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Evaluation of Cellulase Complex of *Penicillium funiculosum* and Its Mutant UV–49 in Relation to Practical Saccharification

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ABSTRACT

UV–49, the mutant of *Penicillium funiculosum*, showed higher production of cellulase although the specific activity with regards to filter paper activity was unaltered (0.7 U/mg). Fractionation of culture filtrates by isoelectric focusing indicated the presence of three endoglucanases and two β-glucosidases in the parent strain, whereas one endoglucanase and one β-glucosidase for UV–49. The thermostability of activity towards filter paper, CM-cellulose and *p*-nitrophenyl-β-D-glucoside of the parent strain was about 30–50% more than the corresponding activities of the mutant. The saccharification of bagasse with the enzymes from parent (66%) and mutant (62%) was comparable. However, the recovery of enzyme from residual cellulose was 10–20% less in case of the mutant. The formation of higher oligosaccharides in the hydrolysates of UV-49 on prolonged incubation indicated the presence of transglycosylation activity in the enzyme complex. In contrast the parent strain produces glucose; the desired end product of the practical saccharification.

Index Entries: Cellulase; hemicellulase; *P. funiculosum*; saccharification; cellulose bioconversion; cellulose-enzyme complex.

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INTRODUCTION

The commercialization of the enzymatic saccharification of cellulose to glucose is hampered because of the high cost of cellulase production. Cellulases are composed of three distinct types of enzymes: exoglucanase, endoglucanase, and β-glucosidase, which act synergistically for the efficient biodegradation of cellulose to glucose. One of the ways to reduce the cost of enzyme production is to select for hypercellulase secreting mutants. Improvements in the yields of cellulase have been reported by the isolation of mutant strains of *Trichoderma reesei* (*1–3*) or by optimization of fermentation conditions (*4*). The cellulases secreted by parent and mutant strains of *T. reesei* during controlled fermentations were characterized with respect to their enzyme composition and their ability to hydrolyze cellulose to glucose (*5,6*). The diversity in the control of cellulase production and in the types and levels of individual cellulase components of the mutants of *T. reesei* was studied by Shoemaker et al. (*7*). Although *T. reesei* and its mutants are the most extensively studied of the known cellulolytic organisms, cellulase produced by the parent and mutant strains have low β-glucosidase activity. An adequate amount of β-glucosidase activity is important from the point of view of practical saccharification (*8*). *Penicillium funiculosum* secretes a complete cellulase complex with high β-glucosidase activity and is capable of efficiently degrading native cellulose to glucose (*9,10*). The mutant UV–49 isolated from *P. funiculosum* exhibited a distinctly superior performance in terms of cellulase production (*11*). The knowledge of biochemical nature of the cellulases from the mutants could be useful to define the enzyme complex in relation to its saccharification efficiency. The objective of the present work is to characterize the cellulase complex produced by *P. funiculosum* and its mutant UV–49 with respect to their enzyme composition, stability, ability to hydrolyze different substrates, and their mode of action.

MATERIALS AND METHODS

Materials

The following materials were purchased from the suppliers indicated: Cellulose powder CP-100 (Cellulose Products of India Ltd., Ahmedabad); Larchwood xylan; 4-nitrophenyl β-D-glucopyranoside (4-NPG); 4-nitrophenyl β-D-xylopyranoside (4-NPX); 3,5-dinitrosalicylic acid (DNS) (Sigma Chemical Co., USA), and ampholine carrier ampholytes (LKB-Productor, Bromma, Sweden). The cellooligosaccharides and xylooligosaccharides used as standard sugars for end product determination were obtained as kind gifts from Elwyn T. Reese (Natick, MA, USA) and Peter J. Reilly (Ames, IA, USA), respectively. The other chemicals were used of analytical grade and obtained locally.

Microorganism

The isolation of *P. funiculosum* and UV–49 mutant has been described previously (*10,11*). The subcultures were maintained on potato dextrose agar (PDA) slants.

Production of Enzyme

Enzyme was produced by growing the organism in 100 mL of modified Mandels and Weber medium containing 0.75% casein and 2.5% cellulose powder in 500 mL Erlenmeyer flasks on a rotary shaker at 220 rpm, at 28°C for 10 d (*11*). The pH was maintained at 5 throughout the fermentation with sterile 1 *M* NaOH or 1 *M* HCl.

Enzyme Assays

Activities towards CM-cellulose (endoglucanase), Walseth cellulose (exoglucanase), filter paper (FP), xylan, 4-NPG (β-glucosidase), and 4-NPX (β-xylosidase) were determined according to recommendation of The Commission on Biotechnology, IUPAC (*12*) and were described previously (*13*).

Unit of Activity

Enzyme activities were expressed in (International) units (U). One unit is the amount of enzyme which releases one μmol of reducing sugar/min under standard assay conditions. In the case of β-D-glucosidase and D-xylosidase, release of 4-nitrophenol was determined. Protein was determined according to the method of Lowry et al. (*14*). Specific activities of the enzymes are expressed as U/mg of protein.

Stability

The stability of enzymes was determined by incubating the enzyme at 50°C in 50 m*M* acetate buffer, pH 4.8. The samples were removed at 2, 4, 6, 16, 24, and 30 h and estimated for enzyme activity.

Pretreatment

50 mesh bagasse (50 g) was treated with 5 *M* NaOH (100 mL) for 18 h at 28°C and washed free of alkali with water.

Hydrolysis

Hydrolysis of bagasse as carried out in stoppered flasks in the presence of toluene by incubating substrate (500 mg) with 5 U of FP activity in 50 m*M* acetate buffer (10 mL), pH 4.8 at 50°C.

Recovery of Enzyme

After 24 h of hydrolysis, the reaction mixture was filtered and the residual cellulose was washed three times with 50 m*M* acetate buffer. The filtrate and the washings were pooled, dialyzed, and estimated for enzyme activities.

Determination of End Products

The end products in the hydrolysates were determined by paper chromatography using n-butanol:pyridine:water (6:4:3 v/v) as the solvent system. The paper chromatograms were sprayed with a solution of 10 m*M* p-anisidine and 100 m*M* phthalic acid in 96% ethanol.

Fractionation by Flat Bed Isoelectric Focusing

The culture filtrates were concentrated by ultrafiltration using UM-10 membrane. The concentrated enzyme (10 mg) was subjected to preparative isoelectric focusing in the pH range of 3.5–10 as described by Bodhe et al. (*15*). Ultrodex (Sephadex G-75, superfine, 40-120 μ) was substituted with 24 strips of foam (polyurethane strip) each of which was 5 × 1 × 0.5 cm. These were soaked in 7 mL of 2% (w/v) Ampholine in water and placed horizontally on the LKB tray (24.5 × 11 × 0.5 cm). The anodic strip was soaked in 1 *M* H_3PO_4 and cathodic strip in 1 *M* NaOH. Two strips were removed, squeezed, dipped in the solution containing enzyme sample, and then replaced on the tray. The electrofocusing was run with an initial current of 12 mA and a constant power of 8 W for 18 h at 10°C. After termination of the run, foam strips were squeezed, the material collected in different test tubes, and the enzyme activities and pH determined.

Effect of pH and Temperature

Estimation of enzyme activities at different pH values was carried out with reaction mixture containing 50 m*M* of the following buffers: phthalate-HCl (pH 2.2–2.8); Na-citrate-citric acid (pH 3–4.8); citrate-phosphate (pH 5–7) under standard assay conditions. The optimum temperature was determined in the temperature range of 30°–80°C.

RESULTS

The Component Activities in the Culture Filtrates

UV–49, the mutant of *P. funiculosum* showed enhanced production of endoglucanase, exoglucanase, and β-glucosidase activities than the parent strain (*11*). The specific activity with regards to FP activity was not altered. However, the specific activities of other enzymes showed marginal differences (Table 1).

Thermostability

The longer incubation periods required in the practical saccharification of lignocellulosic substrates substantially inactivate the enzyme components. The thermostability of the enzymes from the parent and the mutant strain was examined at 50°C (Table 2). The cellulolytic components of the parent strain are more stable than the mutant. The cellulases of UV–49 are more unstable than the hemicellulases. The rate of fall in

Table 1
Specific Activity of Parent and Mutant Strain of *P. Funiculosum* Toward Different Substrates

	Specific activity (U/mg^{-1})	
	Parent	Mutant
Filter paper	0.7	0.7
Walseth cellulose	3.0	2.0
CM-cellulose	4.8	6.6
p-nitrophenyl β-D-glucoside	2.2	1.6
Xylan	5.0	6.3
p-nitrophenyl β-D-xyloside	0.04	0.026

Table 2
Thermostability of the Enzymes from the *P. Funiculosum* Parent and UV–49 Mutant[a]

Enzyme Component	Remaining activity, %	
	Parent	UV–49
Filter paper	81	37
Endoglucanase	100	48
Xylanase	60	65
β-Glucosidase (pNPGase)	98	71
Xylosidase	80	77

[a]The culture broths were incubated at 50°C and pH 4.8 for 24 h and were estimated for activity toward different substrates.

activity of the enzymes from UV–49 with respect to time is shown in Fig. 1. Although a pronounced (30–40%) loss of activity on incubation for 4 h was observed, there was no significant loss in activity upon prolonged incubation.

Hydrolysis of Bagasse and Recovery of Enzymes

The extent of hydrolysis of bagasse with the culture filtrate of the parent is comparable to that of mutant. The percent saccharification of bagasse by the parent and mutant was 66 and 62%, respectively, at 50°C after 24 h.

Over 60% of the cost centered in biotechnology of cellulose utilization has been identified with enzyme production which can be lowered by recovering the enzymes after the hydrolysis of cellulose. The recovery of cellulases after hydrolysis was 11–20% lower in case of mutant when compared with the parent strain (Table 3).

Determination of End Products

The end products of hydrolysis of bagasse as examined by paper chromatography indicated apparent differences in the mode of action of

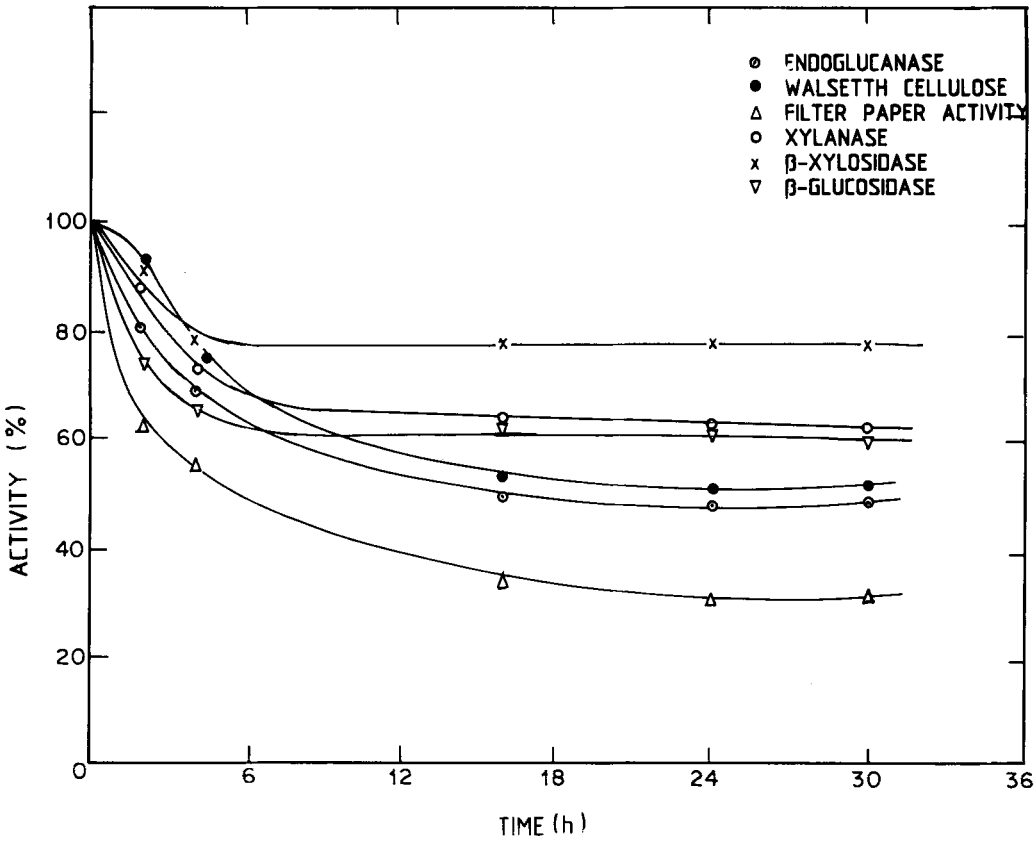

Fig. 1. Stability of UV–49 enzymes: The enzyme was incubated at 50°C and the samples were removed periodically and estimated for different activities.

the enzymes of parent and mutant strains. The enzymes from both strains predominently release glucose, xylose, and xylobiose, up to 4 h of incubation (shown in the case of mutant, Fig. 2). However, the prolonged incubation significantly altered the end products in the hydrolysate of the mutant strain. After 12 h of incubation, additional spots corresponding to cellobiose and xylotriose were observed in case of UV–49. *P. funiculosum* hydrolysate after 24 h showed xylose and glucose

Table 3
Recovery of Enzymes after Hydrolysis of Bagasse[a]

Enzyme Component	Recovery, %	
	Parent	Mutant
FP activity	33	22
Endoglucanase	27	7
β-Glucosidase	31	16

[a]Treated bagasse (500 mg) and enzyme (5 FPU) were incubated at 50°C for 24 h. The enzymes were recovered as described in Materials and Methods.

as major end products with traces of arabinose and cellobiose. However, in addition to these products the mutant showed intense spots corresponding to xylobiose, xylotriose, and cellobiose with traces of xylotetraose.

The Fractionation of Cellulases

The culture filtrates of *P. funiculosum* and UV–49 were fractionated by isoelectric focusing in the broad pH range of 3.5–10. The enzyme components were identified by determining the activities of the eluates towards CM-cellulose, Walseth cellulose, 4-NPG, and xylan. The isoelectric points (pI) of the enzymes from either strains were acidic and ranging from pH 3.0–5.5 (Table 4). The parent strain secreted three endoglu-

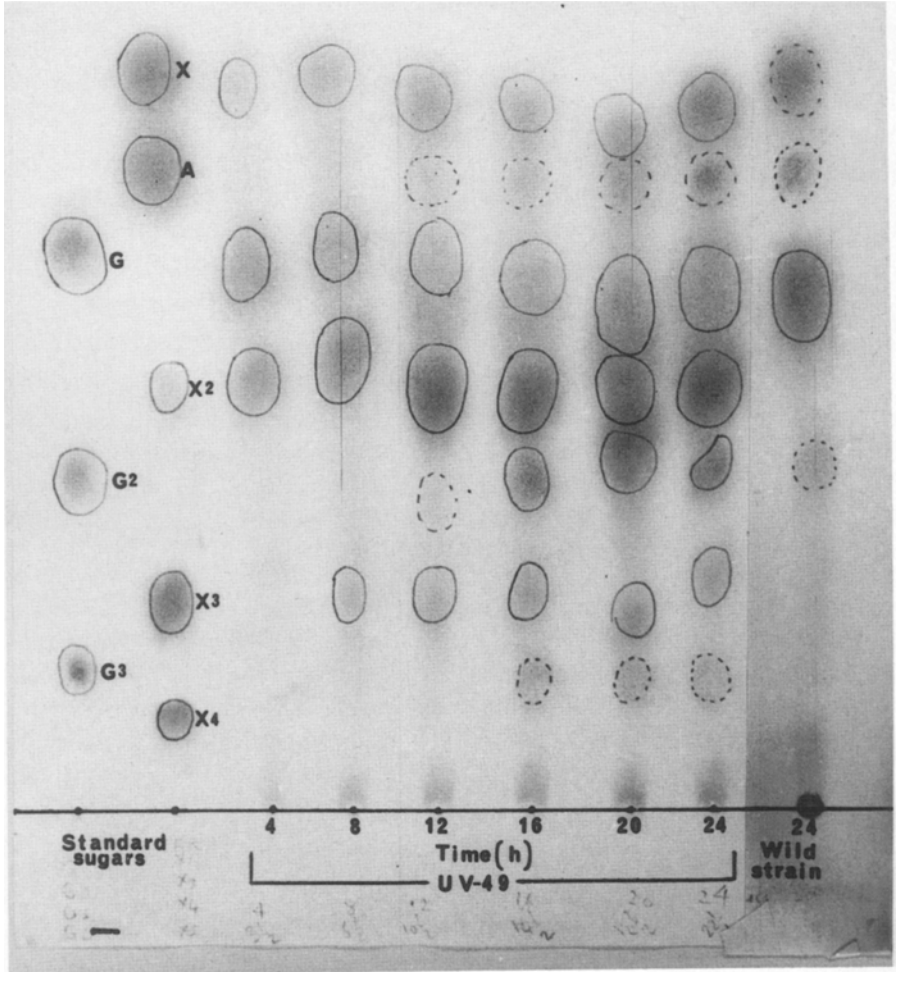

Fig. 2. Paper chromatogram of the hydrolysis products of alkali treated bagasse by the parent (24 h) and UV–49 strain (4, 8, 12, 16, 20, and 24 h). Standard sugars: G, D-glucose; G_2, D-cellobiose; G_3, cellotriose; X, D-xylose; A, L-arabinose; X_2, Xylobiose; X_3 Xylotriose; and X_4, Xylotetraose.

Table 4
Separation of Parent and Mutant Strain of *P. Funiculosum* by Preparative Isoelectric Focusing[a]

Enzyme	Parent		UV–49	
	Enzyme components	pI	Enzyme components	pI
Endoglucanase	Three	5.5	One	4.1
		4.2		
		3.6		
Exoglucanase	Two	3.5	Two	4.2
		3.0		3.7
β-Glucosidase	Two	4.5	One	3.6
		3.0		
Xylanase	One	4.1	One	5.1

[a]Concentrated enzyme (10 mg, 7 filter paper U) were subjected to preparative isoelectric focusing in the pH range 3.5–10.0.

canase and two β-glucosidase components, whereas UV–49 secreted one each of endoglucanase and β-glucosidase. The number of components of exoglucanase and xylanase was identical for both the strains. The pI of the various enzymes in the cellulase complex were altered in the mutant as shown in the Table 4.

Optimum pH and Temperature

Table 5 shows the optimum pH and temperature of the enzymes from *P. funiculosum* and its mutant UV–49. Endoglucanase and β-glucosidase from the parent strain had pH optima in a broad pH range of 3.5–5.0, whereas the mutant enzyme showed sharp pH optima of 4.5 and 4.4, respectively. The enzymes from the parent as well as mutant strain had optimum activity between 50–65°C.

DISCUSSION

Mutant UV–49 of *P. funiculosum* exhibited higher cellulase production than the parent strain. The specific activity of the filter paper degrading enzyme from the mutant remained unaltered. A similar trend was

Table 5
Comparison of the Optimum pH and Temperature of the Parent and Mutant Strain

Enzyme activity	Optimum pH		Optimum Temp.	
	Parent	UV–49	Parent	UV–49
FPase	3.5	4.6	50°	60
Endoglucanase	3–5	4.5	50–60	50
β-Glucosidase	3–5	4.4	60	65
Xylanase	4	4.4	55	55
β-Xylosidase	4	4.2	60	60

observed for *Trichoderma reesei* and its mutants (*16,17*). The cellulolytic enzymes from *T. reesei* mutant viz. RUT C-30 and NG-14 were more stable at 50°C than the parent enzyme, whereas the enzymes from parent strain of *P. funiculosum* are more stable than the mutant. The lower stability of the mutant enzyme at 50°C is also reflected in the poor recovery of the enzyme after saccharification. The fractionation of parent and mutant strains of *P. funiculosum* by preparative isoelectric focusing indicated a shift in pI of the mutant. The number of cellulase components secreted by the mutant was the same as that of parent strain, with the exception of endoglucanase and β-glucosidase components. Sheir-Neiss and Montenecourt have shown by analytical isoelectric focusing that the number of pI of various enzymes in the cellulase complex were unchanged in the mutant (*6*).

The extent of hydrolysis of bagasse by the enzymes from parent and mutant was same. However, a marked difference in the end product formation was observed. The end products of the hydrolysate obtained through UV–49 revealed more intense spots corresponding to cellobiose, xylobiose, xylotriose, and xylotetraose than D-glucose and D-xylose. The intense spots of cellobiose and xylobiose may be resulting from the lower specific activity of β-glucosidase and β-xylosidase with UV–49 enzymes compared to the parent strain (Table 1). The synthesis of higher oligosaccharides following longer incubation periods as revealed on the paper chromatogram also indicates the significantly more transglycosylation activity in the UV–49 enzyme make up than the parent strain.

In a practical saccharification process, it is desirable to get glucose as the major end product. In this respect the enzymes from the parent strain appeared to be more suitable than the mutant. In conclusion, the evaluation of mutants should not be restricted only to the hypercellulase production but emphasis should also be given to other properties of enzymes such as its specific activity, thermostability, and transglycosylation activity.

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