Ann Microbiol (2014) 64:267–274
DOI 10.1007/s13213-013-0659-0

ORIGINAL ARTICLE

Biomass hydrolyzing enzymes from plant pathogen *Xanthomonas axonopodis* pv. *punicae*: optimizing production and characterization

Dolamani Amat · Anju Arora · Lata Nain · Anil Kumar Saxena

Received: 27 September 2012 / Accepted: 2 May 2013 / Published online: 24 May 2013

Abstract *Xanthomonas axonopodis* pv. *punicae* strain—a potent plant pathogen that causes blight disease in pomegranate—was screened for cellulolytic and xylanolytic enzyme production. This strain produced endo-β-1,4-glucanase, filter paper lyase activity (FPA), β-glucosidase and xylanase activities. Enzyme production was optimized with respect to major nutrient sources like carbon and nitrogen. Carboxy methyl cellulose (CMC) was a better inducer for FPA, CMCase and xylanase production, while starch was found to be best for cellobiase. Soybean meal/yeast extract at 0.5 % were better nitrogen sources for both cellulolytic and xylanolytic enzyme production while cellobiase and xylanase production was higher with peptone. Surfactants had no significant effect on levels of extracellular cellulases and xylanases. A temperature of 28 °C and pH 6–8 were optimum for production of enzyme activities. Growth under optimized conditions resulted in increases in different enzyme activities of around 1.72- to 5-fold. Physico-chemical characterization of enzymes showed that they were active over broad range of pH 4–8 with an optimum at 8. Cellulolytic enzymes showed a temperature optimum at around 55 °C while xylanase had highest activity at 45 °C. Heat treatment of enzyme extract at 75 °C for 1 h showed that xylanase activity was more stable than cellulolytic activities. *Xanthomonas* enzyme extracts were able to act on biologically pretreated paddy straw to release reducing sugars, and the amount of reducing sugars increased with incubation time. Thus, the enzymes produced by *X. axonopodis* pv. *punicae* are more versatile and resilient with respect to their activity at different pH and temperature. These enzymes can be overproduced and find application in different industries including food, pulp and paper and biorefineries for conversion of lignocellulosic biomass.

Keywords Lignocellulosic biomass · Cellulases · Xylanase · *Xanthomonas* · Plant pathogen

Electronic supplementary material The online version of this article (doi:10.1007/s13213-013-0659-0) contains supplementary material, which is available to authorized users.

D. Amat · A. Arora (✉) · L. Nain · A. K. Saxena
Division of Microbiology, Indian Agricultural Research Institute, 110012, New Delhi, India
e-mail: anjudev@yahoo.com

Introduction

Ever increasing energy demand, depletion of fossil fuels and increased environmental concern has shifted the focus of energy generation towards renewable alternatives. Bioenergy from biomass is promising and is available in the form of solid fuel, liquid biofuel and biogas. Bioenergy from biomass, especially second generation biofuels, has significant advantages over other types of renewable energies because biomass is the most abundant, renewable material on our planet and helps in C sequestration. Lignocellulose, consisting of cellulose, hemicelluloses and lignin, is the major structural component of all plant biomass. Cellulose, the most abundant organic molecule on Earth, is a glucose homopolymer while hemicelluloses, the second most abundant component of lignocellulosic biomass, are heterogeneous polymers of pentoses (including xylose and arabinose), hexoses (mainly mannose, less glucose and galactose) and sugar acids. The sugars in these two polysaccharides are fermented into fuel molecules; however, prior to fermentation, the polysaccharides have to be hydrolyzed into an assimilable and fermentable form.

Enzymatic hydrolysis of cellulose and hemicellulose to fermentable sugars is achieved using a complex set of enzymes called cellulases and hemicellulases. Bacteria and fungi are good sources of cellulases and hemicellulases. The classical cellulase system includes endoglucanase, exoglucanase, and cellobiase. Hemicellulases include enzymes that break down β-1,4 xylan and various other side chains. Currently, enzymatic saccharification of cellulose is achieved using preparations from saprophytic organisms *Trichoderma* and *Aspergillus*. However, there are several limitations, such as the crystalline recalcitrance of cellulose, product inhibition, enzyme deactivation, etc., which hinder high saccharification yields and make enzymatic hydrolysis the most costly step in biomass to ethanol process. It has been estimated that the greatest returns in cost savings will be realized by improving conversion of biomass to sugars, increasing hydrolysis yields, reducing enzyme loadings, and eliminating or reducing pretreatment (Lynd et al. 2008). A broader suite of enzymes is required for hydrolysis of cellulose and hemicelluloses to fermentable sugars (McMillan et al. 2011). Enzymatic hydrolysis can be carried out effectively if a mixture of different cellulolytic and accessory enzymes is used. Development of low cost, commercial enzyme mixtures with hemicellulases that work synergistically with cellulases is one of the goals of current research activities in this field. Having a diverse library of cellulases and other complementary enzymes will enable tailoring of such cocktails. Many bacterial and plant pathogens are known to produce an arsenal of plant-cell-wall-degrading enzymes, which can be more potent as the pathogens have to overcome plant defenses (King et al. 2011). A number of plant pathogenic fungi (*Fusarium oxysporum*, *Phoma betae*, *Collectotrichum gloeosporioides*, *Myrothecium verrucaria*, etc.) have been reported to elaborate high levels of cellulases (Ortega 1994; Moreira et al. 2005). The pathogenicity of a large number of Gram negative bacteria (*Xanthomonas*, *Erwinia* and *Pseudomonas*) relies on their ability to secrete hydrolytic enzymes into external medium (Kamoun and Kado 1990; Kazemi-Pour et al. 2004). Many plant pathogens, including those belonging to genus *Xanthomonas*, secrete extracellular depolymerizing enzymes, such as cellulases, xylanases, proteases and pectinases (Singh and Verma 1975; Chapon et al. 2001). Although the role of depolymerizing enzymes in necrosis and pathogenicity is well documented, studies on the commercial production of cellulases and xylanases from other plant pathogens, including *Xanthomonas*, are almost lacking. A literature survey revealed scant information regarding purification and characterization of cellulase enzyme from *Xanthomonas*. Thus, the present study aims at production, optimization and characterization of cellulolytic and xylanolytic enzymes produced by *X. axonopodis* pv. *punicae,* a potent plant pathogen of a number of crops like rice, soybean, cotton, pomegranate, etc., for use in saccharification of lignocellulosic biomass.

Material and methods

Organism and strain maintenance

Xanthomonas axonopodis pv. *punicae* strain was procured from the germplasm collection at the Division of Microbiology, IARI, New Delhi. It was grown and maintained on nutrient agar at 28 °C for 3 days and slants were stored at 6–8 °C in a refrigerator.

Determining cellulolytic and xylanolytic abilities

The cellulolytic and xylanolytic potential of *X. axonopodis* was judged qualitatively on the basis of its ability to grow on mineral medium [2.0 g KH_2PO_4; 1.4 g $(NH_4)_2SO_4$, 1.4 g KNO_3; 0.3 g $MgSO_4 \cdot 7\ H_2O$; 0.3 g $CaCl_2$; 5.0 mg $FeSO_4 \cdot 7\ H_2O$; 1.6 mg $MnSO_4 \cdot H_2O$; 2.0 mg $CoCl_2$; 1.4 mg $ZnSO_4 \cdot 7\ H_2O$ in 1 l at pH 7] with 1 % acid swollen cellulose or 0.1 % xylan as sole carbon source (Rautela and Cowling 1966). The ability of *X. axonopodis* pv. *punicae* strain to produce endoglucanases was also assessed by its ability to produce zones of hydrolysis on carboxy methyl cellulose (CMC) agar as proposed by Teather and Wood (1982). The cultures were point inoculated on basal agar medium containing 1 % CMC and incubated at 28 °C for 5 days. Plates were flooded with aqueous congo red solution (1 mg ml^{-1}). After 15 min, dye was drained and plates were washed with 1 M NaCl and observed for production of yellow zones around growth.

For quantitative assay, *X. axonopodis* strain was grown in 25 ml basal mineral medium dispensed in 100 ml Erlenmeyer flasks with 1 % CMC as C source supplemented with 0.1 % peptone and yeast extract under submerged culture at 28 °C. A bacterial overnight culture grown in nutrient broth was used as inoculum at a rate of 10 %. After 7 days growth on a shaker at 150 rpm and 28 °C, the contents of flask were centrifuged at 10,000 rpm for 10 min and the supernatant was used for estimation of different enzyme activities.

Assay of cellulase and xylanase activity

Activity of cellulase complex was assayed in terms of filter paper activity (FPA /exo-β-1,4- glucanase) and CMCase (endo-β-1,4-glucanase) using Whatman no. 1 and CMC as substrate and incubation at 50 °C as described by Ghose (1987). Xylanase activity was assayed by the method described by Ghose and Bisaria (1987) using xylan. Reducing sugars released were measured by the DNSA method (Miller 1959). One unit of FPA or CMCase corresponded to 1 μmol glucose formed per minute during hydrolysis and one enzyme unit of xylanase was expressed as 1 μmol xylose formed per minute during hydrolysis. β- glucosidase assay was performed using p-nitrophenyl-β-D-glucopyranoside as

Table 1 Effect of different carbon sources on the production of enzyme activities by *Xanthomonas axonopodis* pv. *punicae* strain. *CMC* Carboxymethylcellulose, CMCase carboxymethylcellulase, *FPA* filter paper activity, *SEM* standard error of the mean, *CD* critical difference

	Specific activity				
Carbon source	CMCase	FPA	Xylanase	β-Glucosidase	Protein concentration (mg ml^{-1})
CMC	0.071	0.059	0.702	0.082	0.085
α-Cellulose	0.006	0.077	0.022	0.178	0.177
Starch	0.038	0.007	0.023	0.198	0.198
Xylan	0.013	0.004	0.006	0.155	0.155
SEM	0.005	0.005	0.011	0.022	0.015
CD ($P > 0.05$)	0.013	0.013	0.029	0.059	0.040

substrate (Wood and Bhat 1988) and activity was calculated in terms of micromoles of p-nitrophenol produced per milliliter of culture filtrate per minute.

Optimization of cellulases and xylanase production

The one-factor-at-a-time methodology was employed to determine the best C and N source. The effect of different C sources on the production of cellulases and xylanases was studied by supplementing basal medium with 1 % of one of the following C substrates: CMC, cellulose powder, starch or xylan. Cultures were incubated at 28 °C and, after 7 days of incubation, enzyme activities were assayed as described above. Different organic N sources were added to basal medium at 0.5 % concentration to test their effect on enzyme production. Incubation and enzyme assays were performed as mentioned above. The effect on extracellular levels of enzymes produced by *X. axonopodis* pv. *punicae* of addition of surfactants like Tween-20, Tween-80, Triton X-100 was also studied. Basal medium was amended with surfactant at a concentration of 0.1 % (w/v or v/v and, after appropriate incubation, enzyme activities were assayed as mentioned above.

Effect of temperature and pH on cellulolytic and xylanolytic enzyme production

The optimum temperature for production of extracellular enzymes was determined by incubating the cultures at 28 °C or 37 °C. To determine the optimum pH leading to maximum enzyme production, the final pH value of the culture medium was adjusted in the range 4–8 by adding 1 *N* HCl or 1 *N* NaOH. Incubation and enzyme assays were carried out as described above.

Physico-chemical characterization and thermal stability of enzyme activities

To determine the optimum pH for enzyme activity, the enzyme reactions were performed in 0.05 M citrate phosphate buffer with pH ranging from 4 to 8 in which the substrate was dissolved and incubated at 50 °C. The optimum temperature for enzyme activity was determined by incubating the standard reaction mixtures at temperatures between 45 °C and 75 °C.

To test the thermal stability of the enzymes, the supernatant was kept at 75 °C for 1 h in a water bath. It was then cooled and residual activities determined by performing enzyme assays at 50 °C using the standard protocols described above.

Growth of *X. axonopodis* under optimized conditions and levels of enzymes produced

The organism was grown in 1 l Erlenmeyer flasks containing 250 ml medium under optimum conditions using the best C and N source, and enzyme activities were assayed after 7 days.

Saccharification of biologically pretreated paddy straw using enzyme extract produced by *Xanthomonas axonopodis* pv. *punicae*

Enzymatic saccharification of lignocellulosic substrate, i.e., biologically pretreated paddy straw, was carried out following the procedure described by Saritha (2011). Biologically pretreated paddy straw was obtained from the laboratory (i.e., prepared by solid state fermentation of paddy straw with the isolated strain of *Aspergillus niger* for 10 days at 30 °C). Compositional analysis of raw and biologically pretreated paddy straw was performed following the procedure of

Table 2 Effect of nitrogen source on the production of enzymes (IU ml^{-1}) by *X. axonopodis* pv. *punicae* strain

	Enzyme activity			
Nitrogen source	CMCase	FPA	Xylanase	β-Glucosidase
Soyameal	0.004	0.010	0.022	0.012
Yeast extract	0.010	0.008	0.016	0.010
Urea	0.001	0.003	0.008	0.001
Peptone	0.005	0.001	0.028	0.014
SEM	0.005	0.001	0.001	0.001
CD ($P > 0.05$)	0.013	0.002	0.002	0.002

CD critical difference

Table 3 Effect of surfactants on the production of enzyme specific activities by *X. axonopodis* pv. *punicae* strain

Treatment	Specific activity				Protein concentration (mg ml^{-1})
	CMCase	FPA	Xylanase	β-Glucosidase	
Control	0.059	0.070	0.536	0.015	0.100
Tween -20	0.006	0.050	0.079	0.006	0.166
Tween -80	0.020	0.063	0.077	0.009	0.100
Triton X-100	0.039	0.040	0.094	0.005	0.199
SEM	0.005	0.015	0.027	0.004	0.021
CD ($P > 0.05$)	0.013	0.040	0.072	0.010	0.056

CD critical difference

Goering and Van Soest (1970). Biologically pretreated paddy straw had 68 % holocellulose content, 5.21, 7.94, and 18.85 % protein, lignin and ash content, respectively, whereas raw paddy straw had 39.4 % cellulose, 16.2 % lignin, 21.6 % hemicellulose, 3.42 % protein and 19 % ash content.

Saccharification or hydrolysis of the biologically pretreated paddy straw samples was carried out according to the NREL LAP-009 protocol (Brown and Torget 1996), with some modifications. Pretreated samples were used in wet form for the enzymatic digestibility test; 1 g biologically pretreated paddy straw was placed in a 50-ml plastic bottle to which 1 ml enzyme extract was added and the volume made up to 10 ml by adding 0.05 M citrate buffer (pH 4.8) and incubated in shaker water bath at 50 °C. Periodically, 0.5 ml aliquots were withdrawn, heated in a boiling water bath for 2 min to stop the reaction and total reducing sugars estimated by the DNSA method as described above. In another set, biologically pretreated paddy straw samples were incubated with a standard cellulase and β-glucosidase mixture from Megazyme International (10 U E-CELAN, an endo-1,4-β-glucanase from *A. niger* supplemented with 5 U EBGLUC, a β-glucosidase from *A. niger*. This set served as a positive control for studying enzymatic saccharification.

Results and discussion

Xanthomonas axonopodis pv. *punicae*, an original isolate from infected pomegranate fruit during an epidemic in Sholapur, Maharashtra, India, was selected for this study (Supplementary Fig. 1). It showed good growth on basal medium with acid-swollen cellulose and xylan as sole C source, and produced a zone of hydrolysis on CMC agar, indicating its cellulolytic and xylanolytic potential (Supplementary Fig. 2). Quantitative assays to measure extracellular levels of cellulolytic and xylanolytic enzymes revealed that this strain was a good producer of xylanase and FPA, showing high specific activities of 0.743 and 0.168, respectively. Many plant pathogens, including those belonging to the genus *Xanthomonas*, have been reported to secrete extracellular depolymerizing enzymes such as cellulases, xylanases, proteases and pectinases (Singh and Verma 1975; Chapon et al. 2001). These enzymes serve as virulence factors for invading pathogens and contribute to their pathogenicity. The enzymes elaborated by these organisms promise to be more efficient because plants produce proteins as counter defense to inhibit cell wall degrading enzymes (CWDE), and this interaction drives plant pathogens to evolve unique and ever more potent hydrolytic enzymes (Juge and Svensson 2006; Gibson et al 2011). Different authors have advocated that total cellulolytic activities shown on insoluble substrates like filter paper and cellulose powder are more relevant for biomass conversion as compared to activity on soluble and synthetic substrates (Maki et al. 2009; Banerjee et al. 2010).

The effect of different carbon sources on the production of different cellulolytic and xylanolytic enzyme activities was studied (Table 1). Maximum specific activity (IU mg^{-1} protein) of cellulolytic enzymes, i.e., CMCase (0.071), FPA (0.059) and xylanolytic activity, i.e., xylanase (0.702) were observed when 1 % CMC (w/v) was used as a C source, except for β-glucosidase, which was maximum when 1 % starch was used as sole C source. Thus, CMC was a better inducer for xylanases and FPA while starch was found to be a better inducer of β-glucosidase. However, starch and xylan being soluble and are thus an easier C source than CMC, and cellulose powder, supported better growth in terms of higher extracellular proteins. While studying

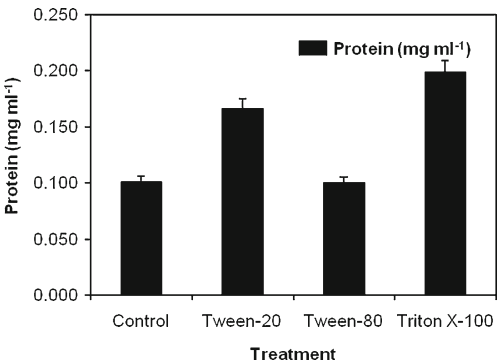

Fig. 1 Effect of different surfactants on the release of extracellular proteins by *Xanthomonas axonopodis* pv. *punicae* strain

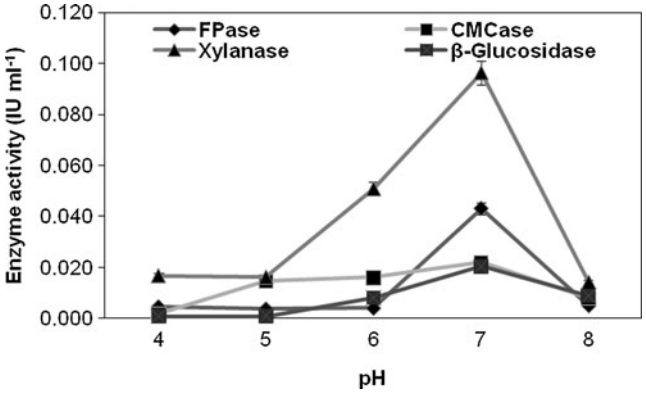

Fig. 2 Optimum pH for cellulolytic and xylanolytic enzymes production by *X. axonopodis* pv. *punicae* strain

production of hydrolytic enzymes from the plant pathogenic fungus *Myrothecium verrucaria*, Moreira et al. (2005) found that, amongst the various C sources tested, wheat bran produced the highest levels of enzyme activities as it contains insoluble carbohydrates. Ortega (1994) reported that, in the plant pathogen *Collectotrichum gloeosporioides*, the highest endoglucanase levels were induced by xylan, whereas in *Fusarium oxysporum* the highest levels were induced on CMC. In the case of *Streptomyces* sp., glucose gave highest levels of endoglucanases indicating its constitutive nature (Jaradat et al. 2008). While producing thermostable cellulases from a *Streptomyces* transformant, Jang and Chen (2003) found CMC to be the best C source to stimulate cellulase production. Wheat bran was found to be the best C source for β-glucosidase production by *Humicola brevis* followed by soyabean protein (Masui et al. 2012). Bakshi (1988) also observed higher levels of β-glucosidase from *Streptomyces griseus* when starch was provided as a C source. Studies have shown that carbon source is a main factor in inducing high levels of lignocellulolytic enzymes and their cost (Gao et al. 2008). Regarding N sources, the data presented in Table 2 showed that soya meal and yeast extract were good nitrogen sources for cellulolytic and xylanolytic enzyme activities, whereas peptone was found to be a good nitrogen source for production of cellobiase and xylanase enzymes. But when urea was used as a nitrogen source, enzyme activities diminished considerably. Jang and Chen (2003) also found that, in the case of *Streptomyces* transformants, nitrogen sources like urea and peptone made no difference to enzyme production. On the contrary, Jaradat et al. (2008) found that, in the case of *Streptomyces* sp., the inorganic nitrogen source ammonium chloride yielded a higher level of cellulases than yeast extract and asparagines.

As shown in Table 3, there was no significant effect of surfactants on the production of extracellular cellulolytic and xylanolytic enzymes. Controls showed highest production of extracellular enzyme activities. Extracellular proteins increased with all the surfactants tested compared to the control (Fig. 1). Although surfactants did cause leaching of proteins, the levels of these enzymes did not increase as they are extracellular by nature. The substrates for endoglucanases (CMCase activity), cellobiohydrolases (FPA) and xylanases are large polysaccharide molecules that cannot enter the cell. Therefore, the organisms excrete most of these activities into extracellular milieu so that they can act on polysaccharide substrates and break them into much smaller units, i.e., oligo-, di- and mono-saccharides. Hulme and Stranks (1970) and Yazdi et al. (1990) also reported no significant effect of surfactants on cellulase production by fungi because of inhibition of oxygen supply. However, Tribak et al. (2002) found a stimulatory effect of surfactants on the production of endoxyloglucanases and their release by cellulolytic fungi.

Maximum enzyme production was observed between pH 6 and 8, with highest at 7 and optimum temperature for growth and extracellular enzyme production at 28 °C, which coincides with its optimum physiological temperature and the pH of the organism (Fig. 2). Enzymes were produced under optimized conditions and the results are presented in Table 4. There was an increase in all enzyme activities. CMCase and FPA increased around 4- to 5-fold, while a 1.72-fold increase was observed in the case of xylanase and cellobiase activities.

The activity profiles of different enzymes as affected by temperature and pH are presented in Figs. 3 and 4. All the enzymes showed activity over a broad range of pH (4–8) with an optimum at 8, except FPA, which showed optimum activity at pH 4 and 6 showing that several isoforms of the enzyme are involved. Xylanase also showed more than one pH optimum (at 5 and 8). Gibson et al. (2011) stated that selected phytopathogenic bacterial genomes revealed diversity among glycosyl hydrolases families, and that *Xanthomonas campestris* was rich in both cellulose- and xylan-degrading enzymes, which appeared to be important virulence factors. Cellulolytic

Table 4 Production of different enzymes under optimized conditions by *X. axonopodis* pv. *punicae*

Enzyme	Normal conditions (IU ml^{-1})	Optimized conditions (IU ml^{-1})	Increase in activity (fold)
CMCase	0.006 ± 0.001[a]	0.030 ± 0.001	5
FPA	0.005 ± 0.001	0.019 ± 0.001	3.8
Xylanase	0.060 ± 0.005	0.104 ± 0.007	1.7
Cellobiase	0.007 ± 0.001	0.012 ± 0.001	1.7

[a]Experiments were carried out in triplicate and data are given as mean ± SD

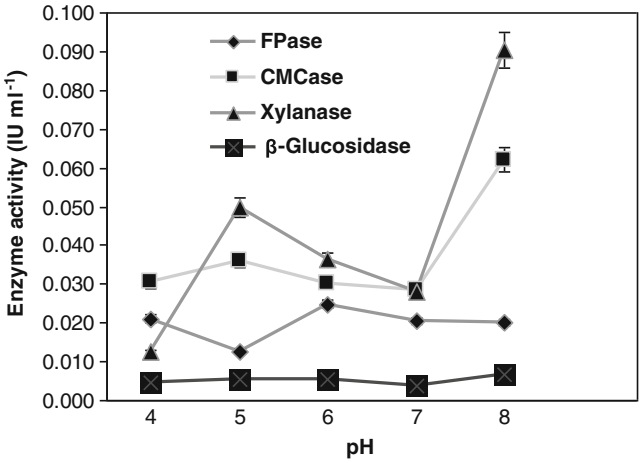

Fig. 3 Optimum pH of cellulolytic and xylanolytic enzymes produced by *X. axonopodis* pv. *punicae* strain

enzymes showed a temperature optimum at around 55 °C, while xylanase activity was highest at 45 °C and declined as the temperature increased. Heat treatment of the supernatant showed xylanase and cellobiase activities to be more thermostable than FPA and CMCase, which indicates that these enzymes are better suited to biomass conversion. Residual activities of 56 % and 80 % were found in xylanase and cellobiase, respectively, after treatment of enzyme extract at 75 °C for 1 h (Table 5), indicating that they are more thermostable than endoglucanase and cellobiohydrolase activities. Song and Wei (2010) reported that cellulases and xylanases produced by *Cellulomicrobium cellulans* grown on pretreated and extracted bagasse can be applied to the saccharification of steam- and alkali-extracted lignocellulosic substrates. Cellulase and xylanase produced by *Cellulomicrobium cellulans* retained 64 % and 87 % of their original activity after 1 h of incubation at 45 °C, and are thus classified as moderately thermostable (Yano and Poulos 2003). Alves-Prado et al. (2010) characterized crude xylanases from

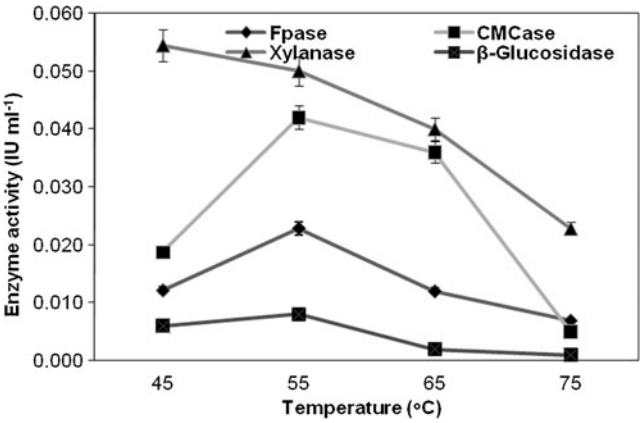

Fig. 4 Optimum temperature of cellulolytic and xylanolytic enzymes produced by *X. axonopodis* pv. *punicae* strain

Table 5 Residual enzyme activities after heat treatment of enzyme extract of *X. axonopodis* pv. *punicae* strain at 75 °C for 1 h

	Enzyme activity (%)			
Treatment	FPA	CMCase	Xylanase	β-Glucosidase
Normal	100	100	100	100
Heat treated	7.7	5.5	56	80

both bacterial and fungal sources and found that bacterial xylanase showed an optimum pH for enzyme activity at 6.0, whereas a fungal xylanase had optimum pH at 5.0–5.5. Bacterial and fungal xylanases were stable in the pH range 5.0–10.0 and 5.5–8.5, respectively. The optimum temperatures for bacterial and fungal xylanase were 55 and 60 °C, respectively, and they were thermally stable up to 50 °C. Bernier et al. (1983) purified multiple forms of xylanases from *Aeromonas* sp. It was found that these xylanases were most active at 50–60 °C. Kang et al. (1996) purified two xylanases, which gave the highest activity at 50 °C and showed relatively high stabilities at this temperature. Kamble and Jadhav (2012) reported that 50 °C is the most favorable temperature for xylanase activity from *Bacillus arseniciselenatis* DSM 15340. Stability of the enzyme was found to be the most important factor when studying the characteristics of this enzyme; it was stable at temperatures of 30 °C and 40 °C for 4 h of incubation, retaining almost 93 % activity. At higher temperatures, xylanase stability gradually declined. Grigorevski de Lima et al. (2005) reported that CMCase from *Streptomyces drozdowiczii* shows optimum activity at temperatures of 50–60 °C. Jang and Chen (2003) also found in the case of a *Streptomyces* transformant that the optimum temperature for CMCase was 50 °C, with optimum activity at pH 7. Moreira et al. (2005) reported that xylanase produced by

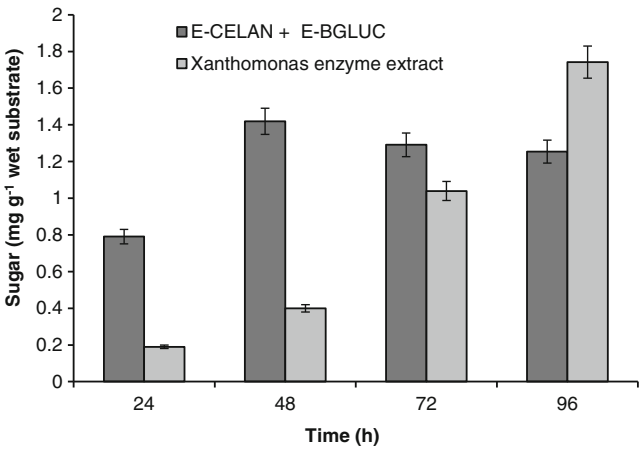

Fig. 5 Saccharification of biologically pretreated paddy straw using *Xanthomonas* enzyme extracts and standard cellulase and β-glucosidase mixture (E-CELAN endoglucanase 10 U + E-BGLUC β-glucosidase 5 U)

the plant pathogenic fungus *Myrothecium verrucaria* in submerged cultures has maximum activity from pH 4.5 to pH 5.5. Masui et al. (2012) suggested that thermal stability of xylanase in crude extracts of *Humicola brevis* var. *thermoidea* was a notable feature that can be useful when preparing efficient enzymatic cocktails for lignocellulosic treatment.

Saccharification of biologically pretreated paddy straw with enzyme extracts from *Xanthomonas* resulted in release of sugars that increased with time of incubation; after 96 h the levels of sugars released from *Xanthomonas* extracts were higher than those obtained with the standard enzyme mixture (Fig. 5). Vlasenko et al. (1997) observed that generally, the optimum conditions for hydrolysis depend on the properties of enzyme and substrate. Enzyme dose, inactivation and inhibition by-products are the main factors affecting carbohydrate conversion. Teymouri et al. (2005) observed that enzyme loading in the saccharification step determines the rate and extent of polysaccharide hydrolysis. In the present study, since crude preparation was used to include all the enzymes, the units of enzymes loaded vs substrate were low therefore the sugar yields were not very high, although they were comparable to yields from the standard enzyme mixture.

This *X. axonopodis* strain produced endoglucanases, cellobiohydrolases, β-glucosidase and xylanases. Berlin et al. (2005) reiterated that endogenous levels of β-glucosidase and xylanase appear to be important for increasing the ability of cellulase preparations to hydrolyze lignocellulosic biomass. Thus, the *X. axonopodis* pv. *punicae* strain tested here has the potential to be exploited for production of biomass-hydrolyzing enzymes.

Conclusion

The *X. axonopodis* strain used in the present study produced high levels of CMCase, cellobiase and xylanase. The enzymes were versatile with respect to their pH and temperature optima and were thermostable. They provide an indication of the biomass-hydrolyzing ability of the enzyme preparation. The enzymes could be overproduced after further optimization and could find application in different industries including the food, pulp and paper industries and in biorefineries. Accessory hemicellulase enzymes such as xylanases can have a significant effect on the performance of cellulases, presumably by increasing cellulose accessibility. Supplementation of cellulase preparations with xylanases is a useful strategy for cellulase improvement.

Acknowledgment D.A. is thankful to the Post Graduate School, Indian Agricultural Research Institute, New Delhi, India for providing a fellowship towards an MSc programme.

References

Alves-Prado HF, Pavezzi FC, Leite RSR, De Oliveira VM, Sette LD, Dasilva R (2010) Screening and production study of microbial xylanase producers from Brazilian cerrado. Appl Biochem Biotechnol 161:333–346

Bakshi A (1988) Lignocellulytic activity of a soil actinomycete and its mutant. MSc thesis, PanjabUniversity, Chandigarh, India, pp 47–50

Banerjee G, Scott-Craig JS, Walton JD (2010) Improving enzymes for biomass conversion: a basic research perspective. Bioenergy Res 3:82–92

Berlin A, Gilkes N, Kilburn D, Bura R, Markov A, Skomarovsky A, Okunev O, Gusakov A, Maximenko V, Gregg D, Sinitsyn A, Saddler J (2005) Evaluation of novel fungal cellulase preparations for ability to hydrolyze softwood substrates—evidence for the role of accessory enzymes. Enzyme Microb Technol 37(2):175–184

Bernier R, Desrochers M, Jurasek L, Paice MG (1983) Isolation and characterization of a xylanase from *Bacillus subtilis*. Appl Environ Microbiol 46(2):511–514

Brown L, Torget R (1996) Enzymatic saccharification of lignocellulosic biomass. NREL Laboratory Analytical Procedure LAP #009, National Renewable Energy Laboratory, Golden, CO, pp 1–13

Chapon V, Czjzek M, El Hassouni M, Py B, Juy M, Barras F (2001) Type II protein secretion in gram-negative pathogenic bacteria: the study of the structure/secretion relationships of the cellulase Cel5 (formerly EGZ) from *Erwinia chrysanthemi*. J Mol Biol 310(5):1055–1066

Gao J, Weng H, Zhu D, Yuan M, Guan F, Xi Y (2008) Production and characterization of cellulolytic enzymes from the thermoacidophilic fungal *Aspergillus terreus* M11 under solid-state cultivation of corn stover. Bioresour Technol 99(16):7623–7629

Ghose TK (1987) Measurement of cellulase activities. Pure Appl Chem 59:257–268

Ghose TK, Bisaria VS (1987) Measurement of hemicellulase activities. Part 1: xylanases. Pure Appl Chem 59:1739–1752

Gibson DM, King BC, Hayes ML (2011) Bergstrom GC (2011) Plant pathogens as a source of diverse enzymes for lignocellulose digestion. Curr Opin Microbiol 14:264–270

Goering HK, Van Soest PJ (1970) Forage Fibre Analysis (Apparatus, Reagents, Procedures and Some Applications). Agriculture Handbook No. 379 Agriculture Research Service, USDA, Washington DC

Grigorevski de Lima AL, do Nascimento RP, da-Silva Bon EP, Coelho RR (2005) *Streptomyces drozdowiczii* cellulase production using agro-industrial by –products and its potential use in the detergent and textile industries. Enzyme Microb Technol 37:272–277

Hulme MA, Stranks DW (1970) Induction and the regulation of production of cellulase by fungi. Nature 226(5244):469–470

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

Jaradat Z, Dawagreh A, Ababneh Q, SaadounI (2008) Influence of culture condition on cellulase production by *Streptomyces* sp. (Strain J2). Jordan J Biol Sci 1(4):141–146

Juge N, Svensson B (2006) Proteinaceous inhibitors of carbohydrate-active enzymes in cereals: implication in agriculture, cereal processing and nutrition. J Sci Food Agric 86:1573–1586

Kamble RD, Jadhav AR (2012) Isolation, purification, and characterization of xylanase produced by a new species of *Bacillus* in solid state fermentation. Int J Microbiol 2012:1–8

Kamoun S, Kado CI (1990) A plant-inducible Gene of *Xanthomonas campestris* pv. *campestris* encodes an exocellular component required for growth in the host and hypersensitivity on non hosts. J Bacteriol 172(9):5165–5172

Kang MK, Maeng PJ, Rhee YH (1996) Purification and characterization of two xylanases from alkaliphilic *Cephalosporium spp.* strain RYM-202. Appl Environ Microbiol 62(9):3480–3482

Kazemi-Pour N, Condemine G, Hugouvieux-Cotte-Pattat N (2004) The secretome of the plant pathogenic bacterium *Erwinia chrysanthemi*. Proteomics 4(10):3177–3186

King BC, Waxman KD, Nenni NV, Walker LP, Bergstrom GC, Gibson DM (2011) Arsenal of plant cell wall degrading enzymes reflects host preference among plant pathogenic fungi. Biotechnol Biofuels 16(4):4

Lynd LR, Laser MS, Bransby D, Dale BE, Davidson B, Hamilton R, Himmel M, Keller M, McMillan JD, Sheehan J, Wyman CE (2008) How biotech can transform biofuels. Nat Biotechnol 26(2):169–172

Maki M, Leung KT, Qin W (2009) The prospects of cellulase-producing bacteria for the bioconversion of lignocellulosic biomass. Int J Biol Sci 5(5):500–516

Masui DC, Zimbardi AL, Souza FH, Guimaraes LH, Furriel RP, Jorge JA (2012) Production of a xylose-stimulated β-glucosidase and a cellulase free thermostable xylanase by the thermophilic fungus *Humicola brevis* var. *thermoidea* under solid state fermentation. World J Microbiol Biotechnol 28(8):2689–2701

McMillan JD, Jenning EW, Mohagheghi A, Zuccarello M (2011) Comparative performance of precommercial cellulases hydrolyzing pretreated cornstover. Biotechnol Biofuels 4:29

Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 31(3):426–428

Moreira FG, Reis S, Costa MAF, De Souza CGM, Peralta RM (2005) Production of hydrolytic enzymes by the plant pathogenic fungus *Myrothecium verrucaria* in submerged condition. Braz J Microbiol 36:7–11

Ortega J (1994) Cell wall degrading enzymes produced by phytopathogenic fungus *Colletotrichum gleosporioides*. Tex J Sci 46(4):329–335

Rautela GS, Cowling EB (1966) Simple cultural test for relative cellulolytic activity of fungi. Appl Environ Microbiol 14(6):892–898

Saritha M (2011) Microbial treatment of lignocellulosic substrate for enhanced enzymatic digestibility. MSc. Thesis, Indian Agricultural Research Institute, New Delhi, India

Singh RP, Verma JP (1975) Cellulase, pectinase and protease of Indian isolates of *Xanthomonas malvacearum*. Indian Phytopathol 28(3):379–383

Song JM, Wei DZ (2010) Production and characterization of cellulases and xylanases of *Cellulomicrobium cellulans* grown in pretreated and extracted bagasse and mineral nutrient medium M9. J Biomass Bioenergy 34:1930–1934

Teather RM, Wood PJ (1982) Use of congo red-polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen. Appl Environ Microbiol 43(4):777–780

Teymouri F, Laureano-Perez L, Alizadeh H, Dale BE (2005) Optimization of ammonia fiber explosion (AFEX) treatment parameters for enzymatic hydrolysis. Bioresour Technol 96(18):2014–2018

Tribak M, Ocampo JA, García-Romera I (2002) Production of xyloglucanolytic enzymes by *Trichoderma viridae*, *Paecilomyces farinosus*, *Wardomyces inflatus* and *Pleurotus ostreatus*. Mycologia 94(3):404–410

Vlasenko EY, Ding H, Labavitch JM, Shoemaker SP (1997) Enzymatic hydrolysis of pretreated rice straw. Bioresour Technol 59(2):109–119

Wood TM, Bhat KM (1988) Methods for measuring cellulase activities. Methods Enzymol 160:87–112

Yano KJ, Poulos TL (2003) New understanding of thermostable and peizostable enzymes. Curr Opin Biotechnol 14(4):360–365

Yazdi MT, Woodward JR, Radford A (1990) The cellulase complex of *Neurospora crassa*: activity, stability and release. J Gen Microbiol 136:1313–1319