PRODUCTION OF XYLANASE-CELLULASE COMPLEX BY *BACILLUS SUBTILIS* NS7 FOR THE BIODEGRADATION OF AGRO-WASTE RESIDUES

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A locally isolated strain of *Bacillus subtilis* NS7 was found to produce xylanase-cellulase complex in submerged-state fermentation. A nutrient broth supplemented with xylan, soybean meal, NaCl, and KH_2PO_4 produced the highest enzyme yields after 72 h of incubation at 37 °C and pH 6.5, revealing xylanase, CMCase, and FPase activities of 353 IU/ml, 1.90 IU/ml, and 1.8 IU/ml, respectively. The enzyme mixture had compatible pH and temperature optima of 6.5-7.0 and 50-55 °C, respectively. The enzyme components were active over a broader range of pH (5.0-9.0) and temperature (40-70 °C). The enzymes appeared to be metalloproteins and could effectively hydrolyze various delignified agro-waste residues into reducing sugars.

Keywords: Xylanase; Cellulase; CMCase; FPase; Bacillus subtilis; Submerged fermentation; Biodegradation

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INTRODUCTION

Lignocellulose, which is mainly comprised of two renewable components, cellulose and hemicelluloses, covalently linked with lignin, amounts to more than 60% of plant biomass produced on this planet (Tengerdy and Szakacs 2003). Cellulose is a homopolysaccharide of β-1,4-linked D-glucose residues, whereas hemicellulose is a complex of polymeric carbohydrates with xylan as its major component. Cellulose is the most abundant biomass on the earth. It is the primary product of photosynthesis in terrestrial environments and the most abundant renewable bioresource produced in the biosphere, at 100 billion tons/year (Zhang and Lynd 2004). A great deal of research on the enzymatic degradation of cellulose and hemicelluloses has been carried out over the past 30 years, but the major hurdles in this area have been difficulties in the delignification and disintegration of the hemicellulose fraction in the residues and low yields of cellulose-degrading enzymes. Cellulose can be hydrolyzed by cellulolytic enzymes, including randomly acting endoglucanase (EC 3.2.1.4), which cleaves internal β-1,4-glycosidic bonds, exoglucanase (EC 3.2.1.91), which releases cellobiose from reducing and non-reducing ends, and β-glucosidase (EC 3.2.1.21), which hydrolyses the cellobiose (a disaccharide) into glucose units. The glucose thus produced can be easily fermented to ethanol and other desirable products. Although high yields of cellulolytic enzymes have been reported from several fungi, few bacteria are capable of producing cellulases (Bajaj *et al.* 2009; Immanuel *et al.* 2006). Xylan, the second most abundant

polysaccharide in nature and the most important component of hemicellulose, mainly consists of β-1,4-linked xylopyranosyl residues, which are further substituted, depending on the plant source (Shallom and Shoham 2003). Endoxylanase (EC 3.2.1.8), the most important xylan-degrading enzyme, primarily cleaves β-1,4-linked xylan backbones and β-xylosidase (EC 3.2.1.37) hydrolyses xylo-oligomers. Debranching enzymes like α-arabinofuranosidase, α-glucouronidase, acetyl esterases, and acetyl xylan esterases, are also required for cooperative and effective hydrolysis of the hemicellulosic fraction, leading to the formation of a mixture of pentoses and hexoses. Cellulases and hemicellulases thus find a significant place in biotechnology for the bioconversion of lignocellulosic waste residues into ethanol, which is gaining significance as a liquid biofuel due to depleting petroleum reserves.

Cellulose and hemicellulose residues co-exist in agricultural residues and are covalently linked with lignin, which makes them difficult to hydrolyze. Therefore, it is desirable to delignify the residues first and then use a cocktail of cellulases and hemicellulases for simultaneous bioconversion into fermentable sugars. Diversified generic microorganisms, especially *Bacillus* spp., have proven to be a rich source of extracellular xylanases (Mullai *et al.* 2010), many of which also produce cellulases whose yields are affected by various inducers. Efficient inducers of xylanase production include xylan, xylan hydrolysis intermediates (xylobiose, D-xylose), lactose, L-sorbose, sophorose, and even cellulose (Xu *et al.* 1998), while sophorose is the most powerful inducer of cellulases (Jeong *et al.* 1985). Good cellulase induction has also been reported with lactose (Messner *et al.* 1988) and L-sorbose (Nogawa *et al.* 2001). Cellobiose, a disaccharide of two β-1,4-linked D-glucose molecules and an intermediate of cellulose hydrolysis, has also been proposed as a natural inducer of cellulases. The present study is aimed at optimizing the nutritional and environmental parameters for the co-production of extracellular xylanase and cellulase components by a locally isolated bacterial strain for exploitation in biomass conversions.

EXPERIMENTAL

Microorganism

The *Bacillus* sp. NS7 used in the present study was isolated from the local soil of Chandigarh city. It was identified according to its morphological, cultural, and biochemical characteristics, based on Bergey's Manual of Systematic Bacteriology (Claus and Berkeley 1986) as well as by molecular characterization using 16s rRNA sequencing by taking the services of Chromous Biotech, India.

Xylanase-Cellulase Production in Submerged Fermentation

Different sets of 100 ml Erlenmeyer flasks were inoculated with 1 ml of the 12-h-old shake culture of *Bacillus* sp. NS7 with a viable cell count of 2.1×10^8/ml. Each flask contained 20 ml of nutrient broth used as the basal production medium, consisting of 0.5% peptone, 0.3% beef extract, 0.9% NaCl, and pH 7.0 The flasks were incubated in a New Brunswick water bath shaker (150 rpm) for 120 h at 37 °C. The time course for the production of enzyme components was studied by withdrawing the flasks at regular time

intervals and analyzed for growth (A_{600}) and pH. The contents of each flask were then centrifuged (10,000 rpm, 15 min, 4 °C) and the cell free supernatant was analyzed for xylanase and cellulase activities.

Enzyme Assays

The cell free supernatant was assayed for xylanase and cellulase activities at 50 °C and pH 6.5. Xylanase was determined in terms of endo-β-1,4-xylanase, using xylan (Bailey *et al.* 1992) as the substrate, by determining the amount of xylose liberated using a dinitrosalicylic acid reagent (Miller 1959). Cellulase was measured in terms of endo-β-1,4-glucanase and exo-β-1,4-glucanase which have been expressed in terms of CMCase and FPase activities, using CMC and Whatman filter paper strips (1×6 cm), respectively, as the substrates (Ghosh 1987), by determining the glucose liberated using dinitrosalicylic acid reagent (Miller 1959). The activities are expressed in terms of international units (IU), equivalent to the number of µmoles of the product liberated in 1 min.

For the xylanase assay, 0.5 ml of appropriately diluted cell free supernatant was mixed with an equal volume of 1% oat spelt xylan (HI-Media, India) solution, made in 0.1 M phosphate buffer, pH 6.5, and incubated at 50 °C for 30 min. The reaction was stopped by adding 1.5 ml DNSA reagent and the colour was developed by boiling for 10 min. The amount of xylose produced during reaction was determined by measuring the absorbance at 540 nm. CMCase was determined in a similar manner, by using 1% carboxymethyl cellulose (CMC) as the substrate and determining the reducing sugar in terms of glucose. FPase activity was assayed using Whatman filter paper strips immersed in 0.5 ml phosphate buffer (pH 6.5) as the substrate. The amount of glucose liberated in a 1 h reaction at 50 °C was determined.

Effect of Cultural and Environmental Conditions on Enzyme Production

Xylanase, CMCase, and FPase production was optimized in submerged fermentation by studying the effect of various cultural and environmental factors in 100 ml Erlenmeyer flasks, containing 20 ml nutrient broth as the basal production medium with pH 7.0 after 72 h of incubation at 37 °C in shake cultures (150 rpm).

1. The effect of carbon sources was studied by supplementing the basal medium, separately, with a variety of carbohydrates, sugar alcohols, polyols and alkali treated agro residues. These included glucose, galactose, lactose, cellobiose, xylose, arabinose, trehalose, inulin, inositol, erythritol, xylan, carboxymethyl cellulose (CMC) at a concentration of 0.5% (w/v) and corn cobs, jute, wheat bran, rice bran, rice husk, wheat straw, sugarcane bagasse, oat meal, tomato puree, at a concentration of 2% (w/v).
2. The effect of various nitrogen sources was studied by supplementing the xylan-containing basal medium with 0.5% (w/v) of different organic and inorganic nitrogenous compounds, including corn steep liquor, tryptone, soyabean meal, yeast extract, beef extract, peptone, ammonium nitrate, ammonium sulphate, sodium nitrate, ferric nitrate, diammonium hydrogen phosphate, and urea.
3. The effect of metal ions was studied by incorporating either of NaCl, $MnCl_2$, $ZnSO_4$, $CoCl_2$, $CaCl_2$, KH_2PO_4, $FeSO_4$, at a concentration of 10 mM, in the basal medium

containing 0.5% xylan and 0.5% soybean meal.
4. The effect of temperature was studied by incubating the optimized production medium in a temperature range of 30-60 °C.
5. The effect of pH was evaluated by varying the pH of the optimized production medium in the range of 4.0-8.0.

Characterization of the Xylanase, CMCase, and FPase Preparations

Purification of the enzyme components is not economically viable because the hemicellulase and cellulase preparations are required in large quantities for the bulk processing of the cellulosic biomass. Therefore, xylanase, CMCase, and FPase components from *Bacillus* sp. NS7 were characterized in the crude extracellular preparation by observing the effect of temperature, pH, and various metal ions on enzyme activity. The temperature activity profile was studied by incubating the assay mixtures of various enzyme components in a temperature range of 35-70 °C at pH 6.5. The pH activity profile was studied by assaying the xylanase, CMCase, and FPase activities in a pH range of 4.0-9.0 using appropriate buffers at 50 °C. The effect of metal ions and EDTA on enzyme activities was studied by supplementing various metal ions and effector molecules, including NaCl, $MnCl_2$, $ZnSO_4$, $CoCl_2$, $CaCl_2$, $MgSO_4$, KCl, and EDTA, at a final concentration of 1 mM each in the reaction mixtures and determining the relative activities under normal assay conditions.

Application of Crude Enzyme Preparation in the Hydrolysis of Biomass Residues

The enzyme preparation from *Bacillus* sp. NS7 was evaluated for the hydrolysis of hemicellulose-cellulose-containing residues obtained after the delignification of various agro-residues, including wheat straw, sugar cane bagasse, coconut fibre, and sawdust. The powdered residues were pretreated by being soaked in 2N NaOH for 24 h at room temperature, repeatedly washed with distilled water, and dried. Enzymatic hydrolysis of pretreated residues was carried out at 2% (w/v) consistency in 0.1 M phosphate buffer, pH 6.5, containing 0.005% (w/v) sodium azide. The flasks containing 200 mg of either of the pretreated substrates dispensed in 5 ml buffer were mixed with 5 ml of the crude enzyme preparation containing 300, 1.6, and 1.8 IU/ml of xylanase, CMCase, and FPase, respectively, and incubated at 50 °C, 150 rpm for 72 h. Samples were withdrawn every 24 h, centrifuged, and analyzed for reducing sugars released by the DNSA reagent (Miller 1959).

Data Analysis

All results represent the mean of at least three independent experiments. For the enzymatic assays and reducing sugar determinations, the standard deviations were less than 5% from samples withdrawn from the same experiment. For the samples taken from different experiments, the standard deviations were less than 10%.

RESULTS AND DISCUSSION

The natural isolate of *Bacillus* sp. NS7 produced opaque and pale colonies on nutrient agar with irregular, wrinkled, and slightly raised surfaces. The bacterial rods were Gram positive with spores capable of growing in a pH range of 4.0-8.0 and a temperature range of 30-60 °C. The biochemical characterization of the strain NS7 showed a resemblance to *Bacillus* sp. The 16S rDNA of the strain was successfully amplified by PCR to a size of about 1.5 kbp. The aligned sequence of this amplified segment was submitted to GenBank. After homology searching against the GenBank, it was found to share 99% similarity with *Bacillus subtilis* subsp. *subtilis*.

Time Course of Xylanase and Cellulase Production

Enzyme production in microorganisms is growth-associated. Maximum enzyme productivities in various organisms have been found during the stationary phase of growth, declining thereafter during the death phase (Damiano *et al.* 2003). In the present study, *Bacillus subtilis* NS7 grew logarithmically from 2 to 48 h and then entered the stationary phase (Fig. 1).

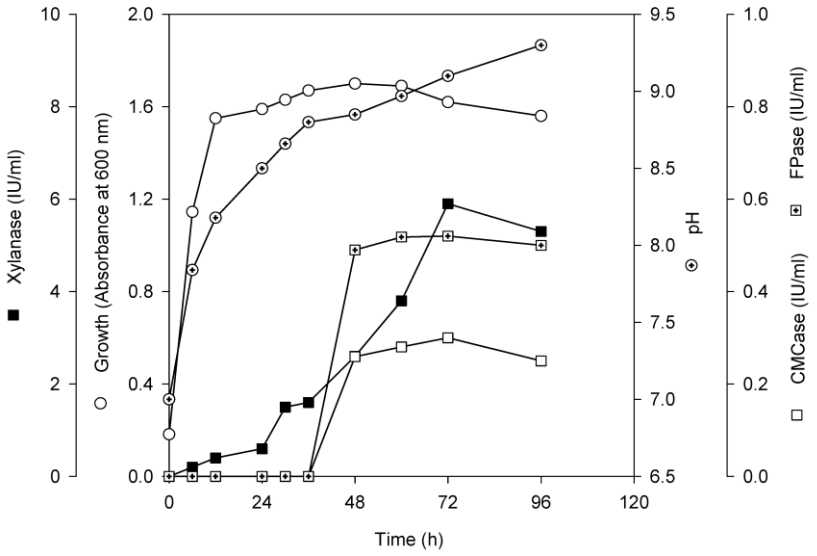

Fig. 1. Pattern of growth, pH, and xylanase, CMCase, and FPase production by *Bacillus subtilis* NS7 in nutrient broth at 37 °C under submerged fermentation

Xylanase, CMCase, and FPase formation started during the *log* phase, and their levels increased gradually during the incubation period, exhibiting the highest activities of 5.9 IU/ml for xylanase, 0.30 IU/ml for CMCase, and 0.52 IU/ml FPase, respectively, after 72 h of incubation. Further incubation brought about a slight decline in the biomass and enzyme yields, which is probably a consequence of random lethal events, including cellular fragmentation in the death phase, leading to a release of intracellular material, including protease, into the fermentation broth (Papagianni and Moo-Young 2002).

Effect of Various Cultural and Environmental Factors on Enzyme Production

Of the various carbon sources in the present study, xylan induced the highest xylanase activity of 103 IU/ml, followed by wheat bran and xylose, which produced 95 and 60 IU/ml, respectively (Fig 2a). These results are in agreement with previously published reports, in which xylan had a strong inducing effect on xylanase production (Nagar *et al.* 2010). CMC induced the highest cellulase yields, amounting to 0.84 IU/ml of CMCase and 0.98 IU/ml of FPase, followed by xylan, which produced 0.76 IU/ml and 0.92 IU/ml of CMCase and FPase, respectively. Wheat bran also produced comparable yields of 0.70 IU/ml of CMCase and 0.85 IU/ml of FPase. Xylan, wheat bran, and xylose produced appreciable amounts of all the three enzyme components when used separately. Thus, xylan was also used in combinations with wheat bran and xylose. These combinations worked very well and brought about a pronounced increase in xylanase yield. Xylan, along with wheat bran, induced the highest productivities amounting to 143, 1.0, and 1.2 IU/ml of xylanase, CMCase, and FPase, respectively. Xylan, along with xylose, produced comparable yields of 132, 0.9, and 1.19 IU/ml respectively of the three components (Fig 2a).

The nitrogen source in the medium had a marked effect on the growth of the organism and enzyme production. Organic nitrogenous compounds exhibited better enzyme yields, with soybean meal producing the highest xylanase (194 IU/ml), CMCase (1.02 IU/ml), and FPase (1.4 IU/ml) activities when used in combination with xylan in the basal medium (Fig. 2b). The presence of ammonium nitrate also proved to be beneficial in producing appreciable enzyme yields. Some earlier studies reported that yeast extract (Katapodis 2007) and peptone (Ding 2004) are the best nitrogen sources for the maximum induction of xylanase. In contrast, fishmeal, cotton seed, soya meal, and corn steep powder reported low xylanase activities compared to yeast extract (Singh *et al.* 2000). Microorganisms require some metal salts for optimal growth and enzyme production. KH_2PO_4 caused the maximum increase in xylanase and CMCase production of 240 IU/ml and 1.20 IU/ml, respectively (Table 1). However, sodium chloride brought about the maximum increase in FPase activity (1.80 IU/ml).

The growth of *B. subtilis*. NS7 over a temperature range of 30-60 °C revealed appreciable levels of xylanase, CMCase, and FPase, with 37 °C displaying the highest levels of the enzyme components (Table 2). pH is one of the most important factors for any fermentation process that is dependent on the composition of the medium. The organism grew in a pH range of 4.0 to 8.0, producing maximum xylanase at pH 6.5 (353 IU/ml), followed 348 IU/ml at pH 6.0 (Table 2). CMCase production was maximal at pH 7 (1.90 IU/ml), followed by pH 6.5 (1.81 IU/ml). FPase production was maximal at pH 6 (1.8 IU/ml), followed by pH 6.5 (1.46 IU/ml).

The enzyme productivities achieved in the present study are quite high compared to many published reports (Damiano *et al.* 2003; Taneja *et al.* 2002; Maheswari and Chandra 2000; Mishra and Pandey 2007). Subramaniyan *et al.* (2001) isolated and characterized several xylanase- and cellulase-producing cultures, of which *Bacillus* SSP-34 produced 100 IU/ml of xylanase activity with low yields of cellulolytic activities (CMCase, 0.4 IU/ml; FPase, 0.2 IU/ml). Most of the other bacteria that degraded

hemicellulosic materials were reported to be potent cellulase producers, including *Cellulomonas* sp., which produced 9.33 IU/ml of xylanase and 0.94 IU/ml of CMCase.

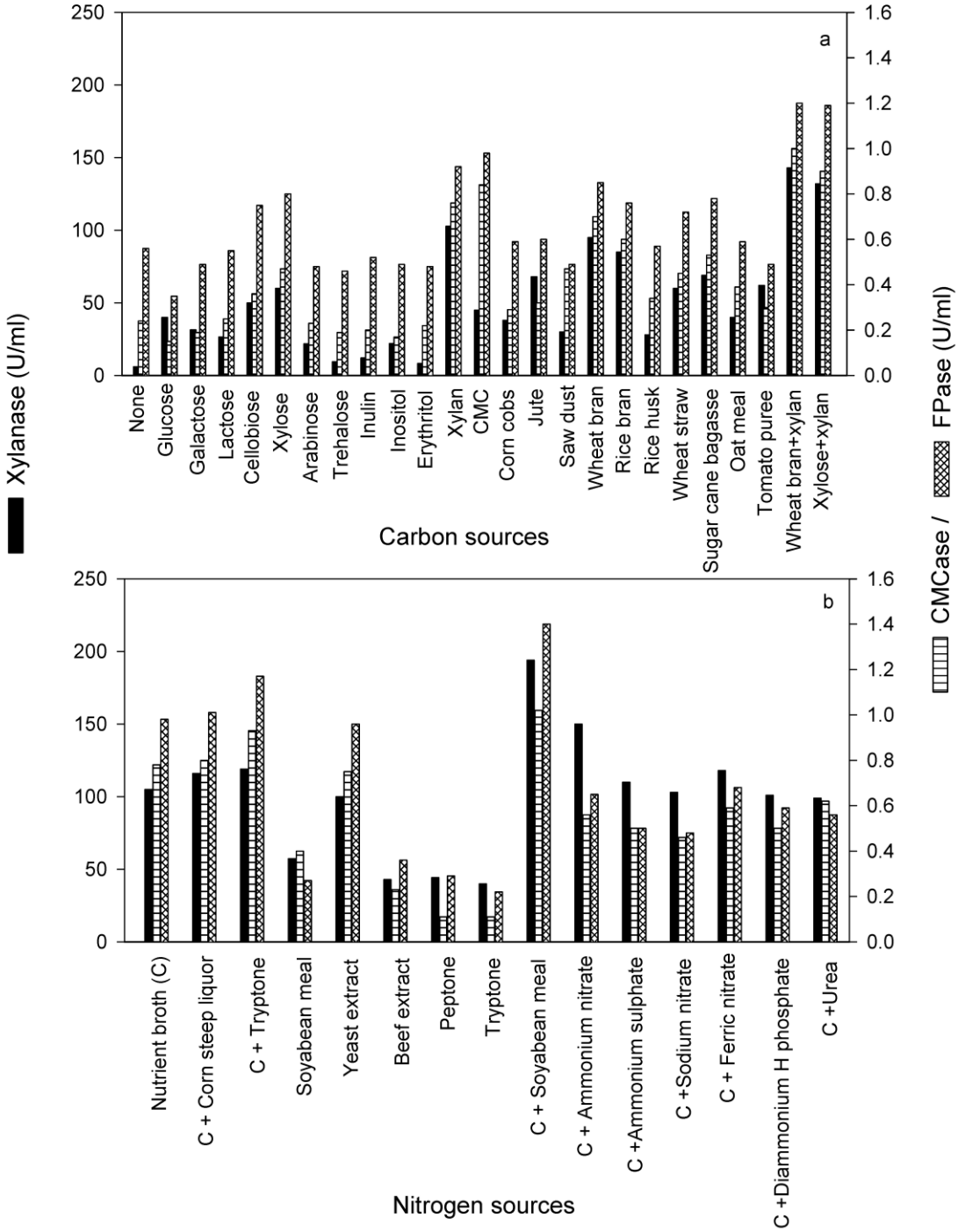

Fig. 2. Effect of various carbon (a) and nitrogen (b) sources on xylanase and cellulase production by *Bacillus subtilis* NS7.

Table 1. Effect of Various Metal Salts on Xylanase and Cellulase Production by *Bacillus subtilis* NS7

Parameter	Enzyme activity (IU/ml)		
Metal salts	Xylanase	CMCase	FPase
None	200.0	1.12	1.59
NaCl	220.0	1.19	1.80
$MnCl_2$	208.0	1.10	1.72
$ZnSO_4$	180.0	1.07	1.42
$CoCl_2$	195.0	1.02	1.60
$CaCl_2$	232.0	1.18	1.69
KH_2PO_4	240.0	1.20	1.70
$FeSO_4$	186.0	1.18	1.62

Table 2. Effect of Incubation Temperature and pH of Production Medium on Xylanase and Cellulase Production by *Bacillus subtilis* NS7

Parameter	Enzyme activity (IU/ml)		
	Xylanase	CMCase	FPase
Temperature (°C)			
30	220.0	1.72	1.20
37	290.0	1.89	1.32
45	281.0	1.86	1.30
50	278.1	1.79	1.27
55	250.2	1.75	1.20
60	198.1	1.20	0.87
pH			
4.0	261.0	0.99	0.12
4.5	291.6	0.67	1.08
5.0	306.0	0.90	1.23
5.5	325.0	1.03	1.23
6.0	348.2	1.68	1.80
6.5	353.1	1.80	1.46
7.0	288.0	1.90	1.28
7.5	241.4	1.70	1.25
8.0	243.2	0.95	0.09

Characterization of Xylanase, CMCase, and FPase Activity

The three enzyme activities increased progressively with a rise in assay temperature, up to 50 °C for xylanase and 55 °C for CMCase and FPase. Any further increase in the temperature decreased the enzyme activities (Fig. 3a). The activities also increased with pH, up to 6.5 for xylanase and CMCase and 7.0 for FPase (Fig. 3b). The enzymes also displayed appreciable activities at pH 7.0, 8.0, and 9.0, revealing 74, 59, and 42% relative activities for xylanase and 78, 47, and 44% relative activities for CMCase. FPase, whose pH optimum was at pH 7.0, had 54 and 47% activities at pH 8.0 and 9.0 (Fig. 3b). The optimum temperature of xylanase was also reported to be 50 °C in the case of *Bacillus subtilis* PAP115 (Bernier *et al.* 1983) and *Bacillus* sp. YJ6 (Yin *et al.* 2010), while it was 40 °C in *Bacillus* sp. MX47 (Chi *et al.* 2012), 60 °C in *Bacillus* sp. BP-7 (Gallardo *et al.* 2004), *Bacillus subtilis* (Saleem *et al.* 2012), *Bacillus* sp. K-1

(Ratanakhanokchaim *et al.* 1999), and *Bacillus* sp. M-9 (Bajaj *et al.* 2009), and 70 °C in *B. subtilis* CCMI966 (Sa-Pereira *et al.* 2002). Optimal activity for CMCase was found to occur at 50 °C and a pH 7.0 for *B. mycoides* S122C (Balasubramanian *et al.* 2012). pH 6.0 and temperature 60 °C were optimal for *B. pumilus* EB3 (Ariffin *et al.* 2006), and pH 7.0 at 60 °C were optimal for *B. subtilis* YJI (Yin *et al.* 2010). *B. amyloliquefaciens* DL-3 was most active at a temperature of 50 °C and pH 8.0 (Lee *et al.* 2008), however, high activity between 40 and 65 °C and pH 6.5 and 7.0 occurred for *B. pumilus* (Kotchoni *et al.* 2006). Most xylanases are active at an acidic or neutral pH (Bastawde 1992). Even xylanases from many alkaliphilic microbial strains are optimally active around neutral conditions (Gessesse and Mamo 1999). Microbial strains producing xylanases with optimum activity at and above pH 8.0 have also been reported (Gessesse and Gashe 1997; Chi *et al.* 2012; Saleem *et al.* 2012).

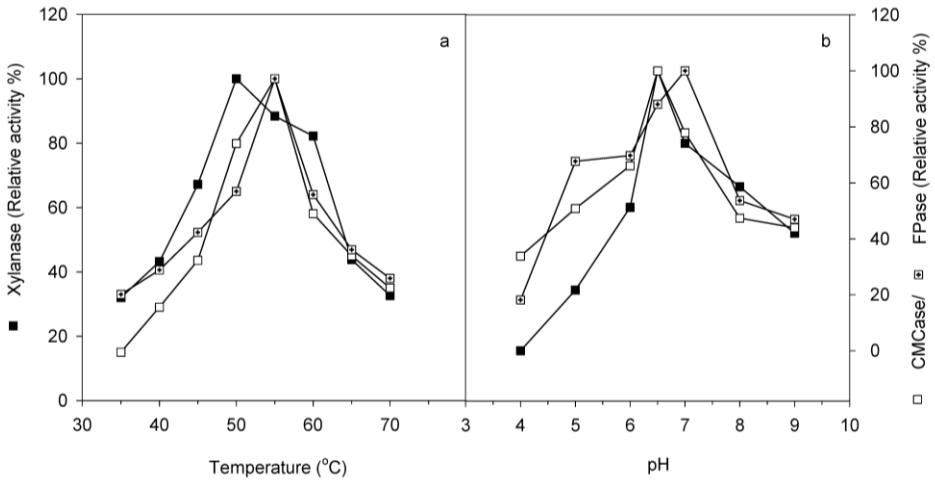

Fig. 3. Temperature (a) and pH (b) activity profiles of xylanase and cellulases from *Bacillus subtilis* NS7

Most of the metal salts supplemented to the reaction assay mixture proved to be essential, as indicated by significant improvements in the various activities. Maximum improvement of xylanase activity was caused by NaCl, which improved the activity by 43%. $CaCl_2$ also caused 35% improvement, while $CoCl_2$ improved xylanase activity by 22%. A pronounced improvement of 492% was observed in the case of CMCase in the presence of $MnCl_2$, followed by a 250% improvement caused by $MgSO_4$. In the case of FPase, the highest improvement of 359% occurred in the presence of $MgSO_4$, followed by 158% in the case of $MnCl_2$ (Fig. 4). EDTA inhibited all of the enzyme activities, confirming the metalloprotein nature of the enzymes. Chi *et al.* (2012) observed that xylanase activity from *Bacillus* sp. MX47 was severely inhibited by many divalent metal ions, including Co^{2+}, Mg^{2+}, Zn^{2+}, Ca^{2+}, and EDTA. Monovalent metal ions, such as Na^+ and K^+, increased enzyme activity slightly. On the other hand, Saleem *et al.* (2012) noted that divalent ions like Ca^{2+}, Mg^{2+}, and Zn^{2+} enhanced xylanase activity from *Bacillus subtilis*, whereas Hg^{2+}, Fe^{2+}, and Cu^{2+} were inhibitory. CMCase activity from *Bacillus mycoides* S122C is inhibited by $HgCl_2$, $CuSo_4$, $FeCl_2$, $MnCl_2$, and EDTA, while $CoCl_2$ promotes activity (Balasubramanian *et al.* 2012).

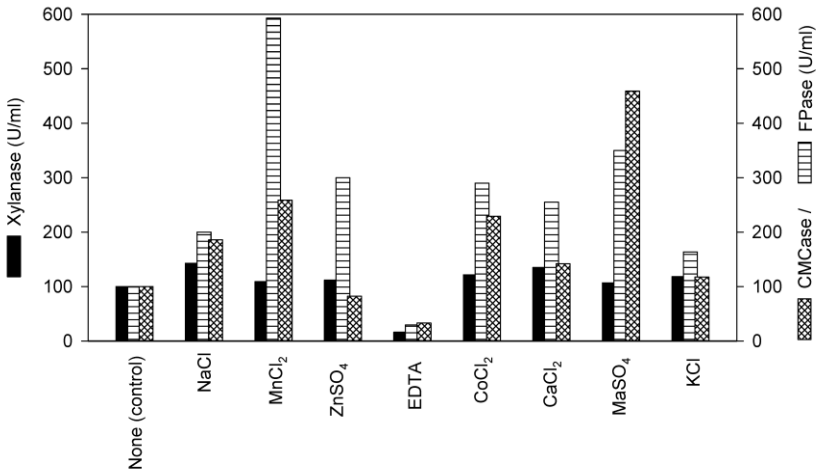

Fig. 4. Effect of metal ions and EDTA on xylanase, CMCase, and FPase activities from *Bacillus subtilis* NS7.

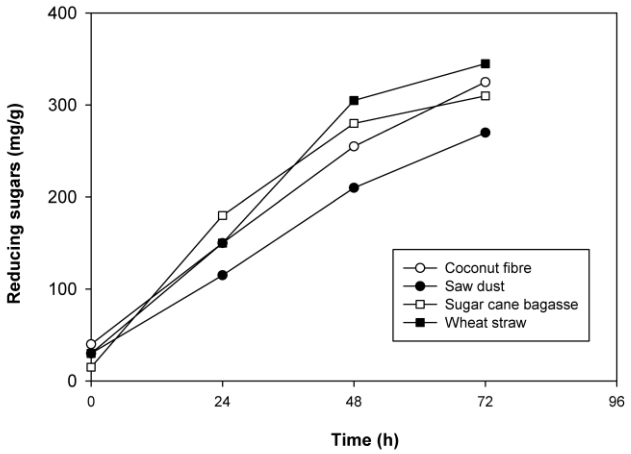

Fig. 5. Enzymatic hydrolysis profile of various defined and alkali-treated agro-residues.

Enzymatic Hydrolysis of Agro-waste Residues

Lignocellulose is a complex substrate and is composed of a mixture of carbohydrate polymers (cellulose and hemicelluloses) and lignin. The carbohydrate polymers are tightly bound to lignin primarily by hydrogen bonds, as well as covalent bonds. The biological process for converting lignocellulose into fermentable sugars requires delignification to liberate cellulose and hemicelluloses from their complex with lignin and depolymerization of the carbohydrate polymers to produce free sugars in the form of pentoses and hexoses. Among the key processes, delignification is the most difficult task. Numerous pretreatment processes for lignocellulosic residues, ranging from hot water and steam explosion treatments, to alkaline and many useful versions of acid, have been tested (Kaar and Holtzapple 2000; Sun and Cheng 2002). The efficacy of crude enzyme preparation from *Bacillus subtilis* NS7 in hydrolyzing delignified agricultural residues was evaluated. The time course of enzymatic saccharification

revealed that irrespective of the substrate, the release of sugars increased with increasing saccharification time (Fig. 5).

Of the various delignified agricultural residues, wheat straw produced the most sugar (345 mg/g), followed by coconut fibre (325 mg/g), sugar cane baggase (310 mg/g), and sawdust (270 mg/g). Interestingly, the enzyme mixture from *Bacillus subtilis* NS7 showed higher saccharification efficiency than many earlier reports. Yoon and Kim (2005) reported a release of only 32 mg/g sugar from avicel after 43 h, while Lee *et al.* (2008) observed a sugar release of only 3.53 mg/g when *Pinus densiflora* was enzymatically hydrolyzed.

CONCLUSIONS

1. The xylanase-cellulase complex from a natural isolate of *Bacillus subtilis* NS7 with a compatible pH and temperature range of 5.0-9.0 and 40-70 °C, respectively, effectively hydrolyzed various delignified agro-residues into reducing sugars.
2. This preparation appears to be a potential candidate for simultaneous bioconversions of xylan and cellulose in delignified biomass residues for various biotechnological applications.

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