The cellulase complex of *Neurospora crassa*: activity, stability and release

M. T. YAZDI,^{1†} J. R. WOODWARD¹ and A. RADFORD^{2*}

Biotechnology Unit¹ and Department of Genetics², The University of Leeds, Leeds LS2 9JT, UK

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The temperature and pH optima, and the temperature and pH stability, of crude and purified enzymes of the cellulase complex of the cellulolytic ascomycete fungus *Neurospora crassa* were investigated. The effects of some non-ionic surfactants and fatty acids on the production/release of enzymes of the cellulase complex were also examined. For the different enzymes of the complex, activity maxima occurred between pH 4.0 and 7.0, with pH 5.0 being close to optimal for stability of all. Temperature optima for activity ranged between 45 and 65 °C, with the stability optimum between 45 and 50 °C. The presence of C₁₈ fatty acids and surfactants resulted in increased production of both endoglucanase and exoglucanase in the medium. Oleic acid was the most effective fatty acid tested, and Tween 80 the most effective surfactant. Oleic acid had no detectable effect on production of β -glucosidase, and Tween 80 actually reduced its production.

Introduction

The cellulase complex of cellulolytic fungi consists of at least three extracellular enzymes: endoglucanase (EC 3.2.1.4, endo-1,4- β -glucanase), exoglucanase (EC 3.2.1.91, 1,4- β -cellobiohydrolase) and β -glucosidase (EC 3.2.1.21). The majority of cellulase enzymes described so far have been from mesophilic bacteria and fungi. The most intensively studied cellulolytic fungus is *Trichoderma reesei* (Bisaria & Ghose, 1981). The major problems in utilizing enzymes from mesophiles for production of fermentable substrates from cellulose are the low rate of hydrolysis achieved at the optimum growth temperature (Tansey, 1971), and the relatively poor thermal stability of the enzymes (Linko, 1977). One potential solution is the use of enzymes from thermophilic fungi. Recently an exoglucanase gene has been cloned and sequenced from the thermophilic fungus *Humicola grisea* var. *thermoidea*; it shows a close similarity to the equivalent enzyme from the mesophile *T. reesei* (Azevedo & Radford, 1990). However, the higher optimum growth temperature of thermophiles is not necessarily reflected in the stability of their enzymes (Mandels, 1975).

In other species, it has been reported that surfactants and fatty acids stimulate production of the cellulase

complex (Reese & Maguire, 1969; Reese *et al.*, 1969; Shewale & Sadana, 1978; Deshpande *et al.*, 1987). The effect of fatty acids is thought to be mainly due to their incorporation into the cell membrane phospholipids, with consequent effects on cell permeability (Demain & Birnbaum, 1968). The effect of surfactants has been attributed to at least three causes: (a) action on the cell membrane causing increased permeability (Reese *et al.*, 1969); (b) promotion of the release of bound enzyme (Reese & Maguire, 1969); (c) decrease in growth rate due to reduced oxygen supply (Hulme & Stranks, 1970).

We have shown that *Neurospora crassa* is a true cellulolytic fungus capable of synthesizing and secreting high levels of the cellulase complex, and we have described optimization of induction and characterization of the enzymes (Yazdi *et al.*, 1990a, b). *N. crassa* has the advantages of being the filamentous fungus most thoroughly characterized genetically (Perkins *et al.*, 1982), and of having an efficient and well-characterized transformation system (Fincham, 1989). This paper extends our studies to the determination of pH and temperature optima for the cellulase enzymes of *N. crassa*, the stability of those enzymes, and the effects of surfactants and fatty acids on their production.

Methods

Culture conditions. Cellulase was produced by growing *N. crassa* strain *cell-1* (FGSC no. 4335) in a medium containing 2% (w/v) microcrystalline cellulose (Sigma type 20), 2% (v/v) Vogel's medium N salts solution (Vogel, 1964) and 0.75% yeast extract, at pH 7.0 and

[†] Present address: College of Pharmacy, Tehran University, Tehran, Iran.

Abbreviations: CMCase, carboxymethylcellulase ($\equiv$ endoglucanase); FPA, filter paper activity ($\equiv$ exoglucanase).

25 °C in a 5 l stirred, air-lift fermenter. After 8 d of growth, the supernate was separated by filtration (10 µm pore size). Immediately after separation, 0.5 ml l⁻¹ of 50 mM-phenylmethylsulphonyl fluoride (PMSF) in propan-2-ol and 0.1 ml l⁻¹ of 20 mM-N-tosyl-L-phenylalanine-chloromethyl ketone (TPCK) in propan-2-ol were added to inhibit protease activity. The enzymes were purified from the supernatant as described previously (Yazdi *et al.*, 1990 b).

Fatty acids and surfactants were added to flasks before autoclaving at 15 p.s.i. (103.5 kPa) for 15 min. Flasks were inoculated with 0.5 ml volumes of conidial suspension (about 10⁷ conidia) made up in sterile distilled water. Cultures were grown in an orbital incubator, at 25 °C and 200 r.p.m., for 12 d.

Surfactants and fatty acids. The surfactants used were Tween 20 [polyoxyethylene (20) sorbitan monopalmitate], Tween 80 [polyoxyethylene (20) sorbitan monooleate], 'poly 10' [polyoxyethylene oleic acid (*cis*-9-octadecanoic acid), stearic acid (octadecanoic acid), X-100 (a proprietary polyoxyethylene ether), all obtained from Sigma. The fatty acids were capric acid (decanoic acid), myristic acid (tetradecanoic acid), linoleic acid (*cis*-9,*cis*-12-octadecadienoic acid), oleic acid (*cis*-9-octadecanoic acid), stearic acid (octadecanoic acid), and arachidonic acid (5,8,11,14-eicosatetraenoic acid), also from Sigma.

Enzyme assays. Activities of the three component types of the cellulase complex, endoglucanase (carboxymethylcellulase, CMCase), exoglucanase (filter paper activity, FPA) and β-glucosidase were assayed as described by Yazdi *et al.*, (1990a). All assays were performed in duplicate. Enzyme activities are expressed in units, defined as µmol product liberated by the enzyme in 1 ml of medium in either 15 min (for endoglucanase and β-glucosidase) or 60 min (for exoglucanase). Mycelial biomass was measured by the method of Margaritis & Merchant (1986), and soluble protein by that of Bradford (1976).

For determination of temperature optima for activity, incubations were carried out at temperatures over the range 30–70 °C. For determination of pH optima, the enzymes were dissolved in distilled water, and mixed with equal volumes of 200 mM-phosphate/citrate buffer over the range pH 3.0–9.0 prior to assay.

For enzyme thermal stability determination, enzyme preparations in 100 mM-phosphate/citrate buffer at pH 5.0 were incubated over the range 30–60 °C, samples being taken periodically for assay. For pH stability determination, enzyme solutions in 100 mM-phosphate/citrate buffer at pH values between 3.0 and 8.0 were incubated over the range 30–60 °C. In all stability determinations, the maximum incubation time was 48 h.

SDS-PAGE. Exported proteins were precipitated in acetone,

centrifuged, and redissolved in 100 mM-citrate buffer at pH 5.0 to a concentration of 5–20 mg ml⁻¹. Gel electrophoresis was in 10% (w/v) polyacrylamide slab gels according to the method of Hames (1986). SDS and mercaptoethanol were used for denaturation.

Results

Optimum temperatures for crude and purified enzymes

Under the assay conditions used, the activity of β-glucosidase was optimal, in both crude and purified preparations, at 65 °C. Endoglucanase activity in crude preparations showed high activity over a wide range of temperature between 45 and 55 °C, with the optimum at 55 °C. Exoglucanase in crude preparations gave high activity between 45 and 55 °C, with the optimum above 50 °C. Results are shown in Fig. 1(a).

The endo- and exoglucanase activities were both purified into their different isoenzymes, and each isoenzyme was found to have a distinct temperature profile. Among the endoglucanases, the activity profiles of which are given in Fig. 1(b), the optima were: endo-I, 55 °C; endo-II, 55 °C; endo-III, 50 °C; and endo-IV, 45 °C. The sum of these profiles is consistent with the very wide activity range of the crude extract. Activity ranges of the exoglucanases are shown in Fig. 1(c); the optima were: exo-I, 50 °C; exo-II, 50 °C; and exo-III, 55 °C. The temperature optimum for the purified β-glucosidase was initially 65 °C as in the crude preparation, but decreased to 60 °C after 30 min.

These temperature optima for the *N. crassa* cellulase complex enzymes are compared with those of other cellulolytic organisms, both mesophiles and thermophiles, in Table 1. *N. crassa* β-glucosidase was comparable with the best of both mesophiles and thermophiles. *N. crassa* exoglucanases were comparable with those of other mesophiles. However, the *N. crassa* endoglucanases had comparatively low optima compared with those of the other species shown.

Fig. 1. Effect of temperature on the activity of cellulases from *N. crassa*. (a) Enzymes in crude preparation (■, CMCase; □, FPA; ●, β-glucosidase). (b) Purified endoglucanases (■, endo-I; □, endo-II; ▲, endo-III; ●, endo-IV). (c) Purified exoglucanases (■, exo-I; □, exo-II; ●, exo-III).

Fig. 2. Effect of pH on activity of cellulases from *N. crassa*. (a) Enzymes in crude preparation (■, CMCase; □, FPA; ●, β -glucosidase). (b) Purified endoglucanases (■, endo-I; □, endo-II; ▲, endo-III; ●, endo-IV). (c) Purified exoglucanases (■, exo-I; □, exo-II; ●, exo-III).

Table 1. Comparison of cellulolytic enzymes from *N. crassa* and other species

Species	Optimal temp. for activity			Ref.*
	CMCase	FPA	β -Glucosidase	
<i>Neurospora crassa</i>	55	55	65	
<i>Trichoderma reesei</i>	60	55	65	1
<i>Penicillium</i> sp.	55	55	60	1
<i>Thielavia terrestris</i>	60	60	67	1
<i>Sporotrichum cellulophilum</i>	60	60	60	1
<i>Thermoactinomyces</i> sp.	70	65	55	2
<i>Chaetomium cellulolyticum</i>	55		55	3
<i>Thermoascus aurantiacus</i>	60	60	70	4

* References: 1, Durand *et al.* (1984); 2, Margaritis & Merchant (1983); 3, Fahnrich & Irrang (1982); 4, Tong *et al.* (1980).

Thermal stability of the cellulolytic enzymes

The total *N. crassa* endoglucanase activity in crude preparations was fairly stable over 48 h at temperatures up to 50 °C. At the temperature optimal for activity, approximately 20% of activity was lost over the first 12 h, following which the level stabilized. The exoglucanase activity was fairly stable over 48 h at temperatures up to 45 °C, but declined significantly at higher temperatures, especially over the first 6 h, dropping to approximately 70% at 50 °C, 60% at 55 °C and 40% at 60 °C, after which it remained fairly stable in each case. With the multiplicity of isoenzymes for both endo- and exoglucanases, at least four of the former and three of the latter, the results are readily explicable. In each class, the multiphasic stability curve reflects the sum of activities of isoenzymes of different stabilities. β -Glucosidase activity was fairly stable over 48 h at temperatures up to 50 °C, but was sensitive to higher temperatures, losing 80% of activity progressively over 36 h at 55 °C and all activity after 24 h at 60 °C.

Although *N. crassa* has low temperature requirements for its production of the cellulase complex, in the range

23–25 °C (Yazdi *et al.*, 1990a), thermal stability of these enzymes is certainly comparable to other mesophilic fungi (Coughlan, 1985; Durand *et al.*, 1984; Margaritis & Merchant, 1983). The half-life for its endo- and exoglucanases and β -glucosidase at 50 °C was in excess of 48 h.

pH optima of crude and purified enzymes

The optimal pH values for *N. crassa* cellulases were in the range 4.5–5.5, with pH 4.5 optimal for crude endoglucanase, pH 5.0 for exoglucanase and pH 5.5 for β -glucosidase (Fig. 2a). For purified endoglucanases, optima were: endo-I, pH 4.0; endo-II, pH 4.0; endo-III, pH 4.5; and endo-IV, pH 6.0 (Fig. 2b). For the exoglucanases, pH optima for the purified enzymes were: exo-I, pH 7.0; exo-II, pH 6.0; and exo-III, pH 4.0 (Fig. 2c).

Combined pH and temperature effects on enzyme stability

The stability of the *N. crassa* β -glucosidase and exoglucanase activities at temperatures up to 37 °C was

Table 2. Effect of various surfactants on extracellular levels of enzymes of the cellulase complex

None of these additives had a significant effect on growth

Additive	Exoglucanase		Endoglucanase		β -Glucosidase	
	U ml ⁻¹	% of control	U ml ⁻¹	% of control	U ml ⁻¹	% of control
None	75	100	305	100	5.6	100
Tween 20 (0.1%)	32	42	230	75	2.5	44
Tween 20 (0.2%)	75	100	310	101	3.6	65
Tween 80 (0.2%)	110	147	350	115	3.3	59
Brij 35 (0.1%)	84	112	320	105	4.2	75
Brij 35 (0.2%)	95	125	350	115	3.9	69

virtually independent of pH: the enzymes retained more than 90% of activity at any pH in the range 3.5–9.0. Endoglucanase stability was pH-dependent even at low temperature, with optimum stability in the range pH 4.5–7.0.

At temperatures at or above 45 °C, the stability of all enzymes in the cellulase complex was pH-dependent. For endoglucanase activity at 50 °C, stability was optimal at pH 5.0, but over the range pH 4.0–7.0, about 70% of the activity was retained after 36 h, falling to 40% at pH 8.0. For exoglucanase activity at 55 °C, 60% of activity remained at pH 4.0–5.0 after 36 h, but this fell with increasing pH to only 25% at pH 8.0. For β -glucosidase at 50 °C, 36 h activities were 90% at pH 5.0, 75% at pH 4.0 and 55% at pH 6.0. At pH 7.0, all β -glucosidase activity was lost after 24 h.

Effect of surfactants and fatty acids

Of all the surfactants and fatty acids tested, only Triton X-100 and 'poly 10', both at 0.1% had any inhibitory effect on growth of the mycelium. For all other compounds, growth was quantitatively and qualitatively normal.

The effect of addition of surfactants on the production of the cellulase complex enzymes by *N. crassa* is shown in Table 2. Addition of Tween 80 gave the greatest improvement in production of extracellular exoglucanase. Similar levels of both Tween 80 and Brij 35 gave similar increases in endoglucanase production. All the surfactants tested sharply decreased β -glucosidase production.

To confirm that the influence of the surfactants on activity of the components of the cellulase complex was due to a direct effect on levels of synthesis of the enzymes, the supernate proteins were analysed by SDS-PAGE; a densitometer tracing is shown for both control and Tween-80-treated cultures in Fig. 3. With Tween 80, a 76 kDa peak (β -glucosidase) was reduced to a shoulder as the activity of β -glucosidase was reduced by 41%, and

Fig. 3. Densitometer tracings of SDS-PAGE gels of exported proteins of control culture and cultures grown with the addition of 0.2% Tween 80 or 0.2% oleic acid. Locations of molecular mass markers and known enzymes of the cellulase complex are indicated.

an increase in the 69 kDa peak (exo-I) correlated with a 47% increase in exoglucanase. Tween 80 also induced a band change not directly correlated with the size of any known member of the cellulase complex, an extra protein peak at 27 kDa. The effect of Tween 80 concentration was also investigated. A concentration of 2% (v/v) was optimal for exoglucanase production, and 0.3–0.5% for endoglucanase production.

Addition of a variety of fatty acids at a concentration of 0.2% was investigated with respect to possible effects on production of the cellulase complex. The fatty acids

Table 3. *Effect of various fatty acids on extracellular levels of the enzymes of the cellulase complex*

None of these additives had a significant effect on growth

Additive (0.2%)	Exoglucanase		Endoglucanase		β -Glucosidase	
	U ml ⁻¹	% of control	U ml ⁻¹	% of control	U ml ⁻¹	% of control
None	75	100	305	100	5.6	100
Capric	38	49	190	65	4.0	70
Myristic	29	39	95	31	3.1	56
Linoleic	90	120	300	98	9.8	175
Oleic	116	154	390	128	5.5	98
Stearic	94	125	425	140	3.6	64
Arachidonic	25	33	105	35	1.2	22

and their carbon backbone lengths were capric acid (C₁₀), myristic acid (C₁₄), linoleic, oleic and stearic acids (all C₁₈), and arachidonic acid (C₂₀). The results are given in Table 3. In general only the C₁₈ fatty acids gave any increase in production of endo- and exoglucanase activities, the best being a 50% increase in exoglucanase with oleic acid and a 40% increase in endoglucanase with stearic acid. All of the fatty acids, with the exception of linoleic acid which produced a 75% increase, and oleic acid which had no significant effect, produced a decrease in β -glucosidase activity. The effect of oleic acid concentration was investigated: concentrations of about 0.2% were optimal for production of the complex.

The effects of these fatty acids on protein concentrations in the supernate were investigated by SDS-PAGE, and a densitometer tracing of the results for oleic acid is shown in Fig. 3. As with the surfactant Tween 80, there were marked changes in level of a limited number of specific protein bands. With oleic acid there were changes in the sizes of peaks correlated with known enzymes of the cellulase complex, an increase in the 72 kDa peak (the known size of exo-II and exo-III) with oleic acid correlating with a 54% increase in exoglucanase activity, and an increase in the 47 kDa peak (endo-I) correlating with a 28% increase in endoglucanase activity. There was no change in either β -glucosidase or the corresponding 76 kDa peak. There were also alterations in peaks not known to be directly correlated with members of the cellulase complex, the main ones being an increase in the peaks at about 110, 42 and 24 kDa, and a reduced peak at 28 kDa.

Addition of any of the fatty acids or detergents, at concentrations used in any of the experiments described, directly to the enzyme preparations had no effect on their activity.

Discussion

The study of pH and temperature characteristics of the *N. crassa* cellulase complex has confirmed the isoenzymes postulated earlier (Yazdi *et al.*, 1990b), as the four

endoglucanases and three exoglucanases defined initially on the basis of molecular mass and extent of glycosylation have now been shown to be distinct also in pH and temperature activity optima and stabilities. The present data on β -glucosidase are consistent with the single enzyme species postulated earlier.

The three activity classes of the *N. crassa* cellulase complex show a wide pH range and a fairly high optimum temperature for activity, and good thermostability.

It is clear from the results presented that, under certain conditions, some fatty acids and surfactants elicit an increase in the amounts of certain of the enzymes of the cellulase complex in the medium. The effect of a surfactant such as Tween 80, and of those positively acting fatty acids, may be due to an increase in the permeability of the cell membrane, thus permitting more of the enzymes to be secreted, as postulated for other species by Reese & Maguire (1969) and Demain & Birnbaum (1968). However, this would not explain the dramatic effect of the addition of linoleic acid on β -glucosidase activity.

The positive effect on cellulase production by oleic acid and Tween 80 was only found on endo- and exoglucanases, and Tween 80 actually decreased the β -glucosidase level. This may imply some difference in the means by which the two glucanases and β -glucosidase are released. In most organisms studied, β -glucosidase is an intracellular enzyme, released only by autolysis of the producing cells (Gong & Tsao, 1979). Substrates such as cellobiose are soluble and potentially transportable, and so do not require enzymes for their utilization to be extracellular.

Rather than oleic acid and Tween 80 having a depressive effect on autolysis, the increase in exo- and endoglucanase but not β -glucosidase could be explained by changes in membrane fluidity, as the number of double bonds present in a fatty acid determines its melting point, and hence has an effect on membrane fluidity.

In general, changing the fluidity of the membrane is seen to substantially affect the passage of ions and small molecules through their appropriate transport systems. There has been little work on the effect of these changes in membrane content on protein translocation across the membrane. However, the presence of detergents and changes in fatty acid composition clearly perturb the membrane composition and fluidity, and physical as well as biochemical parameters of membrane function. The selective increase in secretion of some of the cellulase complex enzymes might well be explained by subtle changes in those parts of the membrane which are normally fluid. The selective nature of this secretion suggests that protein secretion is intimately linked to membrane lipid composition. The observation that certain non-cellulase protein peaks also change under the influence of surfactants and fatty acids, while certain members of the cellulase complex do not, is evidence that their effect is not specifically on the cellulase complex, but on protein secretion in general.

The effect of changes in membrane lipid components on the activity of membrane-bound proteins is well known (Hunter & Rose, 1972; Rattray *et al.*, 1975). Many authors have noted changes in the transport of amino acids and sugars associated with changes in cell membrane composition. The most dramatic of these effects is the death of *Saccharomyces cerevisiae* mutants incapable of synthesizing inositol, and hence phosphatidylinositol, under which conditions the cell membrane becomes permeable to cell constituents (Ulaszewski *et al.*, 1978). Fatty acid starvation of yeasts also causes cell death (Henry, 1973). Similar effects have been reported in *N. crassa* (Statkin & Tatum, 1961; Sullivan & DeBusk, 1973). Less dramatic effects on the alteration of membrane lipid composition were reported in *Fusarium* by Miller & Bairan (1984). These authors also noted increases in permeability to water and amino acids on addition of low concentrations of cationic detergents.

It is possible that the secretion mechanism differs between the various enzymes of the cellulase complex. For example, oleic acid has the greatest effect on exoglucanase activity in the medium, stearic acid on endoglucanase, and linoleic acid on β -glucosidase. This means that a membrane rich in stearic acid, having more rigidity and less fluidity, might favour endoglucanase secretion. Conversely, the most fluid membrane, with an enhanced linoleic acid content, might favour β -glucosidase secretion. The effect of Tween 80 would be similar to that of the fatty acids, effecting changes in the cell membrane. It seems unlikely that the effects noted here are due to changes in the growth patterns of the fungus, especially as there was no change in the overall growth rate of the mycelium on addition of any of these compounds.

The negative effect of higher concentrations of Tween 80 and oleic acid becomes detectable at levels where the surfactant or fatty acid could well provide the major carbon source for the organism, thus providing a possible indirect mode of action via carbon catabolite repression.

References

- AZEVEDO, M. DE O. & RADFORD, A. (1990). Sequence of *cbh-1* gene of *Humicola grisea* var. *thermoidea*. *Nucleic Acids Research* **18**, 668.
- BISARIA, V. S. & GHOSH, T. K. (1981). Biodegradation of cellulosic material: substrates, microorganisms, enzymes and products. *Enzyme and Microbial Technology* **3**, 90–104.
- BRADFORD, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248–254.
- COUGHLAN, M. P. (1985). The properties of fungal and bacterial cellulases with comment on their production and application. *Biotechnology and Bioengineering Reviews* **3**, 39–109.
- DEMAIN, A. L. & BIRNBAUM, J. (1968). Alteration of permeability for the release of metabolites from microbial cells. *Current Topics in Microbiology and Immunology* **46**, 1–25.
- DESHPANDE, M. V., SRINAVASAN, M. C. & DESHMAKH, S. S. (1987). Effect of fatty acids on cellulase production by *Penicillium funiculosum* and its mutants. *Biotechnology Letters* **9**, 301–304.
- DURAND, H., SOUCAILLE, P. & TIRABY, G. (1984). Comparative study of cellulases and hemicellulases from four fungi. *Enzyme and Microbial Technology* **6**, 175–180.
- FAHRNICH, P. & IRRANG, K. (1982). Some characteristics of the cellulolytic enzyme system of *Chaetomium cellulolyticum*. *Biotechnology Letters* **4**, 519–524.
- FINCHAM, J. R. S. (1989). Transformation in fungi. *Microbiological Reviews* **53**, 148–170.
- GONG, C. S. & TSAO, G. T. (1979). Cellulase biosynthesis and regulation. *Annual Reports on Fermentation Processes* **3**, 111–140.
- HAMES, B. D. (1986). In *Gel Electrophoresis of Proteins – a Practical Approach*, pp. 1–86. Edited by B. D. Hames & D. Rickwood. Oxford: IRL Press.
- HENRY, S. (1973). Death resulting from fatty acid starvation in yeast. *Journal of Bacteriology* **136**, 49–54.
- HULME, M. A. & STRANKS, D. W. (1970). Induction and the regulation of production of cellulase by fungi. *Nature, London* **226**, 469–470.
- HUNTER, K. & ROSE, A. H. (1972). Lipid composition of *Saccharomyces cerevisiae* as influenced by growth temperature. *Biochimica et Biophysica Acta* **260**, 639–653.
- LINKO, M. (1977). An evaluation of enzymatic hydrolysis of cellulosic materials. *Advances in Biochemical Engineering* **5**, 25–48.
- MANDELS, M. (1975). Microbial sources of cellulase. *Biotechnology and Bioengineering Symposium* **5**, 81–105.
- MARGARITIS, A. & MERCHANT, R. F. (1983). Production and thermal stability characteristics of cellulase and xylanase enzymes from *Thielavia terrestris*. *Biotechnology and Bioengineering Symposium* **13**, 299–314.
- MARGARITIS, A. & MERCHANT, R. F. (1986). Optimisation of fermentation conditions for thermostable cellulase production by *Thielavia terrestris*. *Journal of Industrial Microbiology* **1**, 149–156.
- MILLER, R. W. & BAIRAN, L. R. (1984). Control of membrane fluidity in *Fusarium*. *Biomembranes* **12**, 433–450.
- PERKINS, D. D., RADFORD, A., NEWMAYER, D. & BJÖRKMAN, M. (1982). Chromosomal loci of *Neurospora crassa*. *Microbiological Reviews* **46**, 426–570.
- RATTRAY, J. B. M., SCHIHECI, A. & KIDBY, D. K. (1975). Lipids of yeasts. *Bacteriological Reviews* **39**, 197–231.
- REESE, E. T. & MAGUIRE, A. (1969). Surfactants as stimulants of enzyme production by microorganisms. *Applied Microbiology* **17**, 242–245.

- SHEWALE, J. G. & SADANA, J. C. (1978). Cellulase and β -glucosidase production by basidiomycete species. *Canadian Journal of Microbiology* **24**, 1204–1216.
- STATKIN, A. J. & TATUM, E. L. (1961). The relationship of *m*-inositol to morphology in *Neurospora crassa*. *American Journal of Botany* **48**, 760–771.
- SULLIVAN, J. L. & DEBUSK, A. G. (1973). Inositolless death in *Neurospora* and cellular ageing. *Nature New Biology* **243**, 72–74.
- TANSEY, M. R. (1971). Agar diffusion assay for cellulolytic ability of thermophilic fungi. *Archives of Microbiology* **77**, 1–11.
- TONG, C. C., COLE, A. L. & SHEPHERD, M. G. (1980). Purification and properties of cellulase from the thermophilic fungus *Thermoascus aurantiacus*. *Biochemical Journal* **191**, 83–94.
- ULASZEWSKI, S., WOODWARD, J. R. & CIRILLO, V. P. (1978). Membrane damage associated with inositol-less death in *Saccharomyces cerevisiae*. *Journal of Bacteriology* **136**, 49–54.
- VOGEL, H. J. (1964). Distribution of lysine pathways among fungi: evolutionary implications. *American Naturalist* **98**, 435–446.
- YAZDI, M. T., WOODWARD, J. R. & RADFORD, A. (1990*a*). Cellulase production by *Neurospora crassa*: induction and optimisation of the enzyme complex. *Enzyme and Microbial Technology* **12**, 116–119.
- YAZDI, M. T., RADFORD, A., KEEN, J. N. & WOODWARD, J. R. (1990*b*). Cellulase production by *Neurospora crassa*. Purification and characterisation of cellulolytic enzymes. *Enzyme and Microbial Technology* **12**, 120–123.