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Toner Removal by Alkaline-Active Cellulases from Desert Basidiomycetes

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Two isolates of wood-decay basidiomycetous fungi from the Sonoran Desert were selected from a preliminary screening of an extensive collection of wood-decay fungi. The isolates produced extracellular, alkaline-active carboxymethyl cellulase (CMCase) and filter paper cellulase activity, especially on solid substrates. Cultivation on wheat bran gave a higher cellulase yield than cultivation on corn steep liquor or paper pulp. The fungi produced alkaline-active cellulases when cultivated at pH 7.0 and 8.0, but not at pH 9.0 or above. Surfactants did not affect enzyme production level during cultivation on wheat bran. The fungi grew at 37°C, but enzymes were produced at 27°C. Isolate 2 produced more CMCase activity than did isolate 1 at both pH 7.0 and 8.5. In both isolates, enzyme activity was higher at pH 7.0 than at 8.5. Isolate 1 CMCase was stable at 50°C and isolate 2 CMCase, at 60°C. The crude alkaline enzymes could deink mixed office wastepaper that was 70% coated with toners. More ink was removed after addition of enzymes to paper pulp in the presence of Surfactant.

Recycling of office and other wastepaper is an effective way to extend our nation's timber resource. For this reason, recycling has been given a high priority in Forest Service pulp and paper research. One of the greatest challenges in paper recycling is removal of contaminants; some of the most problematic contaminants are polymeric inks and coating. Toners such as those used in laser and xerographic copy machines are thermally fused to the surface of the printed page, and because they are nylon-based polymers that do not disperse, they are difficult to separate from fiber stocks. This is unfortunate because office copy paper is made of high-value bleached chemical pulp.

In 1991, Kim *et al.* (1) showed that crude cellulases applied to pulps could facilitate the deinking process. In subsequent studies, other researchers (2-5) showed that enzymatic deinking is most effective when cellulases are used during high-consistency fiberization in the presence of non-ionic surfactants. This step removes residual fibers from the toner surfaces. Subsequent flotation and washing steps remove toner or ink particles.

The most effective cellulases are those that exhibit activity on filter paper at neutral and alkaline pH. Activity at pH 8.5 is particularly important because calcium carbonate is often used as a filler and brightener in recycled fibers, and pulping processes leave the fiber with an alkaline pH. Most fungal cellulases are active in the acid region, and as the use of recycled fibers increases, the cost to neutralize the carbonate becomes excessive. Thermal stability is also important for effective processing.

Previous studies showed that the complete cellulase complex from the ascomycetous fungus *Humicola insolens* is highly effective in removing toners from office waste papers at neutral pH (6), but at the alkaline pH (8.5) found with most recycled fibers, deinking activity of this enzyme preparation is negligible. We therefore sought novel cellulases that could remove toners at alkaline pH. Most known alkaline-active cellulases are from *Bacillus*, but they do not produce a complete cellulase complex. *Bacillus* and other cellulases are presently used in the detergent industry (7-9).

The objective of the present work was to determine whether basidiomycetous fungi from alkaline thermal environments would produce useful cellulase complexes. In preliminary studies, we screened 43 strains of wood-decay Sonoran Desert basidiomycetes for production of alkaline-active cellulases. The screening procedure consisted of growing the strains on defined liquid alkaline media containing carboxymethylcellulose (CMC) as the sole carbon source and visualizing its enzymatic activity by zymogram. In this work, we report the solid-substrate cultivation of two selected fungal isolates for the production of alkaline cellulases. This paper also describes some characteristic properties and application of these crude enzyme preparations in enzymatic deinking.

Materials and Methods

Media. The fungal strains were maintained on yeast malt agar and potato dextrose agar (PDA) (Difco, Detroit, MI). (Note: Trade or firm names are for the convenience of the reader and do not represent the endorsement of the U.S. Department of Agriculture of any product or service.) In the preliminary screening of fungal isolates, both Norkran (10) and Ghosh and Kundu (11) liquid cultivation media were employed. The Norkran medium consisted of (per liter) yeast extract, 0.5 g; $\text{NH}_4\text{H}_2\text{PO}_4$, 2 g; KH_2PO_4 , 0.6 g; K_2HPO_4 , 0.4 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g; and CMC (low viscosity; Sigma, St. Louis, MO), 1.32 g. After autoclaving, 10% sterile Na_2CO_3 solution was added to adjust the pH to 7.3. The Ghosh and Kundu medium consisted of (per liter) KH_2PO_4 , 2 g; $(\text{NH}_4)_2\text{SO}_4$, 2 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3 g; CaCl_2 , 0.3 g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 5 mg; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 1.6 mg; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 1.4 mg; CoCl_2 , 2 mg; and CMC, 1.6 g. The pH of the Ghosh and Kundu medium was adjusted the same as that of the Norkran medium.

Screening and Cultivation of Strains. Forty-three strains of Sonoran Desert wood-decay fungi from the culture collection of the Forest Products Laboratory were selected for initial screening. The strains were grown on fresh yeast maltose agar plates. After the fungi had attained confluent growth, spores and mycelia were washed from the surface with 3 to 4 mL of sterile water and used as an inoculum. The cell suspension (0.2 mL) was added to 0.8 mL of both Norkran (10) and Ghosh and Kundu (11) media in 2-mL microfuge tubes. The tubes were placed in a microfuge fermentation tilt-rack at a 45° angle (12) and shaken at 225 revolutions/min, 30°C for 6 days. To identify cellulase producers, the 3,5-dinitrosalicylic (DNS) acid color reagent (13) was used to estimate reducing sugars released from CMC into the media on days 2, 3, 4, and 6. Further attempts to scale up cellulase production were conducted in shake flask and in solid-substrate culture (SSC).

Shake Flask Cultivation. Twenty-five milliliters of CMC liquid Ghosh and Kundu medium in a 125-mL Erlenmeyer flask was inoculated with 5 mL of fungal inoculum and shaken at 225 revolutions/min at 30°C for 6 days. Samples from days 4 and 6 were centrifuged and the supernatant was saved for DNS assay. To enhance CMCase production, the Ghosh and Kundu medium was supplemented with skim milk, 0.2% (14), corn steep liquor, 2.6%, or wheat bran, 1%.

Production of Alkaline Cellulases. The fungal isolates were cultivated on wheat bran, corn steep liquor, and paper pulp in SSC (15). One-hundred grams of solid substrate was adjusted to 60% moisture (wet weight basis) with an aqueous solution of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (60 mg), K_2HPO_4 (1.2 g), and Na_2CO_3 (1.2 g), at pH 7.0. Corn steep liquor (3%) was used as an experimental variable to provide nitrogen and other nutrients. Fifty grams of wet substrate in a 500-mL conical flask was autoclave at 15 psig at 121°C for 1 h. After cooling, the content of the flask was inoculated with 5.0 mL of fungal culture from the isolates grown on PDA plates. The flasks were incubated at 27°C for 10 days. The moldy bran was then air-dried, packed into a cylindrical glass column, and extracted with 125 mL of tap water. The extracted fluid was centrifuged at 3000 x g for 20 min, and the supernatant solution was used as crude enzyme extract.

Enzyme Assays. For determination of CMCase activity, soluble CMC (degree of substitution (DS) = 0.65 to 0.90), 2% (w/v), was prepared by suspending 2 g of CMC in 100 mL of 100-mM $\text{K}_2\text{HPO}_4/\text{KOH}$ buffer, pH 7, or glycine buffer, pH 8.5, and stirring the suspension until completely dissolved. Crude enzyme preparations were appropriately diluted to obtain maximal activity consistent with a linear response.

The reaction mixture (0.5 mL, containing 0.25 mL of substrate, 1% (w/v) final concentration, and 0.25 mL of diluted enzyme in buffer, pH 7.0 or 8.5) was incubated at 50°C for 30 min. The reaction was stopped by adding 1.0 mL DNS color reagent to estimate reducing sugars. Enzymatic activity was expressed as international units (IU) ($\mu\text{mol glucose}$) per $\text{min}^{-1} \text{mL}^{-1}$. All units were corrected with suitable substrate and enzyme controls under identical assay conditions.

Filter paper culture enzyme (FPase) activity was measured (16) in buffer (see previous description) at pH 7.0 and 8.5. To determine the reduction in viscosity caused by added alkaline cellulases, 10 mL of reaction mixture containing 1% CMC solution was incubated with 20 mg of enzyme protein at 50°C for 1 h. After inactivating the enzyme at 95°C for 5 min, the hydrolyzed samples were filtered and cooled to 25°C, and viscosity was measured with an Ostwald viscometer (17). The reaction mixture without enzyme prepared under the same conditions was used as control. The protein contents of the crude enzyme preparation were estimated with the bicinchoninic acid reagent (18).

Buffers used for studying the effect of pH on enzyme activity were 100 mM of phosphate buffer (pH 6 to 8.5), glycine buffer (pH 8 to 10), and 3-cyclohexylamino-1-propane sulfonic acid (CAPS) buffer (pH 9.5 to 11) (Sigma, St. Louis, MO). For thermostability studies, the crude enzyme was pretreated for 30 min at various temperatures prior to CMCase assay.

Electrophoretic Analysis. Isoelectric focusing (IEF) was performed on a Bromma 2117 Multiphor horizontal slab-gel system (LKB, Sweden) using Servalyt precotes (pH 3 to 10, Serva, Heidelberg, Germany). Protein bands were revealed by staining with Serva violet. Zymogram analysis of CMCase and xylanase activity in IEF was performed as reported previously (19, 20). Substrate gel (1 mm) was prepared with 0.1 % (w/v) soluble CMC in phosphate buffer, pH 7.0, containing agar and filtered dye (Congo red, 0.1%). A zymogram was obtained by overlaying the dye-bound substrate gel on native IEF gel after incubation at 50°C for 1 to 3 h.

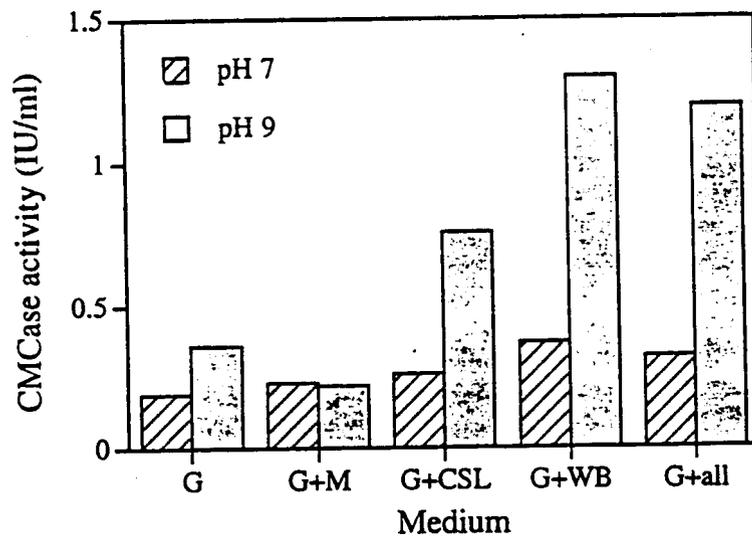


Figure 1. Enhancement of CMCase on isolate 2 grown on Ghosh (G) liquid medium, pH 9. Medium was supplemented with skim milk (M), corn steep liquor (CSL), wheat bran (WB), and all supplements (all). CMCase assays were performed on day 10 enzymes with pH 7 phosphate (dotted bar) and pH 9 glycine buffer (solid bar).

Table I. Production of Alkaline Cellulases and Isolate Activity at pH 7.0 and 8.5

Iso-	Sub-	Growth	CMCase (IU/mL)		Fpase (IU/mL)		Reduction in Viscosity (%)	
			7.0	8.5	7.0	8.5	7.0	8.5
1	wheat	+++	0.78	0.60	0.70	0.95	29.8	19.3
	corn	+++	0.77	0.66	0.78	0.78	12.0	19.9
2	wheat	+++	1.11	0.97	0.39	0.38	46.4	29.0
	corn	-/+	0	0	0	0	0	0

Deinking Trial. Recycled paper pulps were collected from a mixture of photocopier waste papers that were coated with toners. Deinking trials were conducted as reported previously (6). However, surfactant solution (WITCO 5175-26A), 0.02%, was added to the pulp during the fiberization stage. Enzyme treatment was carried out at 14% final consistency at pH 8 to 8.5, 50°C. The enzymes from these isolates and SP 342 Cellulase (Novo Industries, Danbury, CT) were added at three levels: 18, 36, and 72 IU CMCase/200 g oven-dry pulp. Shredded paper was added to water and surfactant in a custom-made, stainless-steel, water-jacketed Hobart mixing bowl. The initial solids content (consistency) was 17% (w/w). The suspension was mixed (fiberized) until paper particles were no longer visible (5 min). At that point, diluted enzyme was added. Enzyme treatment was conducted for 20 min to examine its effect on deinking. All pulping runs were followed by flotation in a 2 L-capacity laboratory flotation unit. The residual ink specks were counted on a Optomax ink scanner (Hollis, NH) and reported as parts per million. The size of the ink specks ranged from 0.02 to 2 mm. The specks were counted from five handsheets (made from enzyme-treated pulp) using 10 scans on each sheet. Suitable water and heat-killed enzyme controls were floated in each batch. The residual ink on handsheets made from enzyme-treated pulp was compared to that on sheets made from heat-killed enzyme.

Results

Identification of Isolates Producing Alkaline-Active Cellulases. Of the 43 fungal strains tested in the preliminary screening, only two strains released high amounts of reducing sugar within 4 days of cultivation. These strains are designated simply as isolates 1 and 2. Isolate 1 grew much slower than isolate 2 on both yeast malt agar and PDA. Isolate 1 does not form spores whereas isolate 2 is a spore-forming strain.

Effect of Shake Flask Culture on Enzyme Production. Low growth accompanied by low levels of CMCase production was observed with both isolates in liquid CMC medium. Alkaline CMCase production improved for isolate 2 in the liquid medium when corn steep liquor or wheat bran, but not skim milk, were incorporated (Figure 1).

Effect of SSC on Enzyme Production. In contrast to results with liquid culture, both growth and production of alkaline cellulases were good in SSC. Enzyme production was improved in SSC with regard to various solid substrates, pH of cultivation, cultivation and incubation time, and effect of surfactants. The best conditions were pH of harvested culture fluid in the range of 8.5 to 9.0 and use of wheat bran supplement. Column extraction was more efficient than batch-wise extraction in a beaker or flask. On wheat bran, both isolates produced alkaline CMCase, Fpase, (Table I). Corn steep liquor supported growth and enzyme production by isolate 1 but not isolate 2. Paper pulp did not support growth or enzyme production (data not

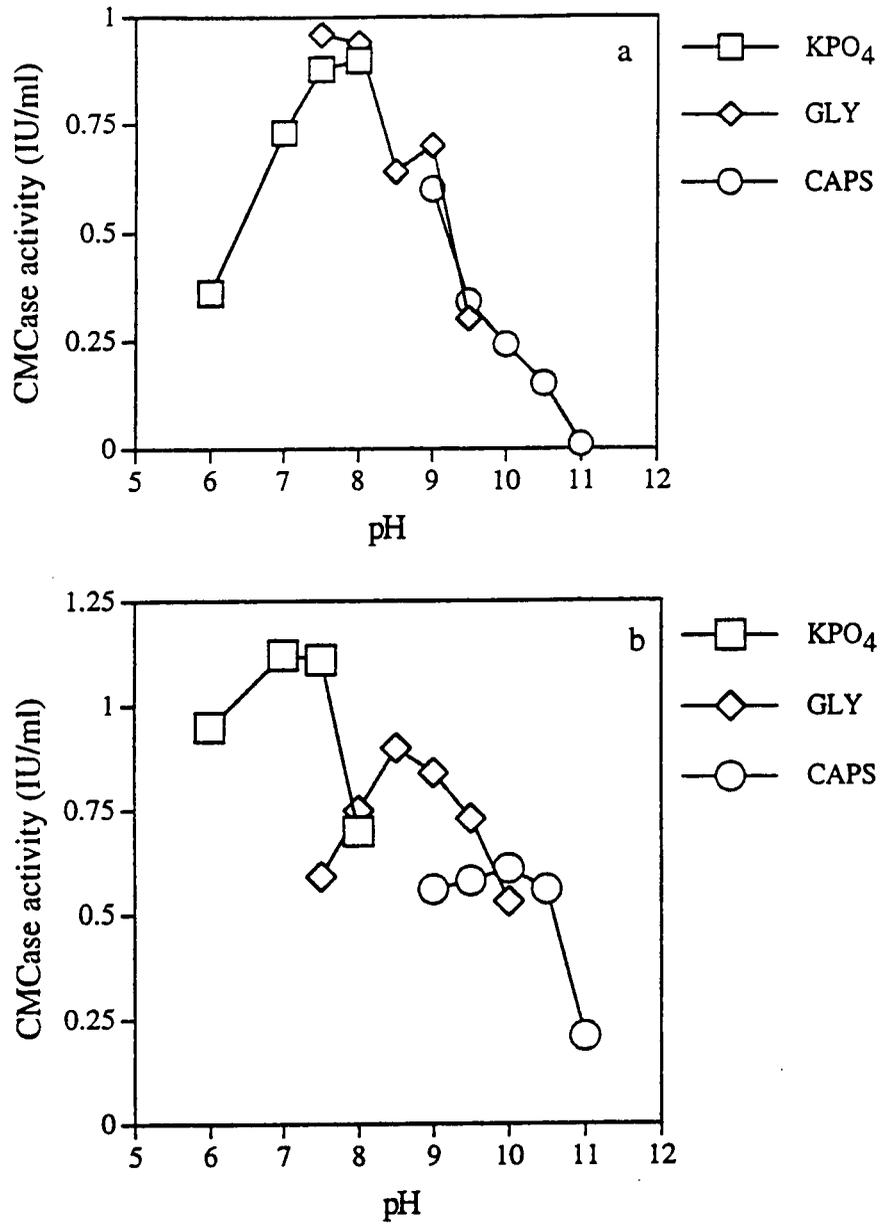


Figure 2. Effect of pH on CMCase activity of isolates 1 (a) and 2 (b). GLY is glycine buffer.

Table II. Effect of SSC pH Production of Alkaline Cellulase on Wheat Bran

<i>Fungus</i>	<i>SSC pH</i>	<i>Growth</i>	<i>CMCase (IU/mL)</i>	
			<i>pH 7.0</i>	<i>pH 8.5</i>
Isolate 1	7.0	+++	0.97	0.58
	8.0	+++	0.46	0.51
	9.0	-	0	0
	10.0	-	0	0
Isolate 2	7.0	+++	1.20	0.79
	8.0	+++	0.71	0.46
	9.0	+++	0	0
	10.0	+++	0	0

Table III. Effect of Incubation Time on Alkaline Cellulase Production

<i>Incubation</i>	<i>CMCase (IU/mL)</i>			
	<i>Isolate 1</i>		<i>Isolate 2</i>	
	<i>pH 7.0</i>	<i>pH 8.5</i>	<i>pH 7.0</i>	<i>pH 8.5</i>
(days)				
0	0	0	0	0
8	0.77	0.70	1.20	0.78
10	0.98	0.89	2.25	1.12
12	0.79	0.60	1.11	0.79
28	0.54	0.17	0.68	0.37

shown). In each isolate, CMCase activity was higher at pH 7.0 than at pH 8.5. At pH 8.5, isolate 2 had 50% more CMCase activity than isolate 1. Isolate 2 produced low amounts of FPase and high amounts of CMCase. CMCase of isolates 1 and 2 caused 19% and 29% reduction in CMC viscosity at pH 8.5, respectively.

The production of alkaline CMCase was influenced by the pH of the SSC medium. Each isolate produced higher CMCase when cultivated at pH 7.0 (Table II). Neither isolate grew on wheat bran when cultivated at pH 9 or 10. The optimum period of cultivation on wheat bran was 10 days (Table III). Increasing cultivation beyond 10 days dramatically reduced alkaline CMCase activity in both isolates. The addition of surfactants to wheat bran cultivation medium did not alter growth or production of alkaline CMCase by isolate 2 (Table IV). However, corn steep liquor was used in most of our SSC studies because it supported favorable growth and higher enzyme production.

Effect of pH, Temperature, and Reaction Time on Crude CMCase. CMCase activity was detected over a wide pH range, from pH 6 to 11. Isolate 1 had optimum activity at pH 8; optimum activity of isolate 2 ranged from pH 7 to 9 (Figure 2). Thermostability was examined by pretreating the crude enzyme at various temperatures for 30 min prior to CMCase assay. Isolate 2 was stable up to 60°C whereas isolate 1 was only stable to 50°C (Figure 3). The CMCase activity of isolate 2 was progressive for at least 30 min at pH 8.5, 50°C (Figure 4).

Electrophoretic Analysis. Isolate 2 enzyme showed a stronger clearance zone on CMC gel than did the isolate 1 enzyme (Figure 5). Hence, it was of interest to

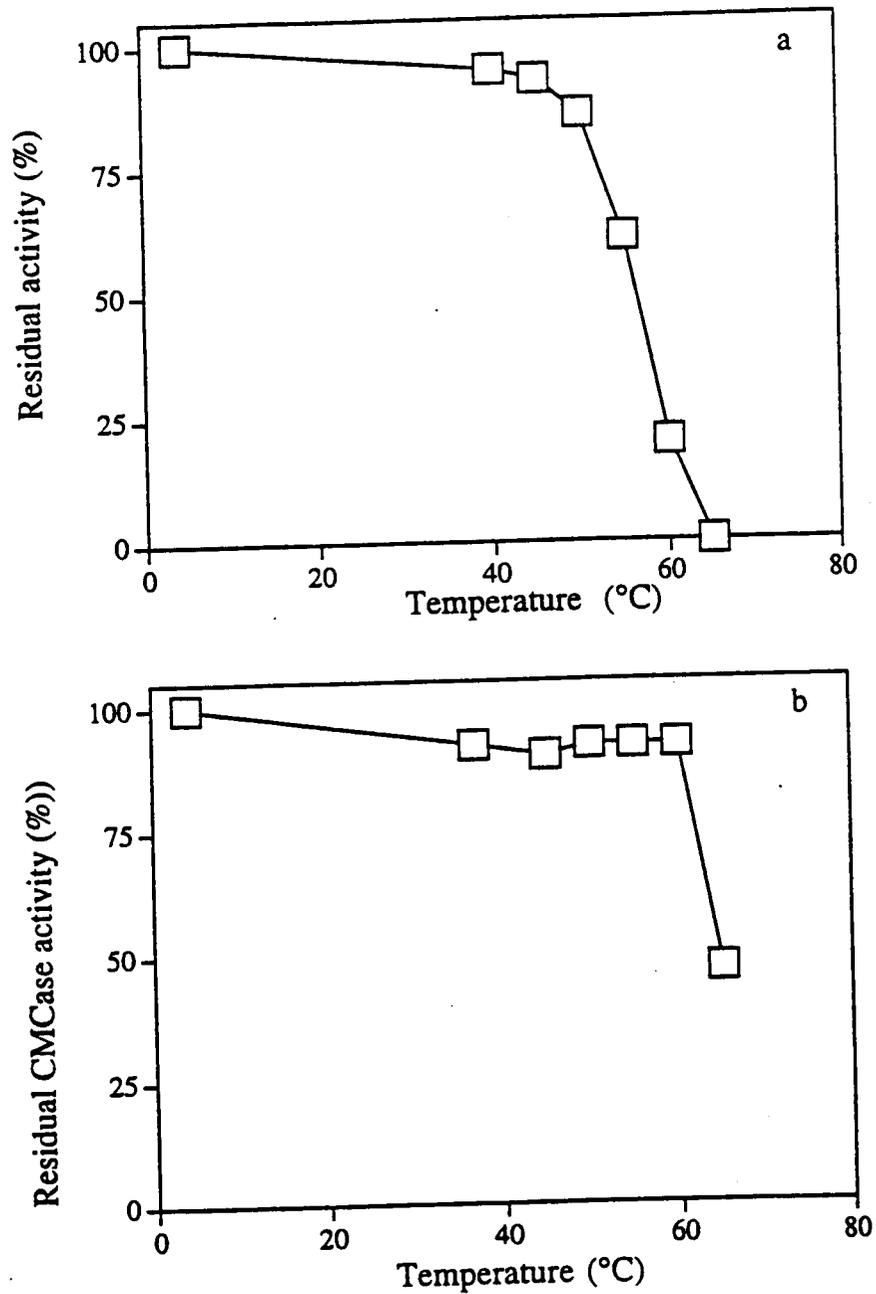


Figure 3. Thermostability of isolates 1 (a) and 2 (b). Crude enzymes were preincubated at various temperatures for 30 min prior to CMCase determination at pH 7. Residual activity is presented as a percentage of the original activity without heat treatment.

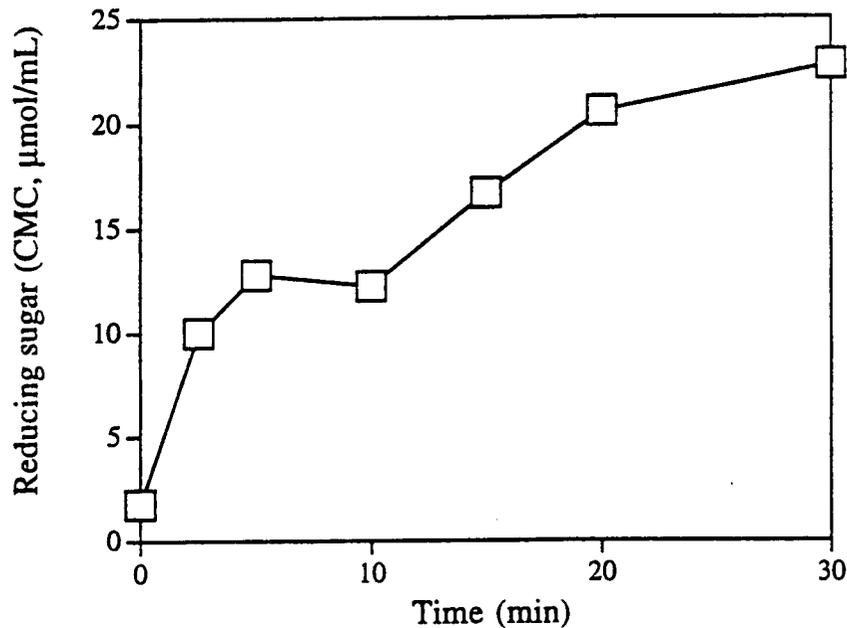


Figure 4. Effect of reaction time on CMCase activity of crude enzyme preparation from isolate 2 at pH 8.5, 50 °C.

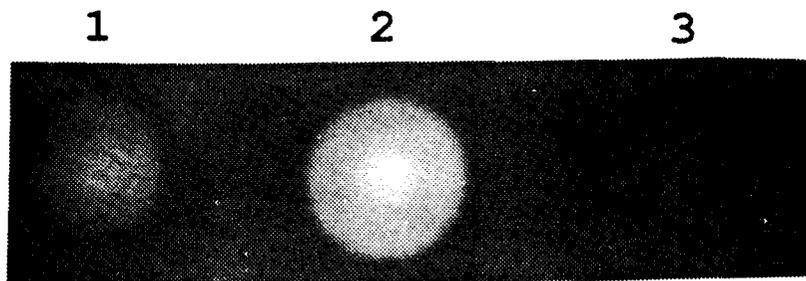


Figure 5. Effect of crude enzymes on CMC gel (pH 7.0) containing 0.1% Congo red. (1) Clearance zone shown by isolate 1 enzyme; (2) clearance zone shown by isolate 2 enzyme; (3) background effect by control sample cultivated at same condition but not inoculated with fungus.

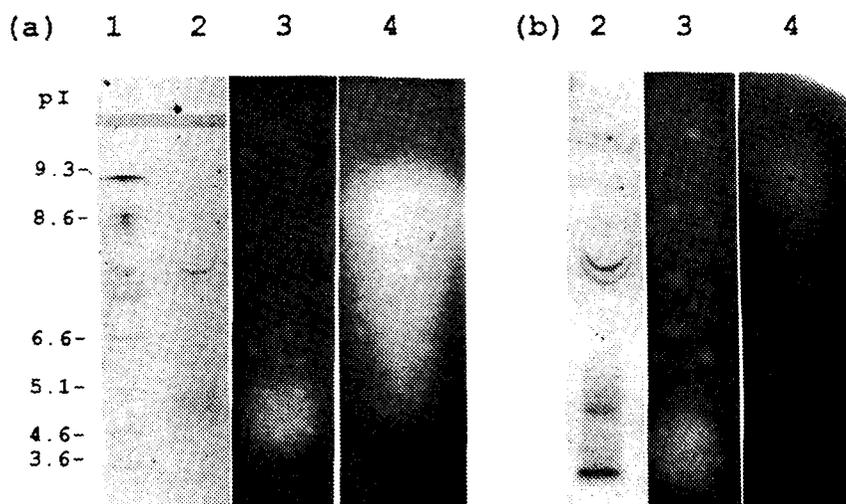


Figure 6. Isoelectric focusing (IEF) and zymogram of crude enzymes of isolate 1 (a) and isolate 2 (b). (1) Position of pI marker proteins; (2) IEF gel (pH 3–10) stained with Serva violet; (3) CMCase zymogram corresponding to lane 2; (4) xylanase zymogram corresponding to lane 2.



Figure 7. Reduction in residual ink from enzymatic deinking at pH 8.5 by SP 342 cellulase, isolate 1, and isolate 2 enzymes. Heat-killed enzyme served as control. Error bars represent standard deviation from replicate trials.

determine if multiple isozymes were formed. Isoelectric focusing and protein staining of each preparation revealed multiple protein bands in crude enzymes from both isolates (Figure 6). Zymograms showed that the pI values were 4.8 and 4.1, for the cellulases of isolates 1 and 2, respectively and 9.0 for the xylanases. Surprisingly, multiple cellulase isozymes did not seem to be present.

Table IV. Effect of Wheat Bran Surfactants on Production of Alkaline Cellulase by Isolate 2

Substrate	Growth	CMCase (IU/mL)	
		pH 7.0	pH 8.5
Wheat bran	+++	2.15	1.11
Wheat + corn liquor	+++	2.26	1.12
Wheat + Tween 80	+++	2.20	1.12
Wheat + triton X-100	+++	2.44	1.20

Effect of Deinking. Enzyme treatment of recycled wastepaper increased ink removal at pH 8.5 in the presence of surfactant (Figure 7). Enzyme from isolate 2 reduced residual ink on handsheets by about two-thirds as compared to that on heat-killed controls. At the same enzyme levels, enzyme from isolate 1 reduced residual ink count by about 50%. In comparison, cellulase from *Humicola insolens* showed no significant reduction in the ink particle count at pH 8.5 even though this fungus was proven to be very effective in pilot plant trials when the pH was adjusted to 7.9 or lower (6, 21).

Discussion

In preliminary screening work, only a few of the 43 fungal isolates cultivated on cellulose medium grew and secreted cellulolytic enzymes. In secondary screening studies, we repeatedly found that the fungal isolate 1 grew slowly on PDA plates as well as on CMC liquid medium; isolate 2 exhibited much better growth. Slow growth could be due to the presence of a growth-limiting substrate like cellulose as an organic carbon source in the fermentation medium (22) or could be intrinsic to the strain. On SSC, these isolates grew well and produced significant levels of alkaline cellulases (Table I). It should be noted that although the activity levels reported are low in comparison to the acid or neutral cellulase activities of *Trichoderma* (23) or *Humicola* (20, 21), they are reasonable when compared to levels observed with other alkaline active cellulases (6-9, 24). Typically, even the best producers of alkaline-active cellulase seldom produce more than a few international units of CMCase activity per milliliter. It is unclear whether this is due to the low turnover number of alkaline-active enzymes or to a low level of enzyme expression.

SSC is a simple technique that is easy to apply in small scale, and it has many advantages compared to submerged fermentation (25,26). SSC is particularly useful for enzyme production by fungi. Most filamentous fungi and especially basidiomycetes perform much better in solid substrate than in liquid cultivation probably because they are adapted to growth on solid surfaces. The carbon source in wheat bran, a typical solid substrate used in SSC, is primarily hemicellulose and cellulose. On the negative side, SSC is generally much more difficult to scale up than is submerged culture.

When cultivated at pH 7.0 by SSC, the fungal isolates produced higher alkaline activity on CMC compared with cultivation at pH 8.0 (Table II). Similarly, in

Bacillus maximum yield of the alkaline endo-1,4- β -glucanase was reached when the cultivation medium was adjusted to pH 7.5 and the enzyme activity decreased over pH 8.0 (24). The surfactants supplemented in SSC medium did not show any negative effect on enzyme production (27) nor did they enhance activity. Both alkaline CMCases from isolates 1 and 2 differed in their activity with regard to optimum pH and temperature (Figures 2 and 3).

The electrophoretic mobility of proteins and activity clearance on CMC gel of the isolate enzymes followed almost identical patterns. Alkaline enzyme activity on soluble CMC, viscosity reduction of CMC, and clearance zone activity on CMC gels on zymogram confirm the cellulolytic nature of the isolates. In deinking applications, the alkaline activities of the fungal cellulases are advantages because neutralization of the recycled fiber is not necessary.

Acknowledgments

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