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Polysaccharides from bagasse: applications in cellulase and xylanase production

M.G. Adsul[a], J.E. Ghule[b], R. Singh[b], H. Shaikh[b], K.B. Bastawde[a], D.V. Gokhale[a,*], A.J. Varma[b,*]

[a]*NCIM, Biochemical Sciences Division, Pune, India*
[b]*Polymer Science and Engineering Group, Chemical Engineering Division, National Chemical Laboratory, Dr Homi Bhabha Road, Pune 411 008, India*

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Abstract

Sugarcane bagasse was chemically treated to generate different bagasse samples with varying quantities of lignin and hemicelluoses, keeping the cellulose fraction intact in all cases. These bagasse samples were evaluated for the production of cellulase and xylanase enzymes by *Penicillium janthinellum* NCIM 1171 and *Trichoderma viride* NCIM 1051 in the production medium. Higher xylanase and β-glucosidase activities were detected in the medium with all bagasse samples as compared to the values obtained with pure cellulose powder 123 (CP-123). Amongst all bagasse samples, sample IV (kappa number 22.9) gave the highest yields of xylanase (130 IU/ml) and β-glucosidase activities (2.3 IU/ml) in case of *P. janthinellum.* There was no increase in cellulase (FPase and CMCase) activities in both strains irrespective of the bagasse sample compared to the values obtained in presence of CP-123. The productivities of all the enzymes except CMCase in medium containing bagasse sample IV are higher as compared to pure CP-123. The productivity of Fpase is slightly lower when *T. viride* NCIM 1051 is grown in a medium containing BS-IV. More studies have to be carried out to generate bagasse samples, which can be used as substrates to produce high levels of cellulases and hemicellulases in proper proportion. This may in turn reduce the cost of enzyme production leading to efficient use of lignocellulosic materials to produce value-added products.

Keywords: Sugarcane bagasse; Bagasse polysaccharides; Chemical treatment; Cellulase; Xylanase; *Penicillium janthinellum*; *Trichoderma viride*

1. Introduction

Lignocellulosic biomass such as agricultural and forestry residues, municipal solid waste and dedicated crops provide a low cost feedstock for biological production of fuels and chemicals, which offer economic, environmental and strategic advantages (Wyman, 1996). These materials generally contain up to 75% of cellulose and hemicellulose, which cannot be easily converted to simple monomeric sugars due to their recalcitrant nature. With the advent of modern genetics and other tools the cost of producing sugars from these recalcitrant fractions and converting them into products like ethanol has been significantly reduced. However additional cost reductions are desirable to achieve competitiveness vis a vis the existing conventional fuels.

Enzymatic hydrolysis of such cellulosic material by cellulase enzymes is the most promising approach to get high product yields vital to economic success (Hinman, Schell, Riley, Bergeron, & Walter, 1992; Lynd, Elander, & Wyman, 1996). The high cost of cellulase enzyme production hinders the application of these enzymes to bioethanol production (Himmel, Ruth, & Wyman, 1999; Wooley, Ruth, Glassner, & Sheehan, 1999). The use of such waste and low cost cellulosic biomass would significantly reduce the cost of enzyme production provided cellulase productivities are comparable to those obtained in presence of other carbon sources like lactose (Kadam, 1996). Such high cellulase productivities can potentially be achieved by the use of chemically pretreated biomass as carbon sources.

Lynd (1996) has summarized the prerequisites for an ideal lignocellulose pretreatment, i.e. it should: (a) produce reactive fibers; (b) yield pentoses in non-degraded form; (c) not release the compounds that significantly inhibit fermentation; (d) work in reactors of reasonable size with

* Corresponding authors. Tel.: +20-2589-3400; fax: +20-2589-3041.
E-mail addresses: ajvarma@che.ncl.res.in, dvg@dalton.ncl.res.in (A.J. Varma).

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moderate cost; (f) produce no solid residues; (g) have a high degree of simplicity, and (h) be effective at low moisture contents. A number of pretreatment options are available: acid pretreatment, alkaline treatment, steam explosion, wet oxidation, organic solvent pretreatment, and hot water. Among all these methods, acid pretreatment is still the method of choice in several model processes.

The present study deals with the evaluation of cellulase enzyme production using bagasse after different pretreatments. This study will also assess whether this route could enhance performance on inexpensive biomass like bagasse and reduce the cost of enzyme production using cellulolytic strains, *Penicillium janthinellum* NCIM 1171 and *Trichoderma viride* NCIM 1051.

2. Materials and methods

2.1. Chemicals

Cellulose powder-123 (CP-123) was obtained from Carl Schleicher and schull co. Dassel, FRG. *p*-Nitro phenyl β-D-glucopyranoside (pNPG), Carboxymethylcellulose (CMC), Xylan from oat spelts, 3,5-dinitrosalysilic acid were obtained from Sigma-Aldrich Co. St Louis, mo. USA. The sugar cane bagasse samples prepared using different chemical pretreatments (Table 1) were used.

2.2. Microorganism and culture media

P. janthinellum NCIM 1171 and *T. viride* NCIM 1051 were obtained from National Collection of Industrial Microorganism (NCIM), National Chemical Laboratory, Pune, India. These cultures were maintained on Potato Dextrose Agar (PDA) and sub cultured once in every three months. PDA contained (g/l) extract from 200 g of potatoes, glucose, 20.0 g; yeast extract, 1.0 g; and agar, 20.0 g. Production medium described by Mandels and Weber (1969) was used for enzyme production.

2.3. Enzyme production

Shake flask experiments were carried out in 250 ml Erlenmeyer flask with 70 ml of production medium containing 1% (w/v) cellulose-123 powder or pretreated sugar cane bagasse samples with different kappa numbers. The flasks were inoculated with spores (approximately 10^7) from 10-day-old culture grown on PDA slant and incubated at 30 °C with shaking at 180 rpm. The samples were removed at various time intervals and centrifuged at 3000 rpm for 10 min. The supernatant was analyzed for extra cellular enzyme activities and soluble protein.

2.4. Enzyme assay

Exoglucanase (FPase; Exo-1, 4-β-D-glucanase, E.C.3.2.1.91) activity was determined according Mandels et al. (1974). Assay mixture (2 ml) consisted of 1.9 ml citrate buffer (50 mM, pH 4.5), filter paper Whatman No.1 (50 mg, 1×3 cm^2) and 0.1 ml suitably diluted enzyme. The reaction mixture was incubated at 50 °C for 60 min.

Endoglucanase (CMCase, Endo-1, 4-β-D-glucanase; E.C. 3.2.1.4) activity was determined according to Mandels et al. (1974) with slight modification. The total reaction of 1 ml contained a 0.5 ml sample of suitably diluted enzyme and 0.5 ml of 1% (w/v) CMC solution in citrate buffer (50 mM, pH 4.5) and incubated at 50 °C for 30 min.

Xylanase (1,4-β-D-xylan xylanohydrolase, E.C. 3.2.1.8) activity was determined under similar conditions as described above, except that 1% xylan solution was used as substrate in place of CMC.

β-Glucosidase (β-D-glucoside glucohydrolase; E.C. 3.2.1.21) activity was estimated according to the method described by Eberhart (1961) using pNPG as substrate. The total of assay mixture (1 ml) consisting of 0.9 ml of pNPG (1 mg/ml) and 0.1 ml of suitably diluted enzyme was incubated at 50 °C for 30 min. The *p*-nitrophenol liberated was measured at 410 nm after developing the color with 2 ml of sodium carbonate (2%).

Table 1
Different bagasse samples with their kappa numbers and lignin contents, generated by different chemical treatments

Sample	Treatment	Condition	Kappa no.	Lignin (%) w/v
I	(a) NaOH	(a) 42.85% NaOH, 70–75 °C, 6 h	0.8	0.1
	(b) $NaClO_2$	(b) Pulp: $NaClO_2$ 1:0.5; pH = 3.7, 70 °C, 4 h		
	(c) H_2O_2 bleach	(c) 10% H_2O_2, pH = 10.5, 70 °C, 4 h		
II	Bagasse Kraft pulp, unbleached	Standard Kraft pulping	12.4	1.9
III	$NaClO_2$ bleaching	Bagasse: $NaClO_2$ = 1:0.5,4 h, 70 °C	14	2.1
IV	$NaClO_2$ bleaching	Bagasse: $NaClO_2$ = 1:0.25, 4 h 70 °C	22.9	3.4
V	NaOH	20% NaOH, 4 h 25 °C	54.7	–
VI	H_2O_2	10% H_2O_2, pH = 10, 4 h, 70 °C	66	–
VII	Untreated sugarcane bagasse	–	79	–

2.5. Enzyme units

One unit (IU) of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol of glucose, xylose or *p*-nitrophenol from the appropriate substrates per min per ml of crude filtrate under assay condition.

2.6. Analytical method

Protein was estimated according to the method of Lowry et al. (1951) using bovine serum albumin as a standard. The reducing sugar was estimated as either xylose or glucose equivalent by dinitrosalicylic acid (DNS) method (Fischer and Stein, 1961). The kappa number of the samples was determined by the method described in Ulmann's Encyclopedia of Industrial Chemistry (2002) and the lignin content was reconfirmed by multiplying the kappa number with 0.15 factor.

3. Result and discussion

Experiments were carried out using holocelluloses derived from different treatments of bagasse introduced individually into the production medium. We also performed fermentation experiments with two control media, one containing untreated bagasse and the second containing pure CP-123 as standard. The enzyme activities were determined up to 15 days and the values are presented in Tables 2 and 3. We also used two different cultures, namely *T. viride* NCIM 1051 and *P. janthinellum* NCIM 1171.

The standard medium with pure cellulose powder gave highest FPase and CMCase activities, indicating the more efficient utilization of pure cellulose than the native and treated bagasse. Bagasse sample IV was generated by selective removal of major part of lignin constituent keeping the other constituents (cellulose and hemicellulose) predominantly intact. The kappa number of bagasse sample of kappa 22.9 works out to a lignin content of 3.4% (Table 1). Apparently, the removal of lignin leads to a more porous and accessible material for microbial attack, which helps in production of significantly enhanced levels of xylanase and β-glucosidase enzymes. FPase activities produced by both strains in a medium containing bagasse sample IV are more or less the same as compared to those obtained in pure cellulose containing medium. However, xylanase and β-glucosidase activities produced by both *T. viride* and *P. janthinellum*are higher when bagasse samples (both treated and untreated) were used as substrates in the medium. The bagasse samples IV yielded 4-fold increase of xylanase (130 IU/ml) and β-glucosidase (2.3 IU/ml) activities in case of *P. janthinellum* NCIM 1171 when compared with

Table 2
Enzyme production by *Penicillium janthinellum* NCIM 1171 in the medium containing 1% w/v bagasse samples or CP-123

Sample no.	Kappa no.	Enzyme activity[a] (IU/ml)			
		Filter paper (FPase)	β-Glucosidase	Endoglucanase (CMCase)	Xylanase
I	0.8	0.3	1.2	6.2	34.8
II	12.4	0.24	1.3	5.7	40.0
III	14	0.38	1.7	8.0	85.0
IV	22.9	0.48	2.3	7.1	130.0
V	54.7	0.33	1.0	6.7	53.0
VI	66	0.31	1.1	6.5	40.0
VII	79	0.27	0.61	5.7	68.0
VIII	CP-123	0.55	0.58	21.5	28.1

[a] All enzyme activities were determined after 8 days of incubation. The values are the average of three independent experiments with 5–% variation.

Table 3
Enzyme production by *Trichoderma viride* NCIM 1051 the medium containing 1% (w/v) bagasse samples or CP-123

Sample no.	Kappa no.	Enzyme activities[a] (IU/ml)			
		Filter paper (FPase)	β-Glucosidase	Endoglucanase (CMCase)	Xylanase
I	0.8	0.41	0.19	7.7	13.1
II	12.4	0.26	0.18	5.4	14.2
III	14	0.81	0.26	10.7	58.2
IV	22.9	0.88	0.33	21.8	69.9
V	54.7	0.80	0.29	12.9	64.6
VI	66	0.62	0.24	9.4	81.4
VII	79	0.28	0.20	4.2	69.7
	CP-123	1.0	0.06	33.8	41.9

[a] All enzyme activities were determined after 10 days of incubation. The values are the average of three independent experiments with 6–% variation.

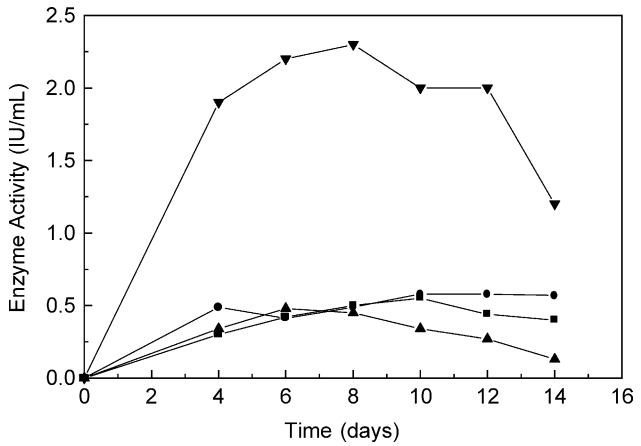

Fig. 1. Enzyme activity profile during growth of *Penicillium janthinellum* NCIM 1171 in 1% CP(FPase,-■-; β-glucosidase, -●-) and in 1% BS-IV (FPase, -▲-; β-glucosidase, -▼-).

the activities produced in presence of pure cellulose powder. The untreated bagasse sample gave lower cellulase (FPase and CMCase) activities. This could be attributed to the much lower accessibility of microorganisms towards the cellulose and hemicellulose in the polymer matrix. Samples I and II (see Table 1) consists of alkali treated bagasse. This treatment leads to significant loss of both lignin and hemicellulose. This is reflected in the lower enzyme activities produced by use of these materials in the medium as carbon source. The difference in cellulase activities may be due to variation in the amounts of utilizable amorphous cellulose present in treated samples. This variation of amorphous cellulose in treated samples could be attributed to capability of each treatment to disrupt the hydrogen bonding of the crystalline form of cellulose (Ball, Godden, Helvenstein, Pennincks, & McCarthy, 1990). These observations led to the conclusion that physical structure of bagasse cellulose and enzyme activities are important factors for determining the efficiency of cellulose conversion to useful products (Azzam, 1987; Broda, 1992).

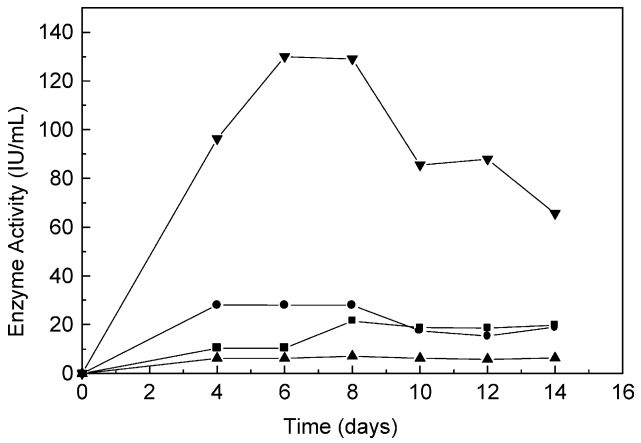

Fig. 2. Enzyme activity profile during growth of *Penicillium janthinellum* NCIM 1171 in 1% CP (CMCase, -■-; xylanase, -●-) and in 1% BS-IV (CMCase, -▲-; xylanase, -▼-).

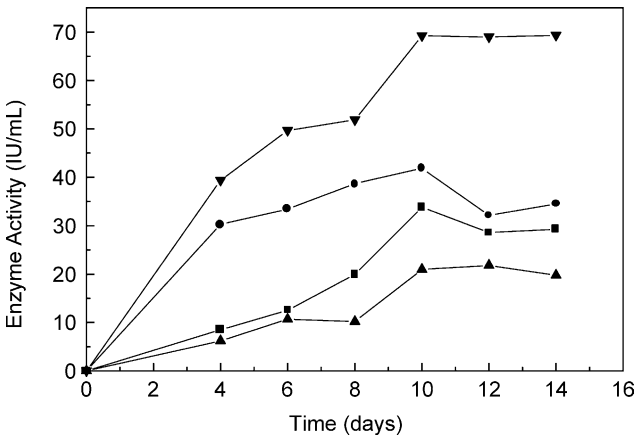

Fig. 3. Enzyme activity profile during growth of *Trichoderma viride* NCIM 1051 in 1% CP-123 (CMCase, -■-; xylanase, -●-) and in 1% BS-IV (CMCase, -▲-; xylanase, -▼-).

We continued to use bagasse sample IV for further studies and this sample is hereafter denoted as BS-IV.

The ratio of cellulase to cellulose substrate is one of the factors, which affects the effectiveness of cellulose hydrolysis. We carried out the fermentation experiments incorporating different concentrations (1.0, 2.5, 4.0%) of BS IV for enzyme production and observed that BS-IV at 1% concentration gave highest enzyme activities (Data not shown).

The results in Figs. 1 and 2 show the activity profiles of enzymes produced by *P. janthinellum* NCIM 1171 during growth cellulose powder 123 and BS-IV incorporated in the production medium individually. The extra-cellular enzymes were released in the medium during exponential phase and maximum activities were observed at the stationary phase. Xylanase and β-glucosidase production peaked on 6th and 8th day, respectively, in a medium containing BS-IV. Figs. 3 and 4 shows the activity profiles of *T. viride* NCIM 1051 during growth in a medium containing cellulose powder or BS-IV. All enzyme activities reached maximum levels on 10th day of incubation

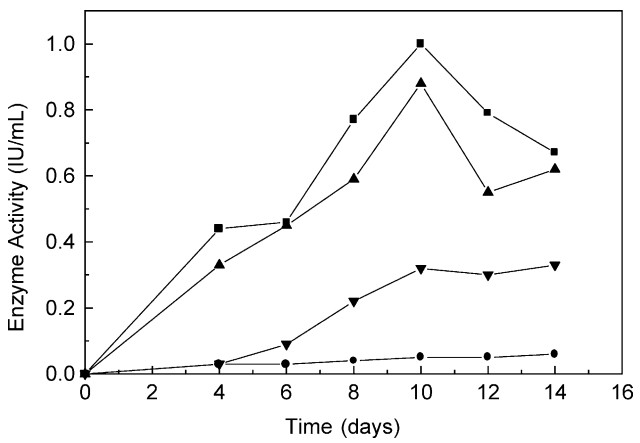

Fig. 4. Enzyme activity profile during growth of *Trichoderma viride* NCIM 1051 in 1% CP-123 (FPase, -■-; β-glucosidase, -●-) and in 1% BS-IV (FPase, -▲-; β-glucosidase, -▼-).

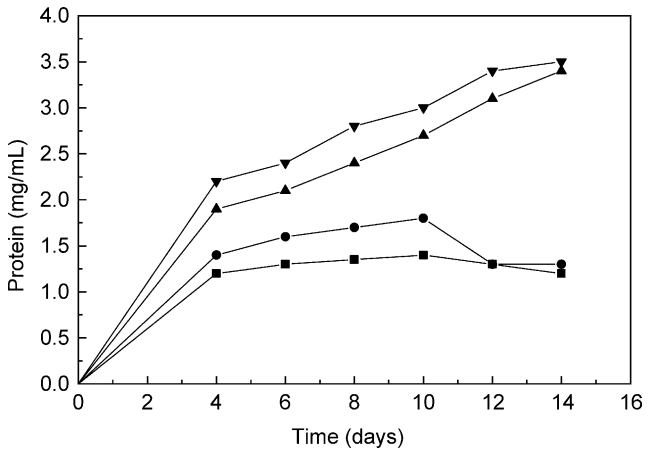

Fig. 5. Extracellular protein profile during growth of *Trichoderma viride* NCIM 1051 (in 1% CP-123, -▼-; 1% BS-IV, -▲-) and *Penicillium janthinellum* NCIM 1171 (in 1% CP-123, -●-; 1% BS-IV, -■-).

irrespective of substrate used in the medium. *T. viride NCIM*1051 produced highest CMCase and Fpase activities while *P. janthinellum NCIM 1171* produced highest xylanase *and* β-glucosidase activities. Kanosh et al. (1999) reported that cellulase production by *T. viride* C-30 was delayed when the organism was grown in medium with treated bagasse samples. This lag in enzyme production was attributed to the presence of lignin in the samples. Such lag in enzyme production was not observed when both the organisms were grown in a medium containing BS-IV.

The results in Fig. 5 reveal the protein production profile during growth of fungal strains in a medium containing CP-123 and BS-IV at 1% concentration. It appeared that the extra-cellular protein production was slightly lower in a medium with BS-IV compared to that with pure CP-123.

Based on the activity measurements, *P. janthinellum* NCIM 1171 produced enzymes except CMCase with much higher productivities in presence of BS-IV than with pure CP-123 (Table 4). The xylanase was produced by *P. janthinellum* NCIM 1171 with productivity of 902 U/h/l, which is three times than the xylanase productivity (353 U/h/l) of *P. janthinellum* CRC87M-115 isolated from decaying wood (Palma, Milagres, Prata, & de Mancilha, 1996). Xylanase and β-glucosidase enzymes were produced with enhanced productivities when *T. viride* NCIM 1051 was grown on BS-IV.

Table 4
Comparison of enzyme productivities during growth of fungal strains on bagasse samples and CP-123

Microrganisms	Substrate (1%) w/v	Enzyme productivity (IU/h/l)			
		FPase	CMCase	β-Glucosidase	Xylanase
Penicillium janthinellum NCIM 1171	CP-123	2.30	111.80	2.40	291.40
	BS-IV	5.00	36.80	11.80	902.30
Trchoderma viride NCIM 1051	CP-123	4.10	140.70	0.17	174.30
	BS-IV	3.60	75.50	0.91	287.20

4. Conclusions

From the results, we conclude that, bagasse treated with $NaClO_2$ generates bagasse sample IV with kappa number 22.9 (lignin content 3.4% w/v, no changes in hemicellulose and cellulose contents) which appears to be an excellent source of carbon for cellulase production by *P. janthinellum* NCIM 1171 and *T. viride* NCIM 1051. The incorporation of this sample in production medium resulted in the production of enhanced levels of xylanase and β-glucosidase enzymes by both *P. janthinellum* and *T. viride* along with substantial levels (but not equal to the values obtained with CP-123) of both FPase and CMCase activities. The productivities of all enzymes except CMCase were higher in media with bagasse sample IV than with pure CP-123. However, *T. viride NCIM 1051*produced both CMCase and FPase with comparatively less productivity. The xylanase productivity was three times than the values obtained with CP-123 in case of *P. janthinellum* NCIM 1171. The use of such enzyme preparations in ligno-cellulose hydrolysis will lead to efficient conversion of cellulose materials to other important products.

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