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Stability and Recovery of *Penicillium funiculosum* Cellulase Under Use Conditions**

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ABSTRACT

The stability of *Penicillium funiculosum* cellulase has been investigated under the conditions used for cellulose hydrolysis. Fifty five percent of filter paper activity (FPA) was inactivated on incubation at 50°C for 24 h, whereas there was no loss in endoglucanase and β-glucosidase activity. The addition of 2% polyethylene glycol (PEG) during incubation stabilized the FPA. The influence of pH during fermentation on the thermal stability of the enzyme is discussed. The recovery of enzymes after hydrolysis of bagasse at 50°C was between 8 and 14%. Under the optimal conditions of elution, the recovery of enzyme was 35% (*1*). Increasing the enzyme to the substrate ratio fivefold and presence of PEG during hydrolysis resulted in 80, 83, and 95% recovery of β-glucosidase, FPA, and endoglucanase activity, respectively.

Index Entries: Stability; recovery of cellulase *P. funiculosum*.

INTRODUCTION

Development of an economic process for the cellulose hydrolysis is determined by the cost of enzyme production. Fungal cultures such as *Trichoderma reesei* (*2*), *Penicillium funiculosum* (*3,4*) and *Sporotrichum pulverulentum* (*5*), are known to be potent cellulase producers. The characteriza-

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tion and the mode of the action of cellulase complex of these organisms and the hydrolysis of various cellulosic substrates by the enzymes have been reported (*6,7*). However, further research is necessary for maximum utilization of these enzymes, since enzyme stability is an important factor in increasing their efficiency. Although celllases are sufficiently stable under conditions of growth, they are sensitive to conditions of application such as high substrate and product concentration, the presence of other materials in culture filtrate, and shear resulting from shaking or agitation (*8*). Thermal inactivation of cellulases has also been reported by several workers (*9,10*). Stability of a cellulase enzyme is important in order to consider its recovery and reuse to improve the economics of enzymatic hydrolysis. The stability of enzymes can be increased by adding stabilizers (e.g., protein substrates or surfactants) to the hydrolysis reactor (*11*) or by enzyme modification through covalent linkages. Immobilization is an attractive means of recovering and reusing cellulases, but the interaction between an insoluble substrate and the immobilized enzyme is severely limited (*12*).

The recovery of enzyme is maximum if the amount of free enzyme in hydrolysis mixture is maximum, which in turn depends on the extent of digestion, agitation, and ratio of enzymes to substrate (*13*). The recovery of cellulases, where initial ratios of components are maintained, depends on the eluant used for desorption of the enzyme (*14*). The addition of Tween-80 was found to increase both hydrolysis and enzyme recovery of *T. reesei* from pulped paper (*15*).

Earlier we reported on the use of *P. funiculosum* cellulase for saccharification of various cellulosic substrates and the recovery of the enzyme after use (*1*). The present article deals with the studies on the stability and recovery of *P. funiculosum* cellulase for its application in practical saccharification processes.

MATERIALS AND METHODS

All chemicals were of analytical grade unless otherwise mentioned. Sodium salt of carboxylmethylcellulose (CMC) (low viscosity (8758), 3,5-dinitrosalicylic acid, polyethylene glycol (PEG) (MW 7,500) were obtained from Sigma Chemical Co., St. Louis, MO. *Penicillium funiculosum* was obtained from the National Collection of Industrial Microorganisms, National Chemical Laboratory, Poona, India (*4*).

Production of Enzyme

The cellulase was produced as described by Rao et al. (*1*) either in New Brunswick fermentor (15 L) with automatic pH and temperature control or in Erlenmayer flasks (500 mL) containing 100 mL medium on a rotary shaker at 220 rpm and 28°C with manual adjustment of pH on alternate days.

ENZYME ASSAYS

Activity toward CMC (endoglucanase) and filter paper (FPA) were determined by incubating 1 mL of reaction mixture containing suitably diluted enzyme with 0.5 mL of CMC (1%) or filter paper (25 mg) in acetate buffer (final concentration 0.05 *M*, pH 4.8) and incubating at 50°C for 30 or 60 min, respectively. The reducing sugar formed was determined by dinitrosalicylic acid method (*2*) using D-glucose as standard. The β-D-glucosidase activity was determined according to Berghem and Pettersson, with *p*-nitrophenyl-β.D-glucoside as the substrate (*16*). One unit of enzyme activity was defined as that amount of enzyme that produces one μmol of reducing sugar in 1 min under the assay conditions. Protein was determined according to the method of Lowry et al. (*17*).

Pretreatment

Fifty mesh sugarcane bagasse was treated with 5 *M* NaOH in a 50% slurry for 18 h at 28°C and was washed free of alkali with water.

Hydrolysis

Hydrolysis of bagasse was carried out in stoppered flasks in the presence of toluene by incubating 500 mg of substrate in a 5% slurry with *P. funiculosum* cellulase in 0.025 *M* acetate buffer pH 4.8, at 50°C.

Recovery

Recovery of enzyme was studied at 50°C with stepwise or multiple addition of substrate. The experimental details, about the amount of additional substrate, elution of enzyme using Tween-80 and grinding of the substrate were according to Rao et al. (*1*).

RESULTS

P. funiculosum cellulase produced under different fermentation conditions showed variations in its stability (Table 1). Enzymes produced in fermentor where pH was maintained around 5.0 were more stable than the enzymes produced in shake flasks where pH was periodically adjusted to 5 and involved repeated fluctuations to acidic pH. The stability of the enzyme produced in shake flasks was improved when the enzyme was precipitated with ammonium sulfate (60 g/100 mL) and dialyzed or ultrafiltered through UM-10 membrane (Table 2).

P. funiculosom cellulase was stable between pH 4 and 5 at 50°C under stationary conditions. Loss in filter paper activity (70%) and β-glucosidase activity (40%) at pH 6.0 was significant, whereas endoglucanase activity remained constant.

Table 1
Influence of pH During Fermentation Conditions on Stability of Enzyme[a]

	Enzyme production	
	Fermentor	Shake flasks
	Residual activity, %	
FPA	81	10
Endoglucanase	100	57
PNP gase	93	34

[a]The pH of the culture broth was adjusted continuously to 5.0 in the fermentor and on alternate days in the shake flasks. Residual activity was measured after incubation at 50° for 24 h under stationary condition. The enzyme activity before incubation at 50°C is considered to be 100% activity.

Table 2
Stabilization of Culture Broth[a]

	Culture broth	Ultra-filtered enzyme	Ammonium sulfate precipitated enzyme
	Activity retained, %		
FPA	10	82	55
Endoglucanase	57	80	97
β-glucosidase	32	91	89

[a]The enzyme (1.5 U FPA, 13.5 U) endoglucanase and 8.0 U β-glucosidase was incubated at 50°C for 24 h under stationary conditions. The enzyme activity before incubation is assumed to be 100%.

Polyethylene glycol (PEG Mr. 7.500), polyvinyl alcohol (PVA), and isopropanol (all at 1% level) activated and improved enzyme stability. PEG was found to improve the stability, especially of the filter paper activity, from 45 to 110%. Figure 1 shows the effect of different concentrations of PEG on the enzyme stability at 50°C under stationary conditions. At 2% concentration, PEG stabilized the enzyme. However, after removal of PEG by dialysis, only 89% enzyme activity was retained (Table 3).

RECOVERY OF ENZYME

P. funiculosum cellulase shows maximum hydrolysis and stability at 50°C (Table 4). Hence, the recovery of cellulase, after saccharification of alkali treated bagasse at 50°C, was studied. When enzyme was eluted

There was no change in the stability of enzyme under stationary or shaken conditions. PEG was found to stabilize the (FPA) even under shaken conditions.

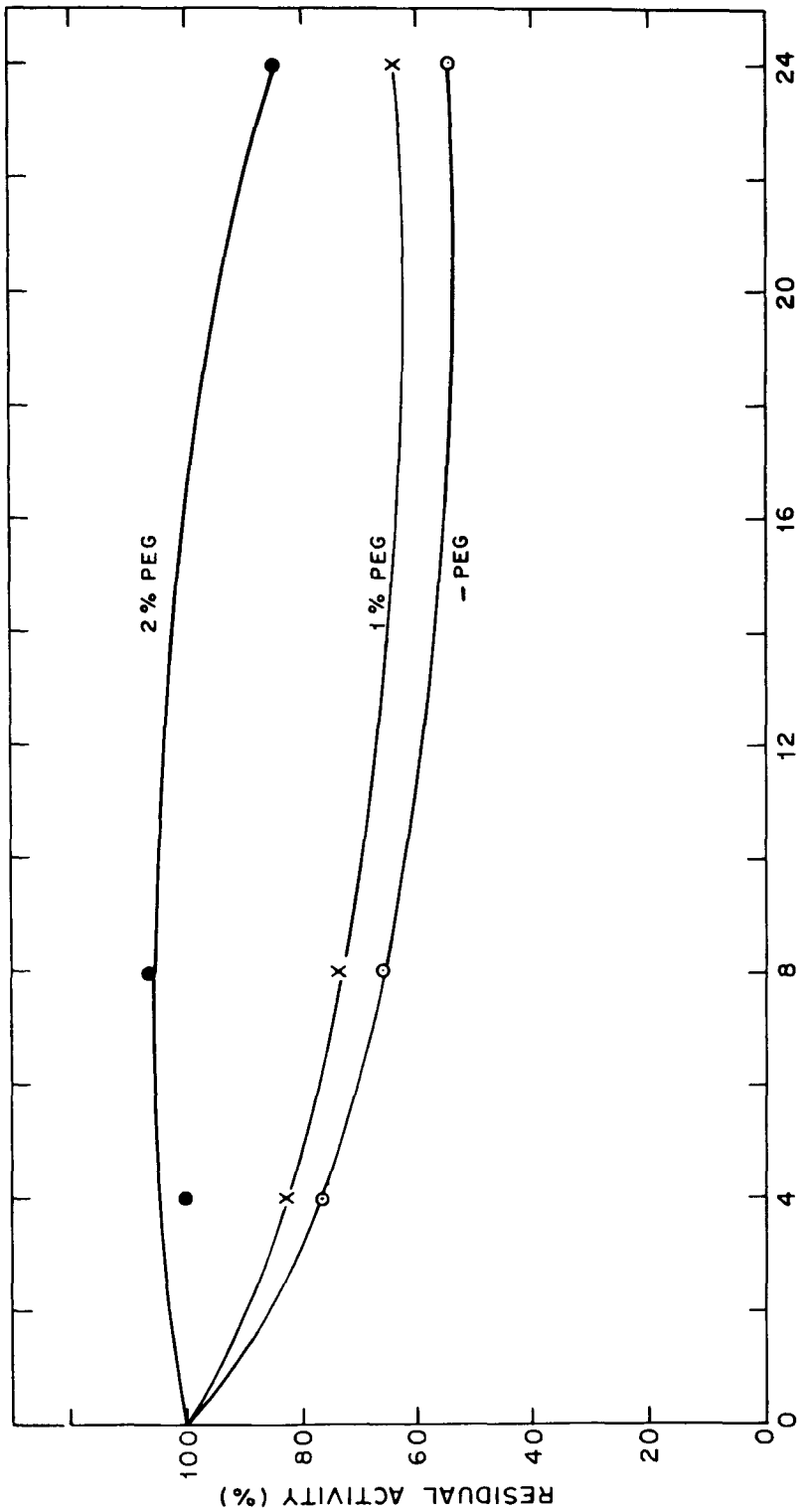

Fig. 1. Effect of PEG on stability of FPA of *P. funiculosum* enzyme. Residual activity was measured after incubation at 50°C under stationary conditions. Enzyme without PEG, ⊙ — ⊙; + 1% PEG, X — X; + 2% PEG, ● — ●.

Table 3
Effect of Removal of PEG on Stability of Enzyme

	Activity retained, %		
	FPA	Endoglucanase	β-glucosidase
Enzyme	55	97	89
Enzyme+PEG, 2%	102	110	96
Enzyme+PEG Dialyzed[a]	89	105	100

[a]Enzyme was treated with 2% PEG at 4°C for 1 h and dialyzed prior to checking the stability.

Table 4
Effect of Temperature on Stability of Cellulase[a]

	Temperature		
	37°C	50°C	60°C
FPA	100	80	50
Endoglucanase	100	80	70
PNP gase	100	88	64
Saccharification, %	51	72	ND

[a]Enzyme was incubated at different temperatures for 24 h at pH 4.8 under stationary conditions. Hydrolysis was carried out using alkali treated bagasse at 5% substrate concentration (0.2 gm with 5FPU) at pH 4.8 for 24 h (ND=not done).

with 50 m*M* acetate buffer by washing the residue twice, the enzyme recovery for different components was in the range of 8–14%. Elution of enzyme with 0.4% Tween-80 followed by grinding the residue with glass powder enhanced recovery of cellulases, especially of filter-paper activity, from 8 to 35%. Although stepwise addition of substrate enhanced the hydrolysis, it did not increase the recovery of the enzyme. The presence of 2% PEG during hydrolysis improved the recovery of the enzyme to 40% (Table 5).

The recovery of the enzyme at 50°C was much lower than at 37°C. Increasing the enzyme to substrate ratio and decreasing the time of hydrolysis to 12 h improved enzyme recoveries. In the absence of PEG and in the multiple mode of addition of substrate, although protein recovery was almost complete, the recovery of enzyme activity was between 55–80%. The presence of PEG increased enzyme recovery up to 95% (Table 6).

DISCUSSION

Enzyme stability is one of the important factors for its recovery and reuse that contributes significantly toward the economics of enzymatic

Table 5
Recovery of Enzyme at 50°C[a]

Mode of addition of substrate	PEG, 2%	Tween-80, 0.4%	Grinding with glass powder	Recovery, %			Saccharification, %
				FPA	Endoglucanase	PNP gase	
Single	–	–	–	7.5	13.8	14.8	44
Single	–	+	+	35.6	28	31.4	42
Single	+	+	+	42.7	30	45	52.6
Multiple	–	+	+	33.3	27.1	34.0	50.6
Multiple	+	+	+	41.4	39	43	63

[a]Hydrolysis was carried out as 5% substrate slurry with E/S ratio to 5 FPU/g at 50°C pH 4.8 under shaking for 24 h. The different modes of addition of substrate are as described in Materials and Methods.

Table 6
Effect of Increased Enzyme to Substrate Ratio on Recovery of Enzyme[a]

Mode of addition of substrate	PEG, 2 %	Recovery, %				Saccharification, %
		FPA	Endoglucanase	PNP gase	protein	
Single	–	56.5	67.1	55.7	93	75
Single	+	59.6	66	68	94	79
Multiple	–	70.2	80.9	55.5	100	73
Multiple	+	83	95	80	98	81

[a]Hydrolysis was carried out at 50°C with an E/S ratio of 25 FPU/g for 12 h with shaking. Elution of enzyme was done by grinding with glass powder with 0.4% Tween-80 in 50 m*M* acetate buffer pH 4.8.

hydrolysis. The different components of cellulase complex have variable stability, filter paper activity being the most susceptible to thermal inactivation (*9*). *P. funiculosum* cellulase is more stable at pH 5.0 than at pH 6.0. The present studies on *P. funiculosum* cellulase show that the stability of enzyme also depends on the conditions used for enzyme production. When the enzyme was produced either in fermentor or in shake flasks using well buffered media, it was found to be very stable, but when it was produced in shake flasks, in poorly buffered medium leading to wide pH fluctuations, the enzyme stability was comparatively lower. Mandels et al. (*7*) also reported similar variations in enzyme stability under different conditions of production. Ultrafiltration of ammonium sulphate precipitated *P. funiculosum* cellulase followed by dialysis improves the stability of the enzyme, probably owning to the removal of low molecular weight impurities.

Various substances such as PEG, PVA, isopropanol, and Tween-80 stabilize the *P. funiculosum* cellulase. PEG protects the filter paper activity against thermal inactivation. The presence of PEG during incubation is necessary for its protective action. *P. funiculosum* celluslase is not inactivated on shaking. Thus, the protective action of PEG is more against thermal inactivation rather than preventing shear inactivation.

Recovery of enzyme after hydrolysis is hindered by irreversible binding of the enzyme to substrate and/or its inactivation during hydrolysis. If 90–95% of the enzyme could be reused the economic viability of the process would be significantly improved. Since hydrolysis of cellulose is a synergistic action of endo- and exoglucanases and β-glucosidase, the recovery of these enzymes in their original proportions is essential for reapplication. The present studies indicate that the recovery of *P. funiculosum* enzyme is influenced by a variety of factors, such as, eluting agent, mode of addition of substrate, E/s ratio and grinding of the residual substrate. Our previous studies showed that it was possible to recover 95–100% of different cellulase components after hydrolysis of bagasse at 37°C. In-

creasing the temperature of hydrolysis to 50°C yielded very poor (40%) recovery of enzyme. Increasing the enzyme substrate ratio and thereby reducing the saccharification period had a remarkable effect, resulting in 90% recovery of enzyme.

The improved stability and recovery in the presence of PEG may be owing to its surface action, which lowers the surface tension of the medium, preventing irreversible adsorption of enzyme on the substrate. The lower recovery of enzyme at 50°C, compared to 37°C under similar conditions, may be owing to the increased affinity of enzyme for the substrate at higher temperature. Under practical saccharification conditions the protein molecules unfold exposing hydrophobic domains. It is possible that PEG reacts with hydrophobic areas of the enzymes and thus minimizes aggregation and subsequent denaturation (*7*).

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