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Factors Affecting Stability of *Sclerotium rolfsii* UV-8 Mutant Cellulase Complex Under Saccharification Conditions

MUKUND V. DESHPANDE

Biochemical Sciences Division, National Chemical Laboratory, Pune 411008, India

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ABSTRACT

Enzyme stability studies in case of *Sclerotium rolfsii* UV-8 mutant have been investigated under the conditions used for saccharification of cellulose (50°C, pH 4.5, 48 h). Avicelase (measure of exoenzymes) and xylanase were found to be less stable than CMCase (endoglucanase) and β-glucosidase. Merthiolate (and other Hg compounds) added as a biocide, inactivated avicelase and xylanase about 60–70%. Of the antibiotics tested, tetracycline, chloramphenicol, and streptomycin sulfate were found suitable as an additive in cellulose hydrolysis system. The optimum hydrolysis of alkali-treated (AT)-rice straw, AT-bagasse, Solka Floc SW40, and Avicel P.H.101 was observed under shaking conditions at pH 4.5, 50°C in CO_2 atmosphere. It is suggested, all the studied parameters could be used for the evaluation of mutant strains.

Index Entries: Cellulase complex; saccharification; *Sclerotium rolfsii* UV-8 mutant; stability.

INTRODUCTION

Enzymatic saccharification of cellulose has very high yields because cellulases catalyze only hydrolysis reactions and not sugar decomposition reactions. However, over the past decade, relevant research has been

performed mainly in areas such as pretreatment of lignocellulosic biomass (*1*); production of more effective enzyme complex and its reutilization (*2*); and product concentration at desired rate (*3*). For practical enzymatic hydrolysis of cellulose, the research on suitability of the enzyme source (*4*) and stability of the enzyme complex is gaining increasing importance, particularly because of the cost of the enzyme (*5*). These enzymes may be sufficiently stable for the growth of the organism but these may not withstand the conditions that are being employed for saccharification, e.g., high substrate and product concentrations; high temperature; shear resulting from vigorous shaking; biocide concentration; and an environment under which saccharification is being carried out.

From a comparison made with other cellulolytic fungi, such as *Trichoderma reesei, Sporotrichum pulverulentum, Aspergillus niger,* the parent strain of *S. rolfsii* produces large amounts of cellulase and hemicellulase enzymes (*6*). A mutant strains of *S. rolfsii,* UV-8, produces 2.5 times more exoglucanase and 1.2–1.5 times more endoglucanase and β-glucosidase activity, as compared to the parent strain (*7*).

The present communication describes the factors affecting the stability of *S. rolfsii* UV-8 cellulase complex under the saccharification conditions.

MATERIALS AND METHODS

Organism

A mutant strain UV-8, of *S. rolfsii* (NCIM 1084) was used in the present study. The organism was maintained on potato dextrose agar (PDA) slants at 30°C.

Cultivation Conditions

The enzyme used in this study was produced by growing the UV-8 mutant on NM-2 medium with the addition of 2% corn steep liquor (*7*) for 14 d under shaking conditions at 28°C. The culture filtrate was adjusted to pH 4.5 with sodium citrate (2 mL, 0.5*M* Na citrate for 100 mL culture filtrate) and stored at 4°C, after addition of merthiolate to a concentration of 0.01%, unless otherwise mentioned.

Enzyme Assays

Carboxymethylcellulase (CMCase, endoglucanase), filter paper degrading activity (FPA), β-glucosidase, and endoxylanase were assayed according to Shewale and Sadana (*8*). Avicelase (measure of exoglucanase activity) was assayed as described by Wood and Bhat (*9*). One international unit of enzyme activity was defined as the amount of enzyme that produces 1 μmol of the product in 1 min under assay conditions.

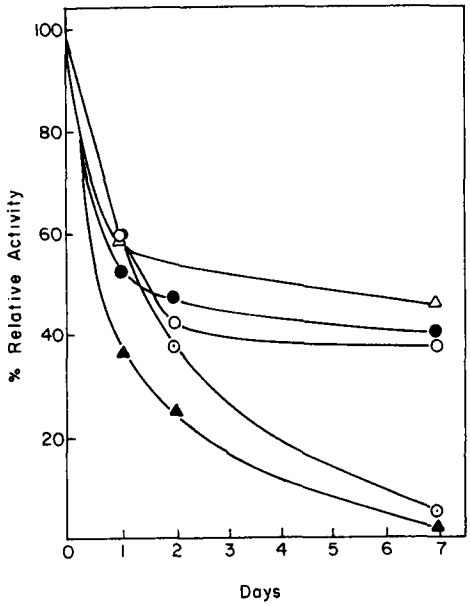

Fig. 1. Stability of cellulase components of *S. rolfsii* UV-8 mutant culture filtrate. Enzyme was incubated at pH 4.5, 50°C, in the presence of 0.01% merthiolate. △, CMCase; ●, β-Glucosidase; ⊙, Xylanase; ▲, Avicelase; ○, FPA.

Saccharification

Hydrolysis of the substrate was carried out by adding 7.5 mL culture filtrate to 1.5 g substrate with 0.5 mL 1*M* citrate buffer, pH 4.5. The reaction mixture was incubated at 50°C in stoppered test tube for different time intervals. The reducing sugars in the aliquots were determined by dinitrosalicylic acid method (*10*).

RESULTS AND DISCUSSION

Stability of Cellulase and Xylanase Components

All stability experiments were carried out under the conditions contemplated for the saccharification (50°C, pH 4.5) of cellulosic material. Effect of saccharification conditions on different enzymes of the cellulase complex and xylanase in the culture filtrate was examined. As shown in Fig. 1, all the cellulase components and xylanase of UV-8 culture filtrate were inactivated. It was shown (*11*) that 90–95% of the enzymes must be recirculated in order to obtain a saccharification process economically

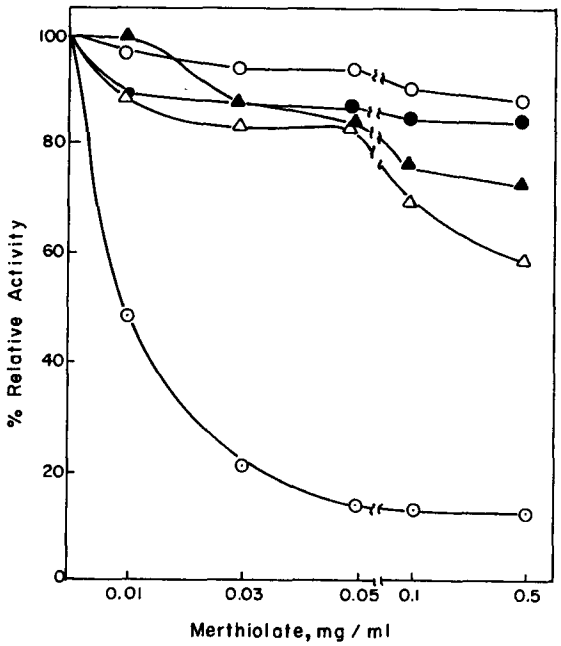

Fig. 2. Effect of merthiolate on stability of cellulase components of *S. rolfsii* UV-8 mutant culture filtrate. Merthiolate was added to enzyme and incubated at 50°C, pH 4.5, 18 h. △, CMCase; ●, β-Glucosidase; ⊙, Xylanase; ▲, Avicelase; ○, FPA.

competitive with acid hydrolysis. However, this could not be possible owing to instability of the enzymes under saccharification conditions.

This led to an evaluation of the incubation conditions, and it was found that the merthiolate used as a preservative was responsible for much of the adverse effects (Fig. 2). Again, greater instability was observed in the case of xylanase at 50°C, pH 4.5, with an increase of merthiolate concentration. The same merthiolate concentration (0.01%) had no effect on enzymes stored in the deep freeze at −15°C for 2–3 yr, and in the refrigerator for 7 d. It seems that at 50°C, the conformation of the affected enzyme may be changed in such a way as to make it more susceptible to merthiolate (*12*). The effect of other mercury compounds was also studied by incubating the culture filtrate with these compounds at room temperature for 15 min and the activities assayed were compared with the culture filtrate incubated simultaneously without any additives (Table 1). Other mercury compounds resemble merthiolate in action. The presence of a number of disulfide linkages in these enzymes may explain the effect (*13*).

The sensitivity of cellulase components to heat and to merthiolate, lead to an investigation of a means of improving the stability, and to a search for a biocide effective in preventing contamination of the hydrolysis

Table 1
Inactivation of Cellulase, Xylanase, β-Glucosidase of *S. rolfsii* UV-8 Mutant Culture Filtrate

Compound	Concentration	% Loss[a]			
		Avicelase	CMCase	Xylanase	β-Glucosidase
p-Chloromercury benzoate	10^{-3} *M*	63	47	55	14
	10^{-4} *M*	37	34	33	6
$HgCl_2$	10^{-3} *M*	68	50	90	23
	10^{-4} *M*	48	38	79	11
Merthiolate	0.1%	50	35	52	30
	0.01%	12	8	20	12
Mercaptoethanol	0.1%	63	15	60	17
	0.01%	31	10	35	8
Dithiothreitol	0.1%	58	15	55	14
	0.05%	28	4.6	28	5.8
Na azide	0.1%	50	38	36	38
	0.01%	14	31	14	17

[a]Inactivation after incubation at room temperature for 15 min.

mixtures without impairing the action of the enzymes. If the saccharification is done batchwise for 24/48 h at 50°C, addition of a biocide may be unnecessary. But in anticipation of a continuous process, in order to avoid contamination with thermophiles, potential biocides for their effects on cellulolytic enzymes have been examined (Table 1). Avicelase and xylanase were found to be much more sensitive to these compounds at concentration required for prevention of microbial growth that CMCase or β-glucosidase. Compounds such as $HgCl_2$, *p*-chloromercury benzoate, and merthiolate revealed the difference between the avicelase and CMCase. These are the agents that usually react with the -SH or $-NH_2$ protein groups. The thiol compounds like dithiothreitol and mercaptoethanol may be acting on the disulfide linkages to modify conformation of avicelase and xylanase (*12*).

A series of antibiotics was also tested for their effect on cellulolytic enzymes. Of the antibiotics tested at concentrations (2–50 μgm/mL) required for preventing the growth of the organisms, tetracycline, chloramphenicol, and streptomycin sulfate were found suitable as an additive in cellulose hydrolysis system. At 50 μgm/mL concentration, 80–100% residual activity of all the cellulolytic enzymes as compared to the control, i.e., culture filtrate incubated at 50°C without biocide for 48 h, was observed, whereas the addition of penicillin G showed greater inactivation (30% at 50 μgm/mL level) of xylanase (Table 2).

Table 2
Effect of Addition of Antibiotics
on the Stability of Cellulase from *S. rolfsii* UV-8 Mutant

		% Residual activity[a]					
	Concentration,	CMCase		Xylanase		β-Glucosidase	
Biocide	μg/mL	18 h	48 h	18 h	48 h	18 h	48 h
Tetracycline	2	100	100	100	85	100	100
	5	100	95	100	80	100	100
	50	88	80	90	75	100	100
Chloramphenicol	2	100	100	100	86	100	100
	5	100	96	100	80	100	90
	50	86	80	92	80	96	90
Streptomycin	2	100	100	100	95	100	98
sulfate	5	100	93	100	92	100	89
	50	88	82	92	90	88	85
Penicillin G	2	100	100	100	72	100	88
	5	100	97	90	70	100	85
	50	89	83	87	70	90	80
Merthiolate	100	84	80	20	10	86	82
	1000	60	50	15	8	65	60

[a]% Residual activity was calculated for each enzyme by taking culture filtrate incubated without biocide as the control.

Effect of Shaking on Enzyme Stability During Saccharification

The cellulose hydrolysis by UV-8 *S. rolfsii* culture filtrate was studied for 48 h at 50°C to see the effect of shaking on the efficiency of saccharification of alkali-treated (2*N* NaOH, 30°C, 48 h) rice straw and bagasse, Solka Floc SW40 (sulfite pulp), and Avicel P.H.101 (microcrystalline cellulose) (Table 3). Cellulase and β-glucosidase activities of the culture filtrate used were (IU/mL): FPA, 2.16; CMCase, 195; xylanase, 185; and β-glucosidase, 13.9. The cellulases were not found to be affected by shaking conditions. In fact, a 10–12% increase in saccharification was observed because of shaking, and this might be attributable to more accessibility to the substrate. However, in the case of cellulases from *T. viride, T. longibrachiatum,* and *A. foetidus,* it was reported by Sinitsyn et al. (*14*) that in absence of cellulose under saccharification conditions (40°C, pH 4.5), agitation caused inactivation. Similar observations were reported in the case of *T. reesei* C-30 cellulase complex (*12*).

Table 3
Effect of Shaking on Enzymatic Hydrolysis of Cellulose by *S. rolfsii* UV-8 Mutant[a]

Shaking	Atmosphere	Substrate, 15%	Reducing sugar mg/mL 12 h	24 h	48 h	% Saccharification[b] 12 h	24 h	48 h
Without shaking	Air	AT-rice straw	71	89	91	42.6	53.4	53.6
		AT-bagasse	56	76	77	33.6	45.6	46.2
		Solka Floc SW40	39	44	47	23.4	26.4	28.4
		Avicel P.H. 101	31	38	41	18.6	22.8	24.6
	CO_2	AT-rice straw	76	99	100	44.6	59.4	60.0
		AT-bagasse	66	83	85	39.6	49.8	51.0
		Solka Floc SW40	44	53	55	26.4	21.8	33.0
		Avicel P.H. 101	33	37	40	19.8	22.2	24.0
	N_2	AT-rice straw	71	88	92	42.6	52.8	55.2
		AT-bagasse	57	76	78	34.2	45.6	46.8
		Solka Floc SW40	39	44	47	23.4	26.4	28.2
		Avicel P.H. 101	31	39	39	13.6	23.4	23.4
With shaking	Air	AT-rice straw	70	96	98	42.0	57.6	58.8
		AT-bagasse	64	83	84	38.4	49.8	50.4
		Solka Floc SW40	40	53	57	24.0	31.8	34.2
		Avicel P.H. 101	34	43	47	20.4	23.8	28.2
	CO_2	AT-rice straw	80	103	105	48.0	61.8	63.0
		AT-bagasse	68	90	91	40.8	54.0	54.6
		Solka Floc SW40	55	66	68	33.0	39.6	40.8
		Avicel P.H. 101	33	41	43	19.8	24.6	25.8
	N_2	AT-rice straw	71	97	99	42.6	58.2	59.4
		AT-bagasse	64	84	85	38.4	50.4	51.0
		Solka Floc SW40	41	54	58	24.6	32.4	34.8
		Avicel P.H. 101	34	44	50	20.4	27.0	30.0

[a] As described under Materials and Methods.

[b] $\%\text{Saccharification} = \frac{\text{Amount of reducing sugars}}{\text{Amount of substrate}} \times \frac{162}{180} \times 100$

Effect of Carbon Dioxide and Nitrogen Atmosphere on Saccharification

A carbon dioxide and a nitrogen atmosphere were used to replace air in hydrolysis tubes in view of Eriksson's claim (*15*) that an oxidative mechanism is involved in cellulose digestion by *S. pulverulentum*. Nitrogen atmosphere had no beneficial effect on the hydrolysis rate, under both shaken and unshaken conditions (Table 3). On the other hand, carbon dioxide did show a favorable effect (5–10% increase in saccharification) in

shaken and unshaken condition. Carbon dioxide atmosphere may be useful in the preservation of the enzymes for greater digestion of cellulose. Reese and Mandels (*12*) reported that the optimum hydrolysis of Avicel with *T. reesei* C-30 enzyme (with added β-glucosidase) was under unshaken conditions at pH 5.0, 50°C in carbon dioxide atmosphere.

All these parameters, such as the effect of perservatives, atmosphere in saccharification vessels, shaking conditions, and so on, should be considered in evaluation of the mutant enzyme complex selected for the hydrolysis. The high β-glucosidase and xylanase production by *S. rolfsii* UV-8 mutant in submerged culture may also be responsible for high cellulose hydrolysis.

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