Trans. Br. mycol. Soc. **90** (4), 577–583 (1988) *Printed in Great Britain*

EFFECT OF POLYMERIC ADDITIVES, ESPECIALLY JUNLON AND HOSTACERIN, ON GROWTH OF SOME BASIDIOMYCETES IN SUBMERGED CULTURE

BY PENELOPE JONES, BATOOL A. SHAHAB, A. P. J. TRINCI AND D. MOORE

Microbiology Research Group, Department of Cell and Structural Biology, Williamson Building, School of Biological Sciences, The University, Manchester M13 9PL

Six species of the Agaricaceae, Coprinaceae, Cortinariaceae and Polyporaceae were caused to grow as finely divided mycelial suspensions in submerged culture by inclusion in the medium of 0·1–0·2 % (w/v) polyacrylic acid or sodium polyacrylate. In the absence of polyacrylates, liquid cultures tended to produce large mycelial clumps whereas cultures containing polymer largely grew as dispersed hyphal filaments (*Phanerochaete chrysosporium*, *Phlebia radiata* and *Phlebia gigantea*) or formed numerous minute pellets (*Bjerkandera adusta*, *Coprinus cinereus* and *Pleurotus ostreatus*). Other polyacrylate salts and acrylamides were less effective in preventing mycelial aggregation and other polymers including alginate derivatives, sorbitans, cellulose and modified celluloses, carrageenan, polyvinyl alcohols and quaternary ammonium compounds were ineffective in promoting filamentous growth. Biomass yields were significantly increased by inclusion of Junlon PW110 (polyacrylic acid) or Hostacerin (sodium polyacrylate) in the medium; yield was doubled in many cases. Increases in yield were probably due to the filamentous cultures maintaining exponential growth for a longer period than when aggregates were formed but growth rates were also improved in medium containing the polymers. The optimum concentration of polymer depends on the species and the constitution of the growth medium; modification of the medium enabled *C. cinereus* to be grown with dispersed filamentous growth both in shake-flask and fermenter cultures.

Despite the length of time during which mushroom fruit bodies of higher basidiomycetes have been cultivated and collected for food, this group of organisms has made remarkably little contribution to other aspects of biotechnology. Yet their appearance as the climax of so many successions, the complexity of their reproductive structures, the wide range of toxins and psychotropic and other agents which have been found in fruit bodies and their ecological activities (which include the ability to degrade lignin) all suggest aspects of basidiomycete biology which could be exploited commercially. However, as far as we are aware, none of these opportunities have been realized. It is difficult to understand why this should be so.

One possible reason is that, like other fungi with filamentous growth, very heterogeneous cultures are formed in submerged culture. The majority of fungi form globose pellets in shaken submerged culture (Burkholder & Sinnot, 1945) and basidiomycetes probably have a greater tendency than other fungi to form large mycelial aggregates even (perhaps especially) in agitated liquid culture. Cellular activities differ in such aggregates because there is only a thin outer layer of growing hyphae; the inner cells suffer oxygen deprivation (Pirt, 1966), the dissolved oxygen tension approaching zero within about 150 μm of the surface of the pellet (Wittler *et al.*, 1986). The respiration rate declines with increase in size of pellets of *Lentinus* (= *Lentinula*) *edodes* grown in submerged culture (Yoshida *et al.*, 1967) and other biochemical processes must also be affected. Pellets formed by *Penicillium chrysogenum* have a layered internal structure, the central mass, even of pellets only about 2 mm diam, showing evidence of hyphal disintegration (Camici, Sermonti & Chain, 1952; Wittler *et al.*, 1986). Thus, when aggregation occurs the whole culture becomes morphologically, physiologically and biochemically very heterogeneous, and consequently very difficult to study, monitor or control (Trinci & Thurston, 1976).

Although various factors influence the degree of aggregation of fungal hyphae in agitated liquids, many workers have found that more homogeneous cultures could be obtained when some sort of polymer was added to the fermentation medium (Metz & Kossen, 1977). Most work on this has been done with the ascomycete *Aspergillus niger*. Natural (alginate, starch, dextran), modified (carboxymethylcellulose) and synthetic (carboxypolymethylene, polyvinyl pyrrolidone) polymers have

all been used. Elmayergi & Scharer (1973) found that addition to the medium of 0·3 % (w/v) of the anionic synthetic carboxypolymethylene polymer Carbopol caused significant enhancement of the growth rate of *A. niger*, and the rate of amylase production. Trinci (1983), also working with *A. niger* but this time with the anionic polyacrylic acid polymer Junlon at 0·2 % (w/v), observed a change in growth form. In the presence of Junlon the mycelium was dispersed and filamentous instead of being aggregated into pellets as it was in the absence of Junlon.

In the study reported here we have extended the observations to a range of basidiomycetes and demonstrated that Junlon is especially effective in preventing aggregation of the mycelium of these organisms in both shake-flask and small-scale fermenter cultures. The significance of the experiments lies in the probability that this additive will prolong the exponential phase, thereby facilitating rapid growth of any basidiomycete, in any medium, in entirely conventional reactor vessels, and will thus allow full advantage to be taken of the considerable promise of this large group of filamentous fungi.

MATERIALS AND METHODS

Organisms

Phanerochaete chrysosporium Burds. was obtained from Dr J. M. MacDonald at UMIST; *Phlebia radiata* (syn. *P. merismoides* Fr.), *Phlebia gigantea* (Fr.: Fr.) Donk and *Bjerkandera adusta* (Willd.: Fr.) Karst. were supplied by Dr A. D. M. Rayner, University of Bath; *Pleurotus ostreatus* (Jacquin: Fr.) Kummer was obtained from Dr O. K. Miller Jr, Bloomingdale, New York. *Coprinus cinereus* (Schaeff.: Fr.) S. F. Gray cultures were from our own collection. Stock cultures were maintained as slopes using the YPG agar medium described below.

Culture media

The medium used routinely (YPG) contained (l^{-1}): yeast extract ('lab m', London Analytical & Bacteriological Media Ltd, Salford M6 6PD), 1·5 g; mycological peptone (Oxoid), 5 g; D-glucose (BDH Ltd, Poole), 10 g. When required, media were solidified with 1·2 % (w/v) Taiyo powdered agar (Davis Gelatine, Ltd, Leamington Spa).

For experiments with polymeric additives the nutrient mixture and polymer solutions were autoclaved separately at 15 p.s.i. for 15 min and combined as sterile solutions. The medium was adjusted to pH 6·5 with sterile 1 M-NaOH. All of the polymeric additives were supplied by Honeywill & Stein Ltd, Greenfield House, 69/73 Manor Road, Wallington, Surrey SM6 OBP; they are all commercial products, generally used for thickening solutions (foods, pharmaceuticals, cosmetics, adhesives) and/or maintaining solids or non-miscible liquids in suspension (paints, inks). They are referred to here by their trade names and the rights of the owners of those names are fully acknowledged.

Some experiments with *C. cinereus* in shake flasks or a 1·3 l (nominal) fermenter vessel (see text) used a defined liquid medium designated SNC (Stewart & Moore, 1974) supplemented with 10 mM D-glucose, 30 mM NH_4Cl and 1 % (w/v) Bacto Casamino acids (Difco). Polypropylene glycol (0·01 % (v/v), MW 2000) was used in all fermenter cultures to control foaming. The final medium had a pH of 6·8 after autoclaving.

Preparation of inocula

Arthrospores of *Phanerochaete chrysosporium*, *Phlebia radiata* and *P. gigantea* were harvested in sterile distilled water from 5-day old cultures on YPG medium agar plates incubated at 25 °C. Suspensions were filtered through sterile lens tissue (Whatman 106) to remove chains of arthrospores that had not disarticulated. Spore suspensions were washed twice with sterile, distilled water, counted using a haemocytometer and their concentration adjusted to give a final concentration in the culture medium of 10^6 ml^{-1}.

Suspensions of mycelial fragments were prepared from *B. adusta*, *P. ostreatus* and the dikaryon of *C. cinereus*. Mycelium scraped from the surface of agar cultures with a sterile spatula and shaken in a 100 ml flask containing 70 g or 40 mesh glass beads (BDH) for 20 min. The slurry was suspended in 1/4 strength Ringer's solution (BDH), and the hyphal fragments decanted when the beads had settled. The suspension was washed by centrifugation. The spectrophotometric turbidity of the suspension was used as a measure of concentration, calibration curves of colony forming units (c.f.u.) being prepared for each organism.

Fermenter cultures of the *C. cinereus* BC9/6,6 monokaryon were inoculated with mycelial fragments prepared by blending shake flask starter cultures in a Waring blender. A Gallenkamp Modular Fermenter was used, the 1·3 l (nominal) vessel being charged with 750 ml of medium. The fermenter culture was maintained at 37 °C with an integral electrical heater + water cooling coil, sparged with sterile air at 500 ml min^{-1}, and stirred with a magnetically coupled power unit.

Monitoring shake-flask cultures

When growth was filamentous, biomass of cultures grown in Nephlos flasks (Trinci, 1972) was esti-

mated by measuring the optical density of the culture using an EEL Colorimeter with a green filter (Evans Electroselenium Ltd, Halstead, Essex). For dry weight measurements, the mycelium was separated from the medium by centrifugation at 1250 g for 5 min. It was then suspended in distilled water and washed onto pre-weighed filter papers (Whatman 105) with 250 ml water. The harvest was dried to constant weight at 60°. Duration of the lag phase was determined by graphical extrapolation of the regression line calculated for data representing the exponential growth phase. Mycelial dry weight yields were determined from cultures incubated at 25° on an orbital incubator operating at 200 strokes min^{-1}, growth being monitored by absorbance measurements or (for the controls) by measuring mycelial dry weight. Flasks were harvested for the final yield determination when culture turbidity (or dry weight) remained unchanged for three successive (hourly) measurements.

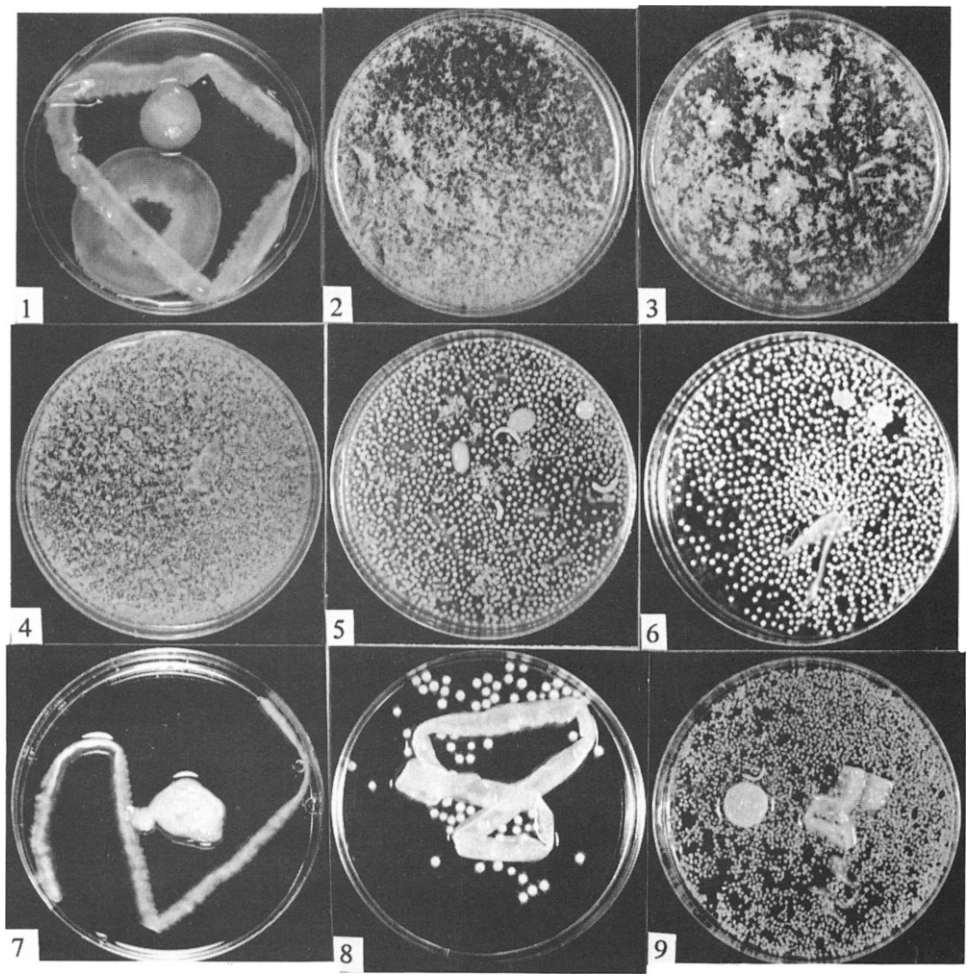
RESULTS AND DISCUSSION

The fundamental improvement sought in the growth form was for submerged cultures to grow as dispersed hyphal filaments as opposed to pellets, clumps or 'crusts' (mycelial mats formed at air-liquid interfaces). The qualitative effects of 0·2% (w/v) final concentrations of different polymers on growth of *P. chrysosporium* in shake-flask culture are summarized in Table 1 and illustrated in Figs

Table 1. *Effects of variety of polymeric additives (all at a final concentration of 0·2%, w/v) on culture morphology of* Phanerochaete chrysosporium *grown in YPG medium*

Additive	Culture morphology	Nature of additive
None	Crust and single large clump	—
Junlon PW110	Dispersed, filamentous growth	Anionic, cross-linked polymers of acrylic acid (–CH·CH(COOH)–)
Junlon PW150	Dispersed, filamentous growth	Anionic, cross-linked polymer of acrylic acid
Hostacerin	Dispersed, filamentous growth	Anionic, sodium salt of polyacrylic acid
Jaypol M40	Small pellets	Anionic, sodium salt of polyacrylic acid
Jaypol A140	Crust and small pellets	Anionic, ammonium salt of polyacrylic acid
Texipol HSM2	Small pellets	Anionic, copolymers of acrylamide, methacrylamide, acrylic acid, methacrylic acid and esters
Manucol Ester M	Large and small pellets	Anionic, polypropylene esters of alginic acid (polymannuronic acid)
Manucol Ester E/RE	Large and small pellets	Anionic, polypropylene esters of alginic acid
Cellobond 300	Crust and large single pellet	Anionic, hydroxyethyl cellulose
Cellobond 7000	Large single pellet	Anionic, hydroxyethyl cellulose
Cellobond 45000	Large and small pellets	Anionic, hydroxyethyl cellulose
Nymcel	Crust and large pellets	Anionic, sodium carboxymethylcellulose
Montanox 20	Crust and small pellets	Non-ionic, polyoxyethylene sorbitan monolaurate ester
Avicel CL611	Crust and small pellets	Non-ionic, microcrystalline cellulose
Gelcarin CIC	Crust and large pellets	Non-ionic, kappa carageenan (polymer of a disaccharide comprised of D-galactose 4-sulphate and 3,6-anhydro D-galactose)
Polyvinyl alcohol 105	Single large clump	Non-ionic, homopolymer of ethenol (–CHOH·CH_2–)
Polyvinyl alcohol 205	Single large clump	Non-ionic, homopolymer of ethenol
Texipol HSED	Clumped, ungerminated spores	cationic, copolymers of acrylamide, methacrylamide, acrylic acid, methacrylic acid and esters
Radiaquat 6470	Clumped, ungerminated spores	Cationic, dialkyl dimethyl quarternary ammonium chloride

Shake-flask cultures (20 ml of medium in 250 ml flasks) were inoculated with 2×10^6 arthrospores of *Phanerochaete chrysosporium* and incubated at 37° for 30 h. Culture morphology was inspected visually or microscopically as appropriate.



Figs 1–9. Culture morphology of *Phanerochaete chrysosporium* grown in YPG medium containing 0·2 % (w/v) polymer additives. All cultures were incubated for 60 h in shake flasks containing 20 ml of medium, at 25° on an orbital shaker operating at 200 strokes min^{-1}. Contents of the flasks were poured into 9 cm Petri dishes for photography. Fig. 1, control medium lacking polymer additive; Fig. 2, Junlon PW110; Fig. 3, Junlon PW150; Fig. 4, Hostacerin; Fig. 5, Texipol HSM2; Fig. 6, Manucol Ester M; Fig. 7, Cellobond 300; Fig. 8, Jaypol A140; Fig. 9, Montanox 20.

1–9. Under these conditions it is evident that the closest approach to the desired improvement in growth form resulted from the particular characteristics of Junlon (polyacrylic acid) and Hostacerin (sodium polyacrylate) and further work concentrated on these products. From the limited information presently available it is not possible to speculate on reasons for the differences observed between different polyacrylate salts tested.

The effects of both polymers on a number of basidiomycetes grown in shake flasks is summarized in Table 2. Morphology of the mycelium was greatly improved in all cases as in the absence of the polymers cultures tended to produce large mycelial clumps and crusts, whereas cultures containing polymer grew as dispersed hyphal filaments (*Phanerochaete chrysosporium*, *Phlebia radiata* and *P. gigantea*) or formed numerous small pellets (*B. adusta*, *C. cinereus* and *P. ostreatus*). In all cases, and despite the higher variability, mycelial dry weight yields were significantly increased by inclusion of Junlon PW110 or Hostacerin in the medium, cultures growing as dispersed hyphal filaments showing the greatest proportional

Table 2. *Mycelial dry weight yields (mg flask^{-1}) and culture morphology of some basidiomycetes grown at 25° in shake flasks containing YPG medium with and without polyacrylate additives*

Organism	Control no additive	Junlon PW110 (0·2 % w/v)	Hostacerin (0·1 % w/v)
Bjerkandera adusta	39 ± 4^a	80 ± 4^b	75 ± 8^b
Coprinus cinereus	27 ± 12^a	79 ± 140^b	77 ± 40^b
Phanerochaete chrysosporium	35 ± 12^a	90 ± 12^c	90 ± 34^c
Phlebia radiata	58 ± 8^a	122 ± 4^c	87 ± 8^c
Phlebia gigantea	69 ± 2^a	136 ± 4^c	109 ± 66^c
Pleurotus ostreatus	56 ± 14^a	74 ± 126^d	72 ± 100^d

Entries show the means ± S.D. of mycelial dry-weight yields of 9 flasks each initially containing 20 ml YPG medium and inoculated with 10^6 spores or mycelial fragments. Culture morphology is indicated by the superscripts: *a*, large mycelial clump and crust; *b*, very small pellets, 1–2 mm diam; *c*, dispersed filamentous growth; *d*, small pellets, 4–6 mm diam. All of the dry-weight yields recorded for cultures containing polymer were significantly greater, at the 95 % level of probability, than controls lacking additive.

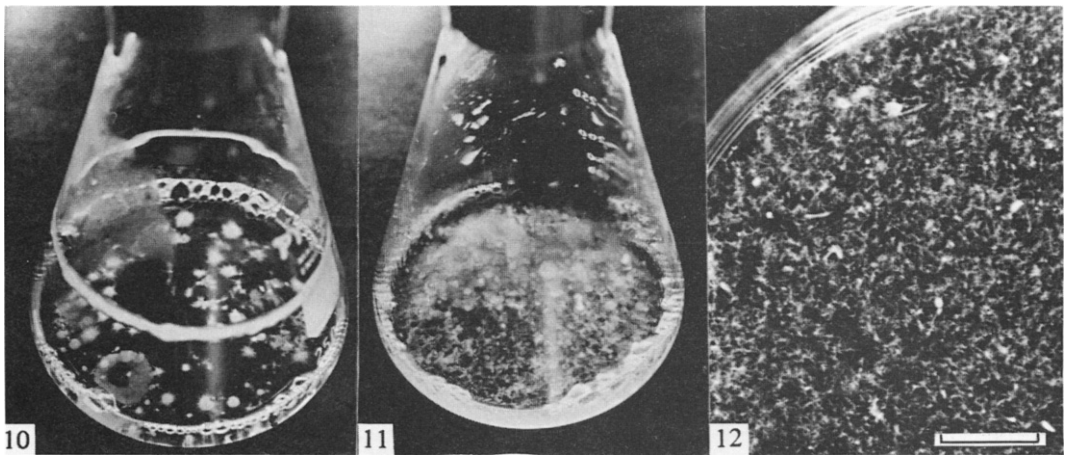
increase in final yield which was doubled in many cases. This yield increase was probably due to the cultures maintaining exponential growth for a longer period than when aggregates were formed. Some of the standard deviations shown in Table 2 are high but, for the most part, the greatest variability in yield was observed between replicates of experiments in which inocula were prepared by fragmenting mycelia. The variability may, therefore, result from a combination of two factors: inherently low reproducibility of fragment numbers between successive aliquots of such suspensions, and the ability of the polymers to maintain dispersion of the fragments and permit each one to make its contribution to growth of the culture rather than aggregate into fewer, larger masses. Variability in yield of biomass was not accompanied by variability in the kinetic aspects of the growth of such cultures. Table 3 shows that growth rates of fungi able to grow as dispersed filaments were also improved in medium containing the polymers.

Although there is some variation in the magnitude of the effect of the polymers on growth of different basidiomycetes it is clear that the growth form of all species can be modified in a way which is highly advantageous for submerged culture. The optimum concentration requirement probably differs between species, and it certainly seems that the nature of the growth medium needs refinement in particular cases. For example, although *C. cinereus* grew as small pellets in the YPG medium

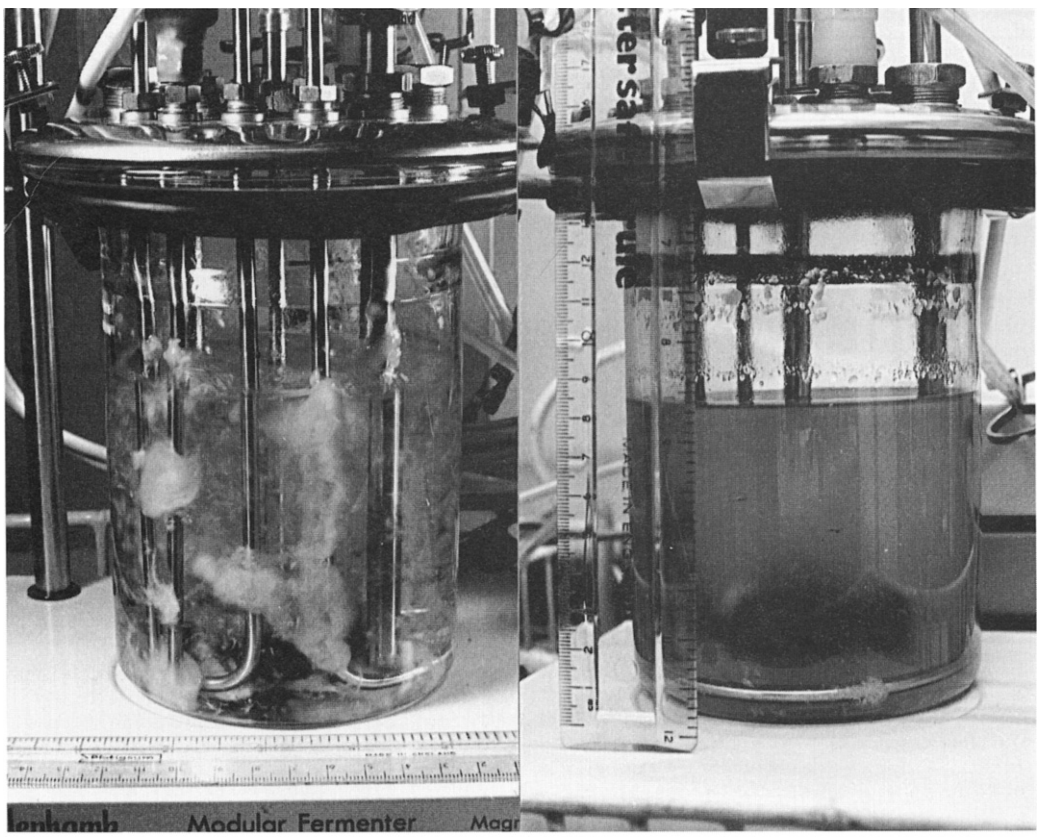
Table 3. *Growth characteristics of three basidomycetes in YPG liquid medium with and without polymer additives*

Organism	Addition to medium	Lag period (h)	Specific growth rate (h^{-1})	Doubling time (h)
Phanerochaete chrysosporium	None	6·5 ± 1·2	0·141 [0·91]	4·93 ± 0·32
	Junlon PW110	10·0 ± 2·7	0·199 [0·99]	3·48 ± 0·34
	Hostacerin	9·8 ± 1·3	0·196 [0·98]	3·53 ± 0·27
Phlebia radiata	None	26·5 ± 2·4	0·035 [0·95]	19·73 ± 0·15
	Junlon PW110	25·0 ± 1·7	0·062 [0·99]	11·20 ± 0·14
	Hostacerin	35·0 ± 2·2	0·057 [0·99]	12·12 ± 0·91
Phlebia gigantea	None	48·0 ± 2·8	0·017 [0·98]	40·77 ± 0·16
	Junlon PW110	51·0 ± 3·1	0·021 [0·99]	32·93 ± 0·28
	Hostacerin	44·0 ± 1·6	0·022 [0·99]	31·38 ± 0·21

All cultures were incubated in shake flasks containing 20 ml of medium, at 25° on an orbital shaker operating at 200 strokes min^{-1}. Junlon PW110 was used at 0·2 % w/v, Hostacerin at 0·1 % w/v. Nine replicates were used; entries show means ± SD, growth rates were determined by regression analysis and the correlation coefficients are shown in square brackets.



Figs 10–12. For captions see opposite.



13 14

Figs 13–14. For captions see opposite.

used to obtain the data reported in Table 2, when this species was grown in a mixture routinely used for its experimental culture supplemented with only 0·1 % (w/v) Junlon PW110, dispersed filamentous growth was obtained both in shake flask (Figs 10–12) and in fermenter cultures (Figs 13, 14). This not only shows that the response of individual species to the polymers can be 'fine-tuned' by modifying both medium and polymer concentration, but demonstrates the applicability of these techniques to monokaryons as well as heterokaryons, and to the conventional stirred tank reactor.

We have tested representatives of the Agaricaceae, Coprinaceae, Cortinariaceae and Polyporaceae and find that all can be caused to grow in submerged culture as finely divided mycelial suspensions by addition of polyacrylic acids or their salts. We believe that this technique promises to be widely applicable to the growth of basidiomycetes in a variety of submerged culture systems (Moore & Trinci, 1986). The exact mode of action of the polymers is unknown, but we believe that the polymers modify the spore surface in some way which prevents aggregation of spores (and hyphal fragments) during germination (Jones, Moore & Trinci, in prep.).

It is a pleasure to acknowledge the help and encouragement of Mr Bert Watkins of Honeywill & Stein Ltd, and the financial assistance of that company and BP Chemicals Ltd which enabled this work to be carried out. BAS thanks the Ministry of Higher Education and Research of the Government of Iraq and the University of Basrah for a Scholarship.

REFERENCES

BURKHOLDER, R. R. & SINNOT, E. W. (1945). Morphogenesis of fungal colonies in submerged shaken cultures. *American Journal of Botany* **32**, 424–437.

CAMICI, L, SERMONTI, G. & CHAIN, E. B. (1952). Observations on *Penicillium chrysogenum* in submerged culture. I. Mycelial growth and autolysis. *Bulletin of the World Health Organization* **6**, 265–275.

ELMAYERGI, H. & SCHARER, J. M. (1973). Physiological studies on *Aspergillus niger* fermentation with polymer additive. *Journal of General and Applied Microbiology* **19**, 385–392.

METZ, B. & KOSSEN, N. W. F. (1977). The growth of molds in the form of pellets – a literature review. *Biotechnology and Bioengineering* **19**, 781–799.

MOORE, D. & TRINCI, A. P. J. (1986). Improvements in the culture of fungi. European Patent Application Number 01916017, filed by BP Chemicals Limited and The Victoria University of Manchester.

PIRT, S. J. (1966). A. theory of the mode of growth of fungi in the form of pellets in submerged culture. *Proceedings of the Royal Society, London*, Series B **166**, 369–373.

STEWART, G. R. & MOORE, D. (1974). The activities of glutamate dehydrogenases during mycelial growth and sporophore development in *Coprinus lagopus* (*sensu* Lewis). *Journal of General Microbiology* **83**, 73–81.

TRINCI, A. P. J. (1972). Culture turbidity as a measure of mould growth. *Transactions of the British Mycological Society* **58**, 467–473.

TRINCI, A. P. J. (1983). Effect of Junlon on morphology of *Aspergillus niger* and its use in making turbidity measurements of fungal growth. *Transactions of the British Mycological Society* **81**, 408–412.

TRINCI, A. P. J. & THURSTON, C. F. (1976). Transition to the non-growing state in eukaryotic micro-organisms. In *The Survival of Vegetative Microbes* (ed. T. R. G. Gray & J. R. Postgate), pp. 55–80. Cambridge, UK: Cambridge University Press.

WITTLER, R., BAUMGARTL, H., LUBBERS, D. W. & SCHUGERL, K. (1986). Investigations of oxygen transfer into *Penicillium chrysogenum* pellets by microprobe measurements. *Biotechnology and Bioengineering* **28**, 1024–1036.

YOSHIDA, T., SHIMIZU, T., TAGUCHI, H. & TERAMOTO, S. (1967). Studies on submerged culture of basidiomycetes. III. The oxygen transfer within pellets of *Lentinus edodes*. *Journal of Fermentation Technology* **45**, 1119–1129.

(*Received for publication 28 July 1987 and in revised form 30 September 1987*)

Figs 10–12. Submerged cultures of the BC9/6,6 monokaryon of *Coprinus cinereus* grown in shake flasks. In each case the medium was a defined salts solution (SNC) supplemented with hydrolyzed casein, glucose and NH_4Cl and the cultures (50 ml volumes in 250 ml flasks) were incubated at 37° for 84 h. Fig. 10, flask containing control medium without polymer additive; Fig. 11, flask containing medium with 0·1 % (w/v) Junlon PW110; Fig. 12, magnified view of the culture from Fig. 11 showing dispersed filamentous growth of the mycelium, scale bar = 1 cm.

Fig. 13. Fermenter-grown batch culture of the BC9/6,6 monokaryon of *Coprinus cinereus* in control medium (supplemented SNC) without polymer additive.

Fig. 14. Fermenter-grown batch culture of the BC9/6,6 monokaryon of *Coprinus cinereus* in medium supplemented with 0·1 % (w/v) Junlon PW110 but otherwise identical to the culture shown in Fig. 13.