APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1987, p. 2831–2834
0099-2240/87/122831-04$02.00/0

Vol. 53, No. 12

Some Properties of Extracellular Acetylxylan Esterase Produced by the Yeast *Rhodotorula mucilaginosa*†

HUNG LEE,‡* REBECCA J. B. TO, ROGER K. LATTA, PETER BIELY, AND HENRY SCHNEIDER
Division of Biological Sciences, National Research Council of Canada, Ottawa, Ontario, Canada K1A OR6

Received 13 April 1987/Accepted 25 August 1987

The red yeast *Rhodotorula mucilaginosa* produced an esterase that accumulated in the culture supernatant on induction with triacetin. The enzyme was specific for substrates bearing an O-acetyl group, but was relatively nonspecific for the rest of the molecule, which could consist of a phenol, a monosaccharide, a polysaccharide, or an aliphatic alcohol. The esterase was more active against acetylxylan and glucose β-D-pentaacetate than were a number of esterases from plant and animal sources, when activities on 4-nitrophenyl acetate were compared. The enzyme exhibited Michaelis-Menten kinetics and was active over a broad pH range (5.5 to 9.2), with an optimum between pH 8 and 10. In addition, the enzyme retained its activity for 2 h at 55°C. The yeast that produced the enzyme did not produce xylanase and, hence, is of interest for the production of acetylxylan esterase that is free of xylanolytic activity.

Hemicellulose is the second most abundant renewable polysaccharide in nature (14), and its microbial degradation is of fundamental importance in the natural carbon cycle. Most studies of hemicellulose biodegradation have focused on xylanases, a group of hydrolytic enzymes which depolymerize xylan (2). However, hemicelluloses in many plant species contain substituents such as arabinosyl, glucuronyl, or acetyl groups (2). Enzymes which remove these substituents, e.g., acetylxylan esterase (4), α-L-arabinofuranosidase (6, 7), and α-glucuronidase (11), have been shown to occur in bacterial or fungal cellulolytic systems. Notably, partially purified preparations of these enzymes could enhance the activity of xylanases on hemicellulosic materials (3, 6, 11). Hemicellulose-debranching enzymes have not been described in yeasts.

In studies and applications of enzymatic deacetylation of acetylxylan, it is of interest to have available a source of esterase that is free of xylanolytic activity. In this study we identify several yeasts that produce the desired esterase activity and characterize the properties of the esterase activity produced extracellularly by *Rhodotorula mucilaginosa*.

MATERIALS AND METHODS

Chemicals. The acetylated birchwood xylan was a generous gift of J. Puls (Institute of Wood Chemistry and Chemical Technology of Wood, Federal Research Center for Forestry and Forest Products, Hamburg, Federal Republic of Germany). It was obtained as a nondialyzable fraction of water-soluble, noncellulosic polysaccharide produced by steaming birch at 200°C for 10 min. It was previously characterized as containing (wt/wt) 62% D-xylose, 3.2% D-glucose, 0.3% D-mannose, and 11% acetyl (3). All other chemicals were of reagent grade and were obtained commercially.

Microorganisms. About 350 yeast strains from the culture collection of the National Research Council of Canada were screened. They included species from the following genera: *Ambrosiozyma*, *Aureobasidium*, *Brettanomyces*, *Candida*, *Cryptoccus*, *Debaryomyces*, *Dipodascus*, *Endomyces*, *Endomycopsis*, *Filobasidium*, *Geotrichum*, *Guilliermondella*, *Hanseniaspora*, *Hansenula*, *Hormoascus*, *Hyphopichia*, *Kloeckera*, *Kluyveromyces*, *Lipomyces*, *Lodderomyces*, *Metschnikowia*, *Pachysolen*, *Phaffia*, *Pichia*, *Rhodosporidium*, *Rhodotorula*, *Saccharomyces*, *Saccharomycopsis*, *Schizosaccharomyces*, *Schwanniomyces*, *Selenotila*, *Torulaspora*, *Torulopsis*, *Trichosporon*, *Trigonopsis*, *Wingea*, *Yarrowia*, and *Zygosaccahromyces*.

Screening methodology. Screening was carried out on agar plates, and glucose β-D-pentaacetate (Sigma Chemical Co., St. Louis, Mo.) was used as the substrate. The plates were prepared by layering a medium containing the acetylated sugar over a medium without the acetylated sugar. The lower layer consisted of 0.67% (wt/vol) yeast nitrogen base (YNB; Difco Laboratories, Detroit, Mich.) without amino acids, 0.2 M potassium phosphate buffer (pH 6.5), and 1.5% agar. The overlay medium contained, in addition to these components, 2% (wt/vol) glucose β-D-pentaacetate. Both media were sterilized by autoclaving. The lower-layer medium was poured into each plate (18 ml per plate). The overlay medium, which was kept at 60°C with constant stirring, was applied at 3 ml per plate after the lower layer had solidified and was spread as quickly as possible. On solidification, the top layer became turbid. With prolonged incubation, the turbidity in the overlay did not diffuse into the lower layer. Incubation was carried out at 30°C. Each plate held 20 to 24 test strains; and they were examined at 3, 5, 7, 9, and 12 days after the start of incubation. Strains with extracellular esterase activity solubilized the substrate, thereby creating clear zones around the point of inoculation. Solubilization of sugar acetyl esters by action of microbial or wheat germ esterases has been reported previously (5, 10, 12).

Growth tests in liquid media. Inocula were prepared by growing a loopful of cells from a YEPD plate in 10 ml of 0.67% YNB without amino acids and 1% glycerol in rotating test tubes (13). After 48 h, 0.1-ml volumes of the inoculum culture were added to tubes containing 10 ml of 0.67% YNB without amino acids in 200 mM potassium phosphate buffer (pH 5.5) and one of the following substrates: 1% glycerol,

* Corresponding author.
† Publication no. 28273 of the National Research Council of Canada.
‡ Present address: Department of Environmental Biology, University of Guelph, Guelph, Ontario, Canada N1G 2W1.

TABLE 1. Yeasts that showed positive response on screening with glucose β-D-pentaacetate

Yeast[a]	Esterase activity[b]	Xylanolytic activity[c]
Aureobasidium pullulans CCY 27-1-32	+ (7)	+
Aureobasidium pullulans CCY 27-1-36	+ (7)	+
Candida sp. strain ATCC 28681	W (7)	−
Candida sp. strain ATCC 20473	W (5)	−
Cryptococcus albidus CCY 17-4-1	+ (7)	+
Cryptococcus laurentii ATCC 10688	W (7)	+
Cryptococcus luteolus ATCC 32044	W (9)	+
Pichia abadieae ATCC 22263	W (12)	−
Pichia lindnerii ATCC 32658	+ (3)	−
Rhodosporidium toruloides ATCC 211008	+ (5)	−
Rhodotorula mucilaginosa NRC 211003	+ (5)	−
Trichosporon cutaneum CCY 30-5-4	+ (7)	+
Trichosporon pullulans ATCC 10677	W (7) + (12)	−

[a] Abbreviations: CCY, Czechoslovak Collection of Yeasts; ATCC, American Type Culture Collection, Rockville, Md.; NRC, National Research Council of Canada Culture Collection

[b] Abbreviations: +, positive strains that produced colonies which were 3 to 4 mm in diameter or larger; W, weakly positive strains that produced colonies which were no greater than 2 mm in diameter. Values in parentheses are the times (in days) of incubation at which activities were measured.

[c] Activities were determined by Lee et al. (8).

1% D-glucose, 1% D-xylose, 1% triacetin, 0.3% acetate, 0.3% propionate, 0.3% butyrate, 1% glucose β-D-pentaacetate, 2% cellulose propionate, or 1% acetylxylan. Growth was monitored in the tubes used for growth by measuring the optical density at 600 nm with a spectrophotometer (model 295; Coleman). In all instances tubes containing uninoculated media served as the blanks.

Preparation of extracellular enzymes. A 1-ml sample of inoculum culture was transferred to 100 ml of medium containing 0.67% YNB without amino acids and 1% glycerol in a 250-ml, loosely capped Erlenmeyer flask. The flask was shaken at 200 rpm on a gyratory shaker at 30°C (9). After 24 h, cells were harvested by centrifugation at 8,000 × *g*, washed once with distilled water, and suspended in 100 ml of medium containing 0.67% YNB without amino acids, 0.1% bovine serum albumin, and 1% triacetin in 200 mM potassium phosphate buffer (pH 5.5). After 48 to 72 h of further incubation at 200 rpm, 50 to 100 ml of the cell suspension was removed and centrifuged at 8,000 × *g*. In time course studies, cells were harvested at various times between 0 and 120 h of incubation with triacetin. The culture supernatant was concentrated 10- to 20-fold by ultrafiltration (Amicon Corp., Lexington, Mass.) by using a membrane with a molecular weight cutoff of 10,000. The retentate was dialyzed against three changes of 5 mM potassium phosphate buffer (pH 6.5) for 3 h. This enzyme preparation was divided into small fractions and stored frozen at −15°C. Activity was retained during storage under these conditions for at least 4 weeks.

Enzyme assays. Acetylxylan esterase activity was assayed by the method described by Biely et al. (4) with some modifications. A 0.1-ml solution of 5 or 10% (wt/vol) acetylxylan in 400 mM potassium phosphate buffer (pH 6.5) was mixed with an equal volume of enzyme solution and incubated at 30°C. At various times thereafter, 10-μl samples were removed, and the free acetic acid content was measured spectrophotometrically by measuring NADH formation by using an enzyme-linked assay kit in which acetyl coenzyme A synthetase, citrate synthetase, and malate dehydrogenase (Boeringer Mannheim Biochemicals, Indianapolis, Ind.) were used. Enzyme and substrate blanks were run concurrently. Identical conditions were used when other acetyl substrates were tested. When ethyl substrates were used, the activity was followed by the release of ethanol, which was determined enzymatically (1). The ethyl substrates tested consisted of 0.5% (wt/vol) ethyl esters of capric acid, caproic acid, caprylic acid, cinnamic acid, butyric acid, heptanoic acid, myristic acid, and palmitic acid. The effect of pH was studied by using phosphate solutions with pHs ranging from 3.0 to 9.2.

Acetylesterase activity was determined by the method described by Biely et al. (4) by using 4-nitrophenyl acetate as the substrate and incubating at 30°C.

Enzymes. Esterases from nonmicrobial sources tested were orange peel acetylesterase (EC 3.1.1.6), pectinesterase (EC 3.1.1.11), porcine liver esterase (EC 3.1.1.1), and electric eel acetylcholinesterase (EC 3.1.1.7). All enzymes were obtained from Sigma.

Analysis. Cell dry weight was determined by drying 20 to 50 ml of cells at 35°C to a constant weight in vacuo.

RESULTS

Yeasts that deesterify glucose β-D-pentaacetate. Seven of the strains screened showed positive responses after 3 to 7 days (Table 1). Six other strains first showed weakly positive responses at 5 to 12 days. Of these, only *Trichosporon pullulans* ATCC 10677 gave an improved response after longer incubation. Of the total of 13 strains listed in Table 1, 6 were previously shown to possess xylanolytic activity (8).

Of the esterase-positive, xylanase-negative strains, *Rhodotorula mucilaginosa* was chosen for detailed study. The others were rejected for such study for a variety of reasons. *Pichia abadieae*, *Trichosporon pullulans*, and the two *Candida* spp. gave weak responses in the screening test. The esterase produced by *Pichia lindnerii* was not active against acetylxylan, although it possessed activity against triacetin and 4-nitrophenyl acetate. *Rhodosporidium toruloides* was rejected for further study because it did not grow as well as *Rhodotorula mucilaginosa* on glycerol, a carbon source that is of interest in testing the inducibility of the enzyme. *Rhodotorula mucilaginosa* grew readily on glycerol, triacetin, acetate, D-glucose, D-xylose, and glucose β-D-pentaacetate in that the absorbance after 3 days increased from ~0.02 to at least 0.32.

Induction and properties of acetylxylan esterase from *Rhodotorula mucilaginosa* NRC 211003. Triacetin induced the production in the extracellular culture fluid of *Rhodotorula mucilaginosa* of an esterase that was active against acetylxylan. Activity was not detected in the absence of triacetin. Extracellular acetylxylan esterase activity increased rapidly with time after an initial lag period of 10 h (Fig. 1). When the induction experiment was carried out, acetic acid was found in the medium shortly after the concentrated washed cell suspension was placed in triacetin. An interpretation of this observation is that the cells grown in glycerol constitutively possess a cell-bound esterase, and that triacetin induces accumulation of the esterase of interest in the medium. The eventual decline of acetic acid was attributed to its utilization by the yeasts.

Extracellular esterase activity against acetylxylan increased linearly with protein concentration up to a concentration equivalent to 0.6 mg of cell dry weight and then increased at a lower rate (Fig. 2). The enzyme was active over a broad pH range (5.5 to 9.2, Fig. 3). The optimal pH value was between 8.0 and 10.0, and it may have been higher

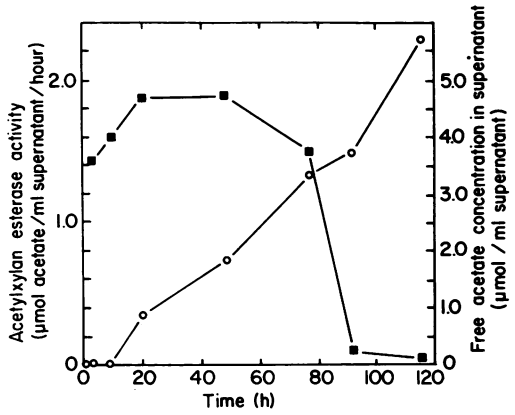

FIG. 1. Time course of induction of extracellular acetylxylan esterase activity by triacetin in *Rhodotorula mucilaginosa* NRC 211003. Symbols: ○, specific acetylxylan esterase activity; ■, acetic acid content in the triacetin medium.

at 30°C than at 40°C. To avoid complications due to nonenzymatic deesterification of substrates in alkaline pH, all enzyme assays other than those in pH dependence studies were carried out at pH 6.5. The maximum enzyme velocity (V_{max}) and the apparent Michaelis-Menten constant (K_m) determined at pH 6.5 and 30°C were 0.56 μmol of acetate released per h per ml of supernatant and 2.6% (wt/vol) acetylxylan, respectively.

The effect of assay temperature on enzyme activity is presented in Fig. 4. Catalysis did not occur at 4°C, and activity increased with rising temperature. At 22 and 30°C, the activity was linear with time. At 40 or 55°C, activity increased with time. At 70°C, the enzyme displayed high activity in the first 0.5 h, but then it decreased and was not detectable at 2 h.

Extracellular acetylxylan esterase activity was not affected by 1 mM EDTA, suggesting that metal ions are not critical to the catalytic activity. The activity was also insensitive to 1 mM phenylmethylsulfonyl fluoride, mercuric chloride, or 5,5'-dithiobis(2-nitrobenzoic acid).

The enzyme was specific for acetyl groups. Ethyl esters of carboxylic acids ranging from *n*-butyric to palmitic and ethyl cinnamate were not hydrolyzed. In contrast, a variety of O-acetyl compounds were susceptible to attack (Table 2).

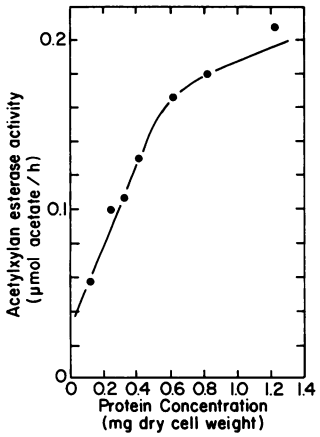

FIG. 2. Dependence of acetylxylan esterase activity on protein concentration.

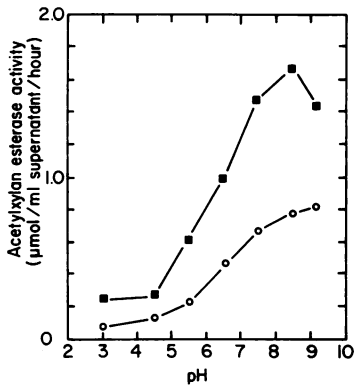

FIG. 3. Dependence of specific acetylxylan esterase activity on pH at 30°C (○) and 40°C (■).

These included a phenol, a monosaccharide, a polysaccharide, and an aliphatic alcohol. The low activity on cellulose acetate may reflect the insoluble nature of this material in aqueous solutions. The specificity of acetylxylan esterase differed considerably from that of electric eel acetylcholine esterase.

The *Rhodotorula mucilaginosa* enzyme exhibited considerable specificity against acetylxylan and glucose β-D-pentaacetate. Specific activities were greater on these substrates than on the others listed in Table 2. This was also apparent from a comparison of the activity ratios, which were computed as the quotient of specific activities on various substrates and that on 4-nitrophenyl acetate. When compared on the basis of activity ratios, the yeast enzyme was more active on acetylxylan and glucose β-D-pentaacetate than were several esterases from animal and plant sources. The activity of the yeast enzyme against acetylxylan relative to that on 4-nitrophenyl acetate was similar to that with material from *Trichoderma viride* (4).

DISCUSSION

Little is known about the functional role(s) of various substituent groups on plant hemicellulose, although they can be envisioned to provide protection against enzymatic attack by plant pathogens. In addition, substituents often introduce heterogeneity, thereby imparting certain physical or structural properties to the hemicellulose polymer. For example,

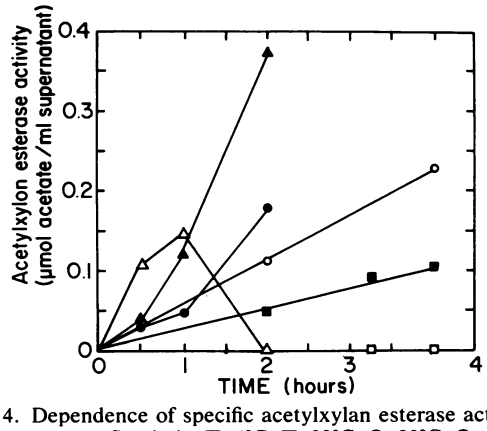

FIG. 4. Dependence of specific acetylxylan esterase activity on assay temperature. Symbols: □, 4°C; ■, 22°C; ○, 30°C; ●, 40°C; ▲, 55°C; △, 70°C.

TABLE 2. Specific activities of various esterases on different substrates and their activity ratios

Substrate	Final concn [wt/vol] (%)	Sp act of: *R. mucilaginosa* NRC 211003 esterase		Orange peel acetyl esterase		Pectin esterase		Porcine liver esterase		Electric eel acetylcholine esterase	
		V[a]	Ratio[b]	*V*	Ratio	*V*	Ratio	*V*	Ratio	*V*	Ratio
4-Nitrophenyl acetate	Saturated	0.18		16.5		10.1		2,072		612	
Acetylxylan	5	0.393	2.2	1.48	0.09	0.282	0.028	0	0	0	0
Glucose β-D-pentaacetate	0.5	0.276	1.53	8.14	0.49	2.5	0.25	7.33	0.004	ND[c]	ND
Cellulose acetate	0.5	0.011	0.006	0	0	0	0	0	0	ND	ND
Butyl acetate	0.5	0.056	0.31	0	0	0	0	1.35	0.0007	ND	ND
Amyl acetate	0.5	0.102	0.57	0.70	0.042	0.92	0.091	4.45	0.0021	ND	ND
Acetylcholine chloride	0.5	0	0	ND	ND	ND	ND	ND	ND	250	0.41

[a] *V* is enzyme velocity which is expressed as micromoles of acetate released per milligram of protein per hour for all enzymes, except *Rhodotorula mucilaginosa* NRC 211003 esterase, which is expressed in micromoles of acetate released per hour per milliliter of supernatant.
[b] Ratio = enzyme velocity on substrate examined/enzyme velocity on 4-nitrophenyl acetate.
[c] ND, Not determined.

the high degree of branching can, in part, account for the amorphous nature of hemicelluloses. Furthermore, the presence of acetyl groups in xylan contributes substantially to its high solubility in water, while deacetylation reduces solubility (2).

Our interest in acetylxylan esterase stems from its potential to aid in efforts aimed at elucidating the structural and functional roles of acetyl groups in hemicellulose. Moreover, since esterases can, in general, function reversibly, acetylxylan esterase may be of use in the synthesis of esters of hemicellulosic materials with novel properties. To achieve full utility, however, the enzymes used should be free of xylanolytic activity. Although such purification has been achieved from fungal cellulolytic systems (3), the procedures were laborious and time-consuming. The identification of a nonxylanolytic yeast *Rhodotorula mucilaginosa* which can produce an extracellular carbohydrate esterase that is active against acetylxylan may be of particular value in such investigations. It is not readily apparent what the functional role of this enzyme might be for this organism. Nevertheless, some of its catalytic properties are desirable when potential applications are considered. These properties include its thermal tolerance and broad pH spectrum.

ACKNOWLEDGMENTS

We thank K. R. Lynn for advice on esterase class enzymes and B. McGavin for typing the manuscript.

P.B. is a visiting scientist from the Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Czechoslovakia.

LITERATURE CITED

1. **Bernt, E., and I. Gutmann.** 1974. Ethanol determination with alcohol dehydrogenase and NAD. Methods Enzymatic Anal. **3**:1499–1502.
2. **Biely, P.** 1985. Microbial xylanolytic systems. Trends Biotechnol. **3**:286–290.
3. **Biely, P., C. R. MacKenzie, J. Puls, and H. Schneider.** 1986. Cooperativity of esterases and xylanases in the enzymic degradation of acetyl xylan. Bio/Technology **4**:731–733.
4. **Biely, P., J. Puls, and H. Schneider.** 1985. Acetyl xylan esterases in fungal cellulolytic systems. FEBS Lett. **186**:80–84.
5. **Fink, A. L., and G. W. Hay.** 1969. The enzymic deacetylation of esterified mono- and di-saccharides. IV. The products of esterase-catalyzed deacetylations. Can. J. Biochem. **47**:353–359.
6. **Greve, L. C., J. M. Labavitch, and R. E. Hungate.** 1984. α-L-Arabinofuranosidase from *Ruminococcus albus* 8: purification and possible role in hydrolysis of alfalfa cell wall. Appl. Environ. Microbiol. **47**:1135–1140.
7. **Kaji, A.** 1984. L-Arabinosidases. Adv. Carbohydr. Chem. Biochem. **42**:383–394.
8. **Lee, H., P. Biely, R. K. Latta, M. F. S. Barbosa, and H. Schneider.** 1986. Utilization of xylan by yeasts and its conversion to ethanol by *Pichia stipitis* strains. Appl. Environ. Microbiol. **52**:320–324.
9. **Lee, H., A. P. James, D. M. Zahab, G. Mahmourides, R. Maleszka, and H. Schneider.** 1986. Mutants of *Pachysolen tannophilus* with improved production of ethanol from D-xylose. Appl. Environ. Microbiol. **51**:1252–1258.
10. **Mandels, M., and E. T. Reese.** 1960. Induction of cellulase in fungi by cellobiose. J. Bacteriol. **79**:816–826.
11. **Puls, J., O. Schmidt, and C. Granzow.** 1987. α-Glucuronidase in two microbial xylanolytic systems. Enzyme Microb. Technol. **9**:83–88.
12. **Reuter, G., and R. Hüttner.** 1977. Physiologie und Biochemie der Streptomyceten. Z. Allg. Mikrobiol. **17**:149–151.
13. **Schneider, H., P. Y. Wang, Y. K. Chan, and R. Maleszka.** 1981. Conversion of D-xylose into ethanol by the yeast *Pachysolen tannophilus*. Biotechnol. Lett. **3**:89–92.
14. **Timell, T. E.** 1964. Wood hemicelluloses: part I. Adv. Carbohdr. Chem. **19**:247–302.