The Tolerance of Fungal Cellulase and Amylase to some Wood Preservatives

Considering the importance of protecting timber from decay there have been surprisingly few studies devoted to the mode of action of preservatives. Such fundamental data must consider the chemical structure of the toxicants, as described by BAECHLER[1] and DA COSTA and OSBORNE[2], and relate this to the metabolic interference of decay microorganisms.

In regard to the biology of preservation there might be a toxic effect on the fungal hyphae, spores, enzymes or the substrate being degraded. Thus for example, CHOU, CHANDLER and PRESTON[3] recorded the uptake of copper by hyphae and SHARP[4] measured its movement in different conditions. CHOU, PRESTON and LEVI[5] noted some hyphae in treated wood were vacuolated, coagulated and disorganized, and eventually they lysed. SOMERS[6] found copper accumulated in the spores of certain fungi. A preservative effect has been obtained by altering the substrate so that nitrogenous nutrients have been removed (SHARP[7]) and thiamine degraded (BAECHLER[8]). Copper might render cellulose unavailable by forming a complex (BELFORD and PRESTON[9]) and similarly tri-*n*-butyl tin compounds might react with hydroxyl groups at the end of the cellulose chain (RICHARDSON[10]). As regards an effect on enzymes, CHOU, PRESTON and LEVI[5] thought that the copper in copper-chrome-arsenate (CCA) mixtures was not only toxic, but also inhibited cellulase, and one author (LEVI) has unpublished data to support a partial inhibition. In contrast BRAVERY[11] found tri-*n*-butyl tin oxide (TBTO) at subtoxic levels allowed the production of extracellular enzymes when some hyphae were being denatured. Indeed LYR[12] found several preservatives, including pentachlorophenol, in low concentrations increased the enzymic activity of 2 test fungi. However, oxidized polyphenols have been demonstrated to inhibit enzymic action (LYR[13]).

As a contribution to the study of action mechanisms the activity of 2 enzymes has been recorded in the presence of some preservatives. Thus amylase and cellulase from a range of fungi have been incubated with starch and a modified cellulose respectively, and any activity, after a preservative was introduced, was determined by measuring the glucose formed over a short incubation period. Thus the in vitro tolerance of enzymes to preservatives was found.

Material and method. Enzymes were obtained in two ways. Firstly by culturing a number of fungi individually on 50 ml portions of a mineral salts medium used by KING[14] with the alteration in amounts of KH_2PO_4. 4.5 g; NH_4NO_3 3 g; and $MgSO_4$, 2 g. The solutions were adjusted to pH 5 with 4 *M* NaOH and poured into 450 *g* medical flats. Scots pine (*Pinus sylvestris* L.) sawdust of 40–80 mesh was added in 0.5 g amounts and the bottles plugged with cotton wool and autoclaved at 15 ψ for 15 min. The bottles were incubated on their sides for 30 days at 25 °C and shaken by hand every 7 days. Afterwards the solutions were filtered and centrifuged

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Table I. Amylase activity of enzyme solutions, with and without preservatives added, measured in mg glucose formed per 100 ml sample

Fungus	Untreated	% w/v of preservative solution					
		C.C.A.		Na. Penta.		T.B.T.O.	
		0.1	1.0	0.1	1.0	0.1	1.0
Enzyme from liquid culture							
Chaetomium globosum Kunze ex Fr.	10.1	10.0	10.3	11.2	19.7	16.2	18.9
Flammulina velutipes (Curt. ex Fr.) Karst	13.4	5.8	9.6	10.1	11.9	12.0	8.6
Fomes pinicola (Swartz ex Fr.) Cooke	16.5	7.7	6.4	13.8	20.3	20.5	25.2
Fusarium sp.	19.9	17.9	10.3	16.4	25.7	14.0	18.1
Heterobasidion annosum (Fr.) Bref.	11.6	11.0	8.5	15.8	13.9	16.4	12.1
Lenzites trabea (Pers.) Fr.	14.5	7.1	8.2	13.2	12.0	9.5	12.1
Penicillium sp.	26.4	11.9	11.0	7.4	13.9	12.2	10.9
Phialophora fastigiata (Lagerberg et Melin) Conant	17.3	4.6	10.3	17.4	13.9	16.4	6.3
Polyporus berkeleyi (Fr.) Bondarzew	20.5	6.9	11.2	15.8	15.9	20.1	13.6
Poria vaporaria (Fr.) sensu. Bres.	12.2	5.8	7.8	19.2	7.7	16.1	6.3
Enzyme from squeezed wood							
Botryodiplodia theobromae Pat.	47.5	23.1	5.9	17.1	15.4	32.3	39.1
Coprinus pseudoradiatus Kuhner et Josserand	13.7	6.2	5.3	8.5	12.2	19.3	23.2
Gliocladium roseum Bain	13.6	16.2	14.5	17.1	15.4	16.8	29.1
Papulaspora sp.	17.5	20.0	7.2	17.1	19.9	17.4	16.6
Sordaria fimicola (Rob.) Ces. et de Not.	8.8	15.6	19.7	12.2	16.6	15.5	6.6
Trichocladium asperum Harz	8.1	21.9	14.5	21.3	18.5	18.7	27.8

Table II. Cellulase activity of enzyme solutions, with and without preservatives added, measured in mg glucose formed per 100 ml sample

Fungus	Untreated	% w/v of preservative solution					
		C.C.A.		Na. Penta.		T.B.T.O.	
		0.1	1.0	0.1	1.0	0.1	1.0
Enzyme from liquid culture							
Aspergillus niger von Tiegh	69.7	51.7	35.8	29.6	39.2	46.3	64.9
Chaetomium globosum	63.4	70.9	58.6	41.4	59.2	58.4	78.0
Coniophora puteana (Schum. ex Fr.) Karst.	62.6	54.6	60.8	43.4	54.4	74.0	49.3
Coriolus versicolar (L. ex Fr.) Quél.	57.0	73.8	65.1	58.0	49.3	45.2	58.8
Flammulina velutipes	81.4	52.7	44.5	48.0	46.3	34.9	55.5
Fomes pinicola	35.8	48.8	67.3	36.0	36.2	70.0	34.9
Heterobasidion annosum	82.7	52.7	65.1	72.0	75.0	59.6	34.9
Polyporus berkeleyi	40.4	51.7	51.0	34.0	25.7	65.7	30.4
Poria vaporaria	60.0	60.3	56.4	46.0	68.6	26.7	51.4
Trichoderma viride Pers. ex Fr.	31.5	45.0	48.9	45.4	61.2	72.5	42.6
Enzyme from squeezed wood							
Botryodiplodia theobromae	28.4	25.0	15.8	35.5	32.7	40.1	35.9
Coprinus pseudoradiatus	21.6	24.4	10.2	34.8	18.5	31.8	29.5
Gliocladium roseum	24.7	17.5	12.7	14.6	17.9	19.1	21.2
Graphium cuneiferum (Berk. et Br.) Mason et Ellis	22.2	15.6	12.9	15.3	11.7	24.2	25.6
Humicola grisea Traaen	13.0	15.0	6.8	10.2	15.4	16.0	20.0
Papulaspora sp.	15.6	11.3	9.3	13.4	19.7	18.6	18.1
Sordaria fimicola	24.4	21.2	31.1	13.4	21.8	25.0	41.3
Trichocladium asperum	23.8	24.5	21.1	24.2	5.8	26.9	31.6

at 4,000 rpm for 5 min and the supernatant removed and stored at 4 °C.

The second method involved inoculating with different fungi 2 cm^3 of beech (*Fagus sylvatica* L.) per species after the cubes had been autoclaved in 3 ml of mineral salts used in the EGGINS and PUGH[15] cellulose medium. The cubes were incubated at 25 °C for 15 days and then compressed in a vice. The squeezed exudate containing enzymes was collected

Aliquots of 0.09 ml of the enzyme containing solutions were mixed in 15 mm diameter test tubes with 0.01 ml of 0.5 *M* sodium azide and either 0.1 ml of 0.2% w/v carboxymethyl cellulose (CMC) ('Cellofas B', grade B50, D.S. 0.78, I.C.I. Nobel division, Stevenston, Ayrshire, Scotland) or the same amount of 0.2% w/v of soluble starch; both substrates being buffered to pH 5 with 0.2 *M* sodium acetate. Either 0.1 ml of water (control) or 0.1 ml of a 0.1% and 1.0% w/v preservative solution in water was added to the untreated enzymes. The preservatives included CCA ('Tanalith CT 106' formula, Hickson's Timber Products, Castleford, Yorkshire), sodium pentachlorophenate (Na penta) and TBTO (needed to be constantly shaken with water before use). The tubes were capped with 'Suba-seal' closures (W. Freeman and Co., Suba-seal works, Staincross, Barnsley, Yorkshire) and incubated at 40 °C for 24 h. Measurements were made using the colorimetric method of WERNER, REY and WIELINGER[16] (The Boehringer Corp., Ealing, London) in which a glucose specific enzyme was used and the product assayed at a wavelength of 640 nm. No glucose was found initially in the enzyme solutions from the liquid cultures or in the reactants employed, and none was formed by the chemical breakdown of the substrates during the incubation. Some glucose was squeezed out of the wooden cubes and this was measured and deducted from the final results. The experiment was repeated and an average of results calculated after they had been converted into mg glucose per 100 ml of sample.

Results and discussion. The amounts of glucose formed with a starch substrate are presented in Table I and the amounts formed with CMC are in Table II.

With every fungus, substrate and method of obtaining enzymes there was some enzymic activity resulting in glucose formation, irrespective of the presence of a preservative. Neither concentration of the 3 preservatives prevented the amylase and cellulase from causing some degradation. Furthermore, because the assay method employs glucose oxidase it can be claimed that the preservatives, albeit in low concentrations in the last stage of assay, had no inhibitory effect on this enzyme. It thus appears that the preservatives do not act by affecting these particular enzymes and must instead have a more subtle action on fungal growth.

Such conclusions have to take into account such possibilities that the effect of the preservatives on the enzymes might be very slow or of a partial nature which cannot be determined with these techniques. They might even act selectively on enzymes not tested in this work, though fungal representatives causing all the types of wood rot known were used. The use of soluble starch and CMC might not reflect conditions in woody materials and not permit much chemical fixation within the substrate.

The lack of an enzyme inhibition by these wood preservatives suggests that solubilized toxicants act in the hyphae. SOMERS[6] thought that copper binds with protein in the cell and LEVI[17] suggested it affected the transport of nutrients and toxicants into and out of cells. Similarly pentachlorophenol and TBTO are thought to inhibit oxidative phosphorylation (LYR and ZIEGLER[18], SONE and HAGIHARA[19]). In regard to insoluble or fixed preservatives the absence of any effect on the enzymes suggests they act primarily as a physical barrier preventig the enzymes from reaching the cell wall constituent.

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Summary. Enzymes from a range of fungi have been obtained from culture filtrates and by squeezing wooden cubes in which fungi had penetrated. They were directly mixed with 2 concentrations of 3 wood preservatives and incubated with either starch or cellulose (CMC) so that any subsequent formation of glucose would indicate the in vitro enzymic tolerance to the toxicants. With every fungus, substrate and method of obtaining the enzymes there was some enzymic activity irrespective of the presence of a preservative. The implications of this tolerance for wood preservation are discussed.

R. F. SHARP[20]

Department of Botany,
Imperial College of Science and Technology,
Prince Consort Road, London, S.W.7 2BB (England),
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Sensitivity to U.V. Treatment and Nuclear Size of Mycoplasma-Like Organism Infected *Humicola* sp.

In a previous paper we reported the presence of a mycoplasma-like organism (MLO) in fungi belonging to the genus *Humicola* Traaen[1]. The MLO was successfully transmitted within some related *Humicola* strains by infecting healthy strains with ultrafiltrates of MLO carrying cultures[1]. The morphological feature of the most interesting strain (named 2–1) in which the MLO infection has been transmitted, has been described elsewhere[2]. The MLO infection greatly influenced its morphology and the infected substrain was named Strain Z-1. This anomalous morphology is actually maintained over a 2-year period of subculturing on malt agar.

On malt agar Strain Z-1 colonies, completely lacking aerial mycelium as opposed to 2-1 colonies with aerial mycelium, have a crusty aspect, and are easily distinguishable from any other *Humicola* colony.

Time after time, but at very low frequency, colony sectors very similar to the well known saltations in fungi arose on Z-1 colonies grown on malt agar (Figure 1). Colonies obtained from sectors (R-type colonies) have a white colour with abundant aerial mycelium. Under optical and electron microscope checks, MLO were still found in R-type subcultures although their frequency was noticeably lowered (unpublished data).

According to these findings, we supposed that Strain Z-1 and R-type strains represent different types of host-MLO interrelationships, their expression being dependent on physiological or biochemical characters of the microfungus, and possibly of the MLO too. As a consequence, mutagenic agents such as U.V. treatment of Strain Z-1 would increase the frequency of R-type colonies.

The present work deals with the U.V. treatment and the nuclear sizing of parental Strain 2-1, of MLO -infected Strain Z-1, and of one of R-type substrains.

Materials and methods. Strain 2-1 has been described elsewhere[2]. Strain Z-1 has been obtained by infecting Strain 2-1 with ultrafiltrates of mycelium of a MLO-carrying microfungus[1]. The colony morphology on malt extract agar (BBL, Maryland) is the following: lack of aerial mycelium; colonies rough, first yellow brown; in old cultures the mycelium becomes brown; pigment not diffused into the agar; hyphae hyaline, wide, often broken and empty, always distorted. Intercalary chlamydospores are abundantly produced, sometimes in chains. In old cultures aleuriospores are formed, first light yellow, later yellow brown, 10 µm in diameter, very irregularly shaped. At the cytomorphological level the strain differs from Strain 2-1 both in the lack of regular aleuriospores and typical phialoconidia, and the morphology of the developing mycelium (Figure 2).

Substrains of R-type have been obtained by subculturing the spontaneous sectorial mutation of Strain Z-1. Strain R-1 is characterized as follows: colonies white with aerial compact mycelium; hyphae hyaline, narrow; few intercalary chlamydospores are present. After 4–5 days aleuriospores are produced, later abundant, irregu-

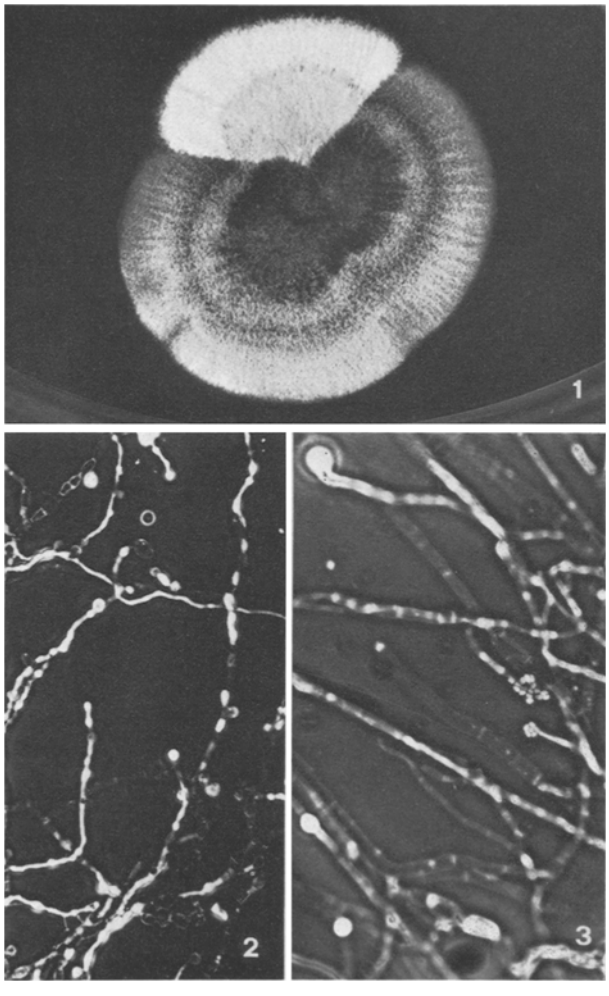

Fig. 1. Sector of R-type arizing on Strain Z-1 colony.
Fig. 2 and 3. Morphological feature of Strain Z-1 and Strain 2-1.

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