CELLULASES FROM *PENICILLIUM JANTHINELLUM* MUTANTS: SOLID-STATE PRODUCTION AND THEIR STABILITY IN IONIC LIQUIDS

Mukund G. Adsul,[a] Asawari P. Terwadkar,[a] Anjani J. Varma,[b] and Digambar V. Gokhale [a*]

The cellulase production by *P. janthinellum* mutants on lignocellulosic material such as cellulose or steam exploded bagasse (SEB) in combination with wheat bran was studied in solid state fermentation (SSF). One of the mutants, EU2D21, produced the highest levels of endoglucanase (3710 IU g^{-1} carbon source) and β-glucosidase (155 IU g^{-1} carbon source). Ionic liquids are so-called green solvents that have become attractive for biocatalysis. Stability of mutant cellulases was tested in 10-50% of the ionic liquid 1-butyl-3-methylimidazolium chloride ([bmim]Cl). FPA and CMCase were significantly stable in 10% ionic liquid after 5h. β-glucosidase showed 85% of its original activity after 5 h incubation in 30% ionic liquid and retained 55% of its activity after 24 h. This enzyme preparation hydrolyzed ionic-liquid-treated SEB completely in 15 h in the presence of 20% ionic liquid. These studies revealed that there is no need of regenerating cellulose after ionic liquid treatment, since cellulase of mutant strain was found to be significantly stable in the ionic liquid.

Keywords: Mutant cellulase; Solid state fermentation; Ionic liquid; Cellulase stability

*Contact information: a: NCIM resource Center, National Chemical Laboratory, Pune 411008, India; b: Polymer Science and Engineering Division, National Chemical Laboratory, Pune 411008, India; *Corresponding author: dv.gokhale@ncl.res.in*

INTRODUCTION

Lignocellulosic materials, which are abundantly available, can be converted to a number of bulk chemicals. Cellulases and xylanases are the key enzymes required for the degradation of lignocellulosic materials into simple sugars, which can be fermented to value-added products. The application of these enzymes for biofuel production is hindered by the high cost of enzyme production and their instability in different physical or chemical environments (organic solvents). Hence, there is a need to improve the cellulase yields, which in turn decreases the production cost. It is also necessary to look for enzyme systems which perform better under different physical and chemical environments (Wyman 2003; Hamelinck et al. 2005; Mtui 2009).

Among the fungi, *Trichoderma* and *Aspergillus* have been extensively studied due to their ability to secrete large amounts of cellulases. These strains produce extracellular cellulase complexes comprised of endoglucanase (EC 3.2.1.4), exglucanase (EC 3.2.1.74), and β-glucosidase (EC 3.2.1.21). *Aspergilli* are superior to other fungal organisms with respect to β-glucosidase production. Earlier we have reported the hyper-production of β-glucosidase (Gokhale et al. 1984) and β-xylosidase (Gokhale et al. 1986) by *Aspergillus niger* NCIM 1207. The *Trichoderma* cellulase system is deficient in β-

glucosidase, causing accumulation of cellobiose, which results in repression and end-product inhibition of enzymes. Thus the conversion of waste cellulose to glucose is not yet commercially feasible. We have reported the isolation of *Aspergillus niger* mutants capable of producing enhanced levels of β-glucosidase (Gokhale et al. 1988). *Penicillium janthinellum* NCIM 1171 produced all enzymes in proper proportion, but their titer was very low (Adsul et al. 2005, 2004). Mutants of *P. janthinellum* NCIM 1171 were isolated that produced enhanced levels of cellulases under submerged conditions (Adsul et al. 2007).

Solid state fermentation (SSF) is advantageous over submerged fermentation because of superior enzyme yields in a shorter time period, better oxygen circulation, and less efforts in downstream processing. It resembles the natural habitat for the filamentous fungi to grow and produce the fermented products. For cellulase production, SSF is most preferred, due to its lower capital investment and lower operating cost (Rodriguez Couto and Sanroman 2005; Pandey et al. 2003). Additionally, SSF finds greater applications in solid waste management, biomass energy conservation, and in the production of secondary metabolites. SSF also allows the use of low-cost agricultural substrates for production of enzymes, which helps in reducing the cost of production. Previously, agricultural residues such as wheat bran, corn stover, wheat straw, rice straw, bagasse, *etc*. were used in cellulase production. Sometimes, chemical and physical pretreatments are necessary before the agricultural residues are used for cellulase production, due to their recalcitrant nature. Physical pretreatment is the most preferred method, in contrast to chemical pretreatment, which causes environmental pollution.

Ionic liquids are novel and chemically inert solvents used in organic synthesis and biocatalysis. Previous reports demonstrated cellulose dissolution in 1-butyl-3-methylimidazolium chloride (Swatloski et al. 2002) and 1-allyl-3-methylimidazolium chloride (Wu et al. 2004). It has also been demonstrated that wood chips can be dissolved in imidazolium-based ionic liquids (Kilpelainen et al. 2007). However, little is known about the function of cellulases in these ionic liquids (van Rantwijk and Sheldon 2007). The lipases and proteases are more studied enzymes in ionic liquids for biotransformation due to their stability. Till date, only endoglucanases (CMCases) were studied for their stability in presence of ionic liquids. Turner et al. (2003) reported that CMCases were irreversibly deactivated in ionic liquid because of unfolding and denaturation. A metagenomic approach was used to select the CMCases stable in ionic liquid, 1-butyl-1-methyl-pyrrolidinium trifluoromethanesulfonate, but the levels of CMCase produced by the selected clones are very low (Pottakamper et al. 2009). No data is available on the stability of β-glucosidase and FPA in ionic liquids. Since the conversion of cellulosic biomass requires the synergistic action FPA, CMCase, and β-glucosidase, it is essential to check the stability of these three enzymes in ionic liquids.

We report here the enhanced production of FPA, CMCase, β-glucosidase, and xylanase by *Penicillium janthinellum* NCIM 1171 mutants in (SSF) using cheap lignocellulosic material (steam exploded sugarcane bagasse). Cellulase preparation of one of the mutants was tested for its stability and biocatalytic performance in different ionic liquids.

EXPERIMENTAL

Materials

1-Hexyl-3-methyl imidazolium chloride, 1-ethyl-3-methyl imidazolium chloride, 1-butyl-4-methyl pyridinium chloride, 1-butyl-3-methyl imidazolium octyl sulfate, 1-butyl-3-methyl imidazolium chloride, cellulose powder-Sigmacell, *p*-nitrophenyl β-D-glucopyranoside (pNPG), carboxymethylcellulose (CMC), xylan (oat spelts), and 3,5 dinitrosalysilic acid were obtained from Sigma–Aldrich Co., St Louis, MO, USA. Media components were procured from HiMedia, India. All other chemicals were of analytical grade and were obtained locally. Sugarcane bagasse was obtained from local sugar factory. Steam exploded bagasse (SEB) was prepared by cutting the bagasse into small shreds of 1-3 mm size, which were pretreated with steam by a proprietary process (under patenting) to remove xylan.

Methods

Microbial strains and cellulase production by solid state fermentation

Penicillium janthinellum NCIM 1171 was obtained from NCIM Resource Center, National Chemical laboratory, Pune, India. Mutants (EMS-UV-8, EU1, EU2D14, EU2D16, EU2D21, EU2D22, and EU2D23) of *P. janthinellum* were generated by exposing the spores of parent strain to ethyl methyl sulfonate, followed by UV irradiation Adsul et al. 2007). These cultures were maintained on potato dextrose agar (PDA) and sub-cultured once every three months. PDA contained (g L^{-1}) extract from 200 g of potatoes, glucose 20.0; yeast extract 1.0; and agar 20.0. Enzyme was produced in 250 ml Erlenmeyer flasks containing 3 or 4 g of wheat bran, and 1g of cellulose or 2g of SEB moistened with 8 ml of production medium. The production medium contained (g L^{-1}) KH_2PO_4 20.0; $CaCl_2$ 2; H_2O 3.0; Urea 3.0; and $MgSO_4 \cdot 7H_2O$ 3.0; $(NH_4)_2SO_4$ 14.0; peptone 2.5; yeast extract 1.0; Tween-80 1 ml; $FeSO_4$.$7H_2O$ 0.05; $MnSO_4.H_2O$ 0.016; $ZnSO_4 \cdot 7H_2O$ 0.014; and $COCl_2 \cdot 6H_2O$ 0.02. The pH of the medium was adjusted to 5.0. The flasks were inoculated with spore suspension (1 ml) containing approximately 10^7 spores from 12-15 days old culture grown on PDA slope. The contents of each flask were mixed thoroughly for uniform distribution of spores in the medium. The flasks were incubated at 30 ^oC in a humidified space with 90% humidity maintained throughout the incubation. After incubation, 50 ml of 0.1% Triton X-100 in distilled water was added in each flask and the mixture shaken on the rotary shaker (180 rpm) for 1 h at room temperature. The mixture was squeezed through a double layer muslin cloth and it was centrifuged at 5000 g for 20 min at 4 ^oC. The supernatant obtained was used as the extracellular enzyme.

Analytical methods

Filter paper activity (FPA), endoglucanase activity (CMCase), xylanase activity, and β-glucosidase activity were determined as reported earlier (Adsul et al. 2007). The reducing sugar was estimated as glucose by the DNS method (Fischer and Stein 1961). 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 standard assay condition.

Enzyme stability in ionic liquid

The crude enzyme preparation derived from mutant EMS-UV-8 was used to check its stability in ionic liquid. Stability experiments were performed in total 2 ml volume containing 0.5 ml of enzyme and various concentrations (10-50%) of ionic liquid in distilled water. The mixture was incubated at room temperature and the aliquots were taken at different time intervals for determination of residual enzyme activity. The crude enzyme in buffer without ionic liquid was treated as a control, and it was found to be stable even after 24 h.

Hydrolysis of steam exploded sugarcane bagasse in presence of ionic liquid

SEB (0.25 g) was suspended with 5 ml of ionic liquid, and the mixture was kept on a hot plate for 4 h with stirring. After complete dissolution of bagasse, 19 ml of 50 mM citrate buffer, pH 4.5 was added, followed by addition of 1 ml of enzyme solution that contained FPA (8 IU), CMCase (400 IU), and β-glucosidase (15 IU). In the control experiment, the reaction mixture consisted of 0.25g of ionic liquid treated and regenerated SEB and 1 ml of enzyme in 24 ml of 50 mM citrate buffer, pH 4.5. These mixtures were incubated at room temperature with shaking at 150 rpm. Reducing sugars were analyzed after different time intervals, and percent hydrolysis was calculated considering the total carbohydrate content in SEB.

RESULTS AND DISCUSSION

Cellulase Production

Mutants were evaluated for cellulase production in SSF, and it was found that all mutants produced enhanced levels (3-5 fold) of FPA, CMCase, and xylanase in comparison to the parent strain during growth on wheat bran and pure cellulose (Table 1).

Table 1. Solid-State Production of Cellulases and Xylanases using Wheat Bran (4g) and Sigmacell Cellulose (1g) as Substrate

Penicillium janthinellum NCIM 1171 mutants	Enzyme activity (IU g^{-1} of carbon source)			
	FPA	CMCase	β-glucosidase	Xylanase
Parent	18	700	100	250
EMS-UV-8	66	3420	150	3040
EU-1	64	3012	84	3630
EU2D14	62	3240	85	4030
EU2D16	55	3000	62	3550
EU2D21	70	3710	155	3230
EU2D22	67	3220	95	2940
EU2D23	60	3402	77	4070

Mutants EMS-UV-8 and EU-2D-21 produced higher levels (1.5 fold) of β-glucosidase. SEB also proved to be a suitable substrate for cellulase production by mutant strains (Table 2). Mutant EU-2D-21 produced the highest FPA (70 IU g^{-1} carbon source),

CMCase (3710 IU g^{-1} carbon source), β-glucosidase (155 IU g^{-1} carbon source) when grown on 1g of pure cellulose and 4 g of wheat bran. Mutants EU1, EU2D14, and EU2D16 produced the highest levels (approximately 4710 IU g^{-1} carbon source) of xylanase activity when grown on 2 g of SEB and 3 g of wheat bran. Only wheat bran or SEB did not give higher yields of cellulases (data not shown).

Table 2. Solid-State Production of Cellulases and Xylanases using Wheat Bran (3g) and Steam Exploded Sugarcane Bagasse (2g) as Substrate

Penicillium janthinellum NCIM 1171 mutants	Enzyme activity (IU g^{-1} of carbon source)			
	FPA	CMCase	β-glucosidase	Xylanase
Parent	16	600	110	800
EMS-UV-8	61	2980	170	3410
EU-1	63	2950	100	4620
EU2D14	55	3000	80	4710
EU2D16	60	2860	88	4600
EU2D21	68	3400	140	3000
EU2D22	62	3010	102	2610
EU2D23	53	3080	78	4060

The effect of different nitrogen sources on cellulase production showed that the yeast extract was the most suitable nitrogen source (data not shown). Increase in yeast extract concentration beyond 0.1% did not enhance the cellulase activity. The time course of cellulase production demonstrated that maximum enzyme activities were obtained on the 6^{th} day and 10^{th} day when the organisms were grown on pure cellulose and SEB respectively. It is very difficult to compare the cellulase activities reported in the literature, as no standard substrates have been used for growing the organism. India is one of the largest sugar cane growing countries, producing 200 million tons per year, which generate about 45 million tons of bagasse. Hence, we used SEB as the cheap source for production of cellulases, which yielded cellulase activities comparable to the values obtained for pure cellulose. The comparison of cellulase activities produced by mutant EU2D21 in SSF with those reported in the literature demonstrated the superiority of the mutant to other fungal strains with respect to CMCase and β-glucosidase production (Table 3). The xylanase activity was also found to be highest compared to the values reported so far for organisms which produce total cellulase complex.

Table 3. Comparison of Cellulase Production by Some of the Fungi using SSF

Microorganisms	Enzyme yield (IU g^{-1} carbon source)				References
	FPA	CMCase	β-glucosidase	Xylanase	
Trichoderma reseei QMY-1	86.0				Chahal 1985
Trichoderma reseei ZU-02	158.0				Xia and Cen 1999
Thermoascus auranticus		1709.0	5.5	79.0	Kalogeris et al. 2003a
Thermoascus auranticus		1572.0	101.6		Kalogeris et al. 2003b
Trichoderma koningi F244	94.0	287.3	184.0		Li et al. 2004
Aspergillus niger KK2	19.5	129.0	100.0		Kang et al. 2004
Melanocarpus sp.		142.4	109.0		Jatinder et al. 2006
Penicillium echinulatum 9A02S1		282.0	58.95	10.0	Camassola and Dillon 2007
Aspergillus terreus M11	243.0	581.0	128.0	NA	Gao et al. 2008
Penicillium janthinellum EU2D21	70.0	3750.0	155.0	3230.0	This work

Stability of Cellulase in Ionic Liquid

Cellulase preparation of EMS-UV8 was evaluated for its stability in five different ionic liquids. Cellulase was found to be equally stable within 1 h of incubation in two ionic liquids at 50% concentration, namely 1-butyl-4 methyl pyridinium chloride and 1-butyl-3-methyl imidazolium chloride (data not shown). The ionic liquid 1-butyl-3-methyl imidazolium chloride is known to solubilize cellulose and it is also available commercially in bulk amount. Hence further stability studies were carried out with 1-butyl-3-methyl imidazolium chloride. FPA, CMCase, and xylanase activities were quite stable in 10% ionic liquid retaining 95%, 90%, and 80% of their initial activity respectively (Fig. 1-4). However, CMCase showed 40% of its original activity after 4 h incubation in 50% ionic liquid. Total inactivation of xylanase and β-glucosidase activities was observed in 50% ionic liquid after 4 h. β-glucosidase remained stable (55%) even at 30% ionic liquid concentration for 24 h (Fig 5). All enzyme activities were inactivated at

40-50% ionic liquid except CMCase after 24 h. An observed increase in activity of CMCase at 40 and 50% ionic liquid concentration cannot be explained.

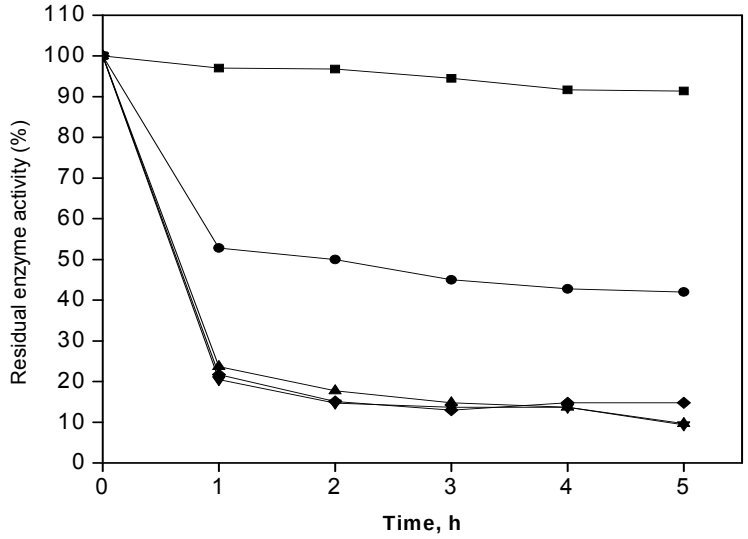

Fig. 1. Stability of FPAase in presence of Ionic liquid 1-butyl-3-methyl imidazolium chloride, 10%(■), 20%(●), 30%(▲), 40%(▼), and 50%(♦). The values are the average of three independent experiments with 5-6% variation.

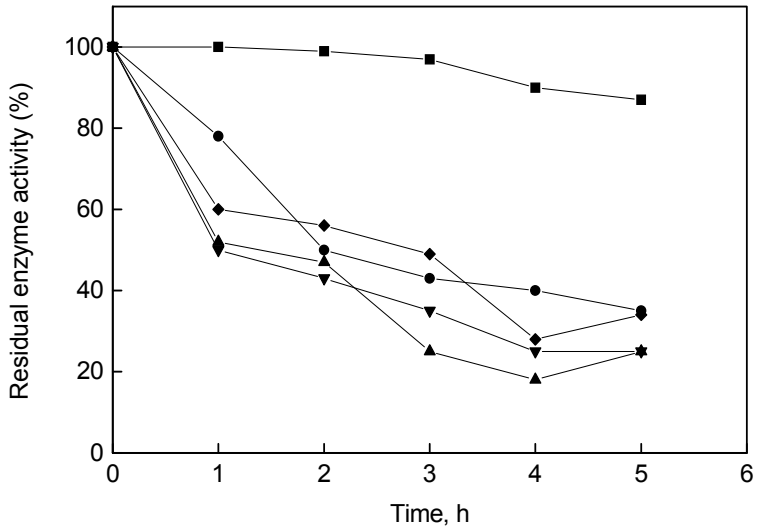

Fig. 2. Stability of CMCase in presence of Ionic liquid 1-butyl-3-methyl imidazolium chloride, 10%(■), 20%(●), 30%(▲), 40%(▼), and 50%(♦). The values are the average of three independent experiments with 5-6% variation.

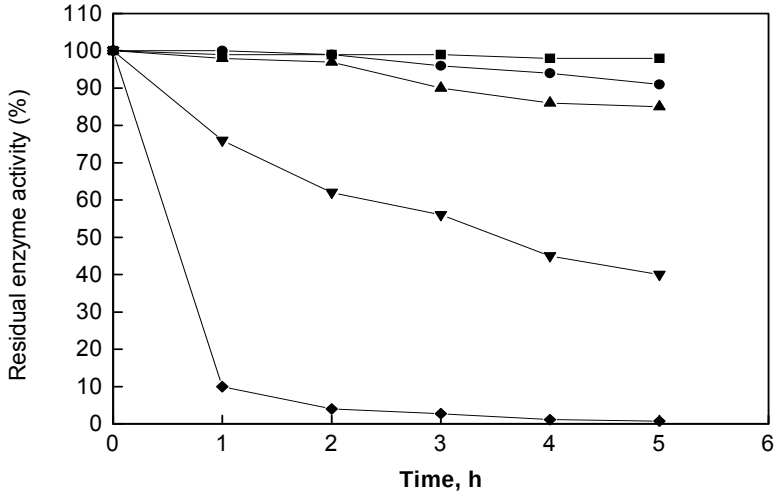

Fig. 3. Stability of β-glucosidase in presence of Ionic liquid 1-butyl-3-methyl imidazolium chloride, 10%(■), 20%(●), 30%(▲), 40%(▼), and 50%(♦). The values are the average of three independent experiments with 5-7% variation.

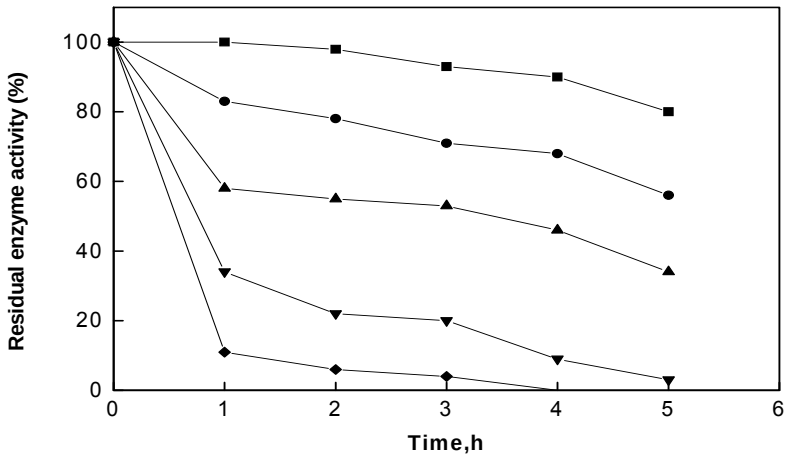

Fig. 4. Stability of Xylanases in presence of Ionic liquid 1-butyl-3-methyl imidazolium chloride, 10%(■), 20%(●), 30%(▲), 40%(▼), and 50%(♦). The values are the average of three independent experiments with 4-6% variation.

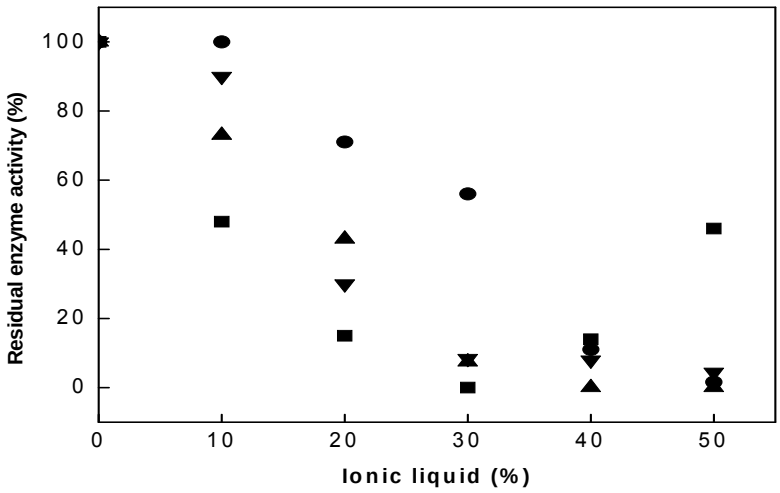

Fig. 5. Stability of CMCase (■), β-glucosidase (●), Xylanases (▲) and FPAase (▼) in presence of Ionic liquid 1-butyl-3-methyl imidazolium chloride (10 to 50%) after 24 hrs of incubation at room temperature. The values are the average of three independent experiments with 6-8% variation.

In recent years the importance of ionic liquid in biocatalysis is increasing. There are very few reports on stability of cellulases, especially endoglucanases, in ionic liquids. However, there are no reports on stability of FPA, β–glucosidase, and xylanase in ionic liquids. Even traces of ionic liquid associated with regenerated cellulose led to cellulase inactivation, suggesting the complete removal of ionic liquid residues after cellulose regeneration (Zhao et al. 2009). Endoglucanases from *Trichoderma sp*. are not stable in very low concentration of [bmim]Cl (Turner et al. 2003). Recently, a metagenomic approach was employed to produce ionic liquid stable endoglucanases. However, they were found to be moderately active and stable in 1-butyl-1-methyl-pyrrolidinium trifluoromethanesulfonate. It is still not known whether this ionic liquid dissolves cellulose (Pottkamper et al. 2009). Although cellulases are not being used commercially to hydrolyze biomass, their ability to hydrolyze biomass in presence of ionic liquids would make them potential biocatalysts to be used further in industrial processes.

Hydrolysis of SEB in Presence of Ionic Liquid

The ionic liquid, 1-butyl-3-methylimidazolium chloride ([bmim]Cl), is known to dissolve crystalline cellulose. Dissolution of cellulose in such ionic liquid followed by its hydrolysis in presence of ionic liquid could lead to increased hydrolysis, when the cellulases are stable in ionic liquids. Therefore, we tested the stability of mutant cellulases in [bmim]Cl and carried out the hydrolysis of SEB in presence of ionic liquid. All enzymes were found to be significantly stable in 20% ionic liquid. Therefore, hydrolysis of SEB was carried out in 20% ionic liquid, and the results are given in Fig. 6. SEB previously treated with ionic liquid was hydrolyzed completely in presence of 20% ionic liquid within 15 h. Ionic liquid treated celluloses are subject to faster saccharification than untreated substrates (Kuo and Lee 2009; Zhao et al. 2009). These results suggest that *P. janthinellum* mutant cellulases are potential sources that can be used to hydrolyze cellulose in presence of higher concentrations of ionic liquid. This

eliminates the key step of separating the ionic liquid treated cellulose for its further hydrolysis to sugars.

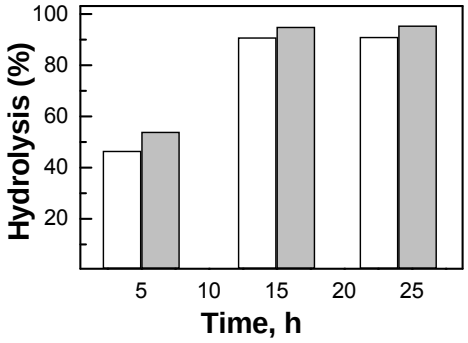

Fig.6. Hydrolysis of ionic liquid treated steam exploded sugarcane bagasse in presence of 20% ionic liquid (open symbols) and without ionic liquid (closed symbols). The values are the average of three independent experiments with 3-5% variation.

CONCLUSIONS

P. janthinellum mutants produced high levels of cellulases in solid state fermentation. The activities were comparable with some of the fungal strains reported so far. However, one of the mutants, EU2D21 was superior to all fungal strains in relation to CMCase and β-glucosidase production in SSF. Ionic liquids show promise in the treatment of crystalline cellulose to make it more amenable to cellulase attack. We studied the stability of cellulase preparation of EMS-UV8 mutant in one of the ionic liquids, [bmim]Cl, and it was found that almost all enzyme activities showed significant stability in 20% ionic liquid. We were able to perform hydrolysis of SEB in presence of 20% ionic liquid, indicating the possibility of exploiting mutant cellulase preparation in hydrolyzing cellulose in the presence of ionic liquids. This is the first report on production of ionic-liquid-stable cellulases that are capable of hydrolyzing cellulose in the presence of ionic liquids.

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