pectinases. The broad-spectrum bacterial pectinases in the present studies with endo-acting nature possess all the necessary attributes for the enzymes that can act as accessory enzymes to aid in cellulase cocktail-mediated plant biomass saccharification.

The proximate composition of three different agrowaste biomass without any pretreatment in terms of % cellulose, hemicellulose, pectin (galactan) and lignin has been reported as follows: BH: 34·8, 30·4, 1·1 and 24·5 (Krawczyk *et al.* 2008); SCB: 41·4, 28·2, 1·3 and 23·6 (Ferreira-Leitão *et al.* 2010); WH: 36, 18, 1·9 and 16 (Bledzki *et al.* 2010). The differences in composition of structural polysaccharides in these agrowaste residues were the reason of their selection for enzymatic saccharification studies.

Low release of end products by all three pectinases during saccharification of BH, SCB and WH by individual enzymes was possibly due to the presence of low amount of structural pectin than xylan or glucan in these agrowastes, whereas less end product release by cellulase as compared to that of higher load of individual xylanases can be possibly attributed to the cellulose-hemicellulose meshwork which was being impenetrable to cellulase, restricted its action on cellulose (Fig. 4a–c). Thus, it is clear that the core group of commercial cellulase require assistance of accessory enzymes essentially to unmask the cellulose fibres from matrix of hemicellulose and pectin.

Further synergism studies with enzyme cocktails consisting of commercial cellulase amended with the crude pectinases and/or crude xylanases from the *Bacillus* isolates were performed as an additive synergism where accessory enzymes are added to constant amount of core enzymes, unlike the substitutive synergism where a portion of one enzyme is substituted with other enzyme keeping total protein load constant (Hu *et al.* 2011). Synergism was clearly observed as the ratio of the end products released by cocktail (X_2P_2, X_4P_4, CP_2, CP_4, CP_2X_2 and CP_4X_4), and cumulative product released by the

individual enzyme when used separately (X_2+P_2, X_4+P_4, C+P_2, C+P_4, C+P_2+X_2 and C+P_4+X_4) was higher than one indicating enhancement in saccharification of biomass by cocktail. Increase in degree of saccharification was observed when the PX, PC (Fig. 5a–f) and CPX (Fig. 6a–c) cocktail combinations from crude enzymes were applied to agrowaste biomass, DS has exceeded 1·0 in all cases (Tables S1–S3).

It was observed that even at increased dosage of crude pectinase (0·4 mg instead of 0·2 mg) in some cases yielded less saccharification during shorter period of incubation possibly due to the structural complexities of the agrowaste substrates. This can be attributed to the fact that availability of sufficient pectin substrate increased as the disintegration of the cell wall complex progressed.

In xylanase-pectinase cocktail of individual *Bacillus* spp., enhanced release of reducing sugars suggested that both accessory enzymes are compatible with each other. Though commercial cellulase showed xylanase activity, supplementation of crude xylanase and crude pectinase enhanced the saccharification, suggesting that both the enzymes play an important role in synergism with commercial cellulase. Synergism between crude xylanases obtained from these *Bacillus* isolates and commercial cellulase for sugar release from the agrowaste used in this study, that is, BH, SCB and WH were also reported in our earlier work (Thite and Nerurkar 2015). In similar studies with fresh, steam exploded and ensiled hemp, when crude preparation of accessory xylanase and pectinase were applied with commercial cellulase released approximately 5·0, 11·5 and 7·0 mg ml^{-1} of reducing sugars after 48 h suggesting synergism of pectinase with xylanase and/or cellulase (Zhang *et al.* 2013). In present studies, enhanced enzymatic saccharification of complex substrates as compared with acid saccharification of the same substrates proves the efficiency of the enzyme cocktail and creates a room for development or further modification and improvement of enzyme cocktail formulations for saccharification.

In conclusion, *B. safensis* M35 and *B. altitudinis* R31 and *B. altitudinis* J208 isolated from three different dung samples viz. camel, bull and buffalo, respectively, produced pectinases. These were together effective over mesophilic range of temperature 30–60°C and mild acidic to alkaline range of pH 6·0–9·0. Moreover, they reduced viscosity by breaking down pectic polymer in endolytic manner and had specificities for different complex pectic substrates. In combination with xylanase and/or cellulase as a cocktail, they enhanced biomass saccharification. Together, all these properties can be an advantage for their application as 'accessory enzymes' in cellulase mediated saccharification of agrowastes. The studies have confirmed the accessory role of pectinase in combination with xylanase and/or cellulase and demonstrated that on involvement in cocktail mediated saccharification, the pectinase can work contributively with xylanase and cellulase to enhance the saccharification.

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Conflict of Interest

No conflicts of interests declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 % Saccharification and fold increase due to enzymatic hydrolysis by combination of pectinase with xylanase and chemical hydrolysis by 1N HCl

Table S2 % Saccharification and fold increase due to enzymatic hydrolysis by combination of pectinase with cellulase and chemical hydrolysis by 1N HCl

Table S3 % Saccharification and fold increase due to enzymatic hydrolysis by combination of pectinase and xylanase with cellulase and chemical hydrolysis by 1N HCl

Figure S1 Production of pectinases on pectin and crude complex polysaccharides by *B. safensis* M35, *B. altitudinis* R31 and *B. altitudinis* J208: (a) polygalacturonate hydrolase, (b) pectin lyase, (c) pectate lyase on wheat bran, pectin and citrus peel containing BHM without any supplementation (grey bar with crossed pattern) and with yeast extract and peptone supplementation (white bar without any pattern