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ORIGINAL PAPER

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Shake flask culture of *Laccaria laccata*, an ectomycorrhizal basidiomycete

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Abstract Large-scale exploitation of the potential benefits of ectomycorrhizal fungi in improving plantation yields means that fermentation techniques for these fungi will be required. Starting with a base performance on a rich, complex medium, the effect of variations in some physicochemical culture parameters on biomass yield was studied. It was possible to reduce the amount of phosphate salts (to 1/9th) and other ingredients (to 1/3rd) in the medium. A shaking speed of either 100 or 200 r.p.m. in an orbital incubator was satisfactory and biomass yield responded to an increase in carbon substrate (glucose, from 10 and 20 g l^{-1}) though $Y_{X/S}$ declined. An increase in inoculum size shortened culture time but decreased biomass yield. The upper limit in incubation temperature was between 25°and 30°C. Biomass yields of about 12 g l^{-1} DW ($Y_{X/S}$ = 0.63) when supplied 20 g L^{-1} glucose was supplied, and about 7 g l^{-1} ($Y_{X/S}$ = 0.74) when supplied 10 g l^{-1} glucose was supplied.

Introduction

Ectomycorrhizal fungi form structures called ectomycorrhiza on the roots of many economically important trees such as pine, spruce, beech and eucalypts (Ruehle and Marx 1979; Warcup 1980). Ectomycorrhiza can increase the growth of host plants by increasing their uptake of nutrients from the soil (Harley and Smith 1983). Ectomycorrhizal fungi are important for the growth and survival of eucalypts (Bowen 1973; Malajczuk *et al.* 1975; Warcup, 1980). The eucalypt is an important plantation tree genus with over 7 million hectares planted world-wide (Cameron and Penna 1988). In Australia, a detailed study has advocated a change from reliance on native forests for eucalypt hardwoods to supply from plantations (Cameron and Penna 1988). Thus, there have been numerous studies on the manipulation of the ectomycorrhizal symbiosis in eucalypts in order to extract an advantage in plantation economics (Grove and Malajczuk 1994; Garbaye *et al.* 1988; Bougher *et al.* 1987; Abouelkhair *et al.* 1986). It is now clear that for many plantation tree species, inoculation at the seedling stage with an appropriate ectomycorrhizal fungus results in faster tree growth.

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Apart from quantitative studies on the enhancement of tree growth attainable with ectomycorrhizal fungi, appropriate technology for the mass culture of the fungi as inocula must be developed so that inoculation becomes an exercisable management option. Various forms of inocula are currently available or advocated. These include spore inocula (Marx *et al.* 1984), inocula produced by solid substrate culture (Marx and Kenney 1982; Le Tacon *et al.* 1988), mycelial slurries (Boyle *et al.* 1988; Gagnon *et al.* 1988), and encapsulated pre-grown mycelium (Le Tacon *et al.* 1985; Mauperin *et al.* 1987; Deacon and Fox 1988). Deficiencies in the efficacy, physical form and manufacturing processes for the inocula forms currently available are revealed when they are assessed using criteria for efficacious and practical inocula which have been proposed (Tommerup *et al.* 1987). The use of fermentation techniques will enable inocula of higher quality to be produced.

Inocula produced by the submerged aerobic culture of mycelia immobilized within hydrogel beads has been found to be of high efficacy (Kuek *et al.* 1992). The production process for hydrogel bead inocula requires the ability to culture mycelium in both free and immobilized states. This is because free mycelium is used as a source of propagules for the production of mycelia immobilized in the hydrogel. Thus, the development of cultural conditions for ectomycorrhizal fungi in submerged aerobic culture is a necessary step towards the production of hydrogel bead inocula. Nutritional studies on the culture of ectomycorrhizal fungi (*e.g.* Ahmad *et al.* 1990; Ohta 1990) provide important information in one of two areas required for their successful mass culture. The other area is that of basic fermentation data such as time-parameter profiles for key indicators such as biomass, residual carbon, and

pH. Fermentation techniques have been discussed (Harvey *et al.* 1988; Harvey 1991), and assorted data from the submerged aerobic culture of various fungi are available (Litchfield and Arthur 1983; Le Tacon *et al.* 1985; Sasek 1989; Pradella *et al.* 1990). On the other hand, some papers refer to mycelial production via fermentation techniques but provide little or no information on either yield or methodology (*e.g.* Kropacek *et al.* 1989). A good fermentation is one where all the major substrates are consumed; there is efficient conversion of substrate to product, and a high yield is obtained in the minimum time. Thus, the attainment of a good fermentation can be determined by how much substrates are provided in relation to actual requirement and by the conditions of the fermentation. When a fungus is liquid cultured for the first time, it is common to provide a medium which is more than adequate in substrate composition and quantity. Similarly, the physical conditions such as agitation and aeration are set high so that they are not a limiting factor. Given such a start, the probability of success with the first culture of the fungus is enhanced. However, in industrial microbiology success in culture often also means the minimization of production inputs or the increase in fermentation efficiency. This paper reports the effects of the sequential manipulation of glucose concentration, shaking speed, incubation temperature, and minimization of added phosphate and non-phosphate nutrients on the growth of an ectomycorrhizal fungus in shake flask culture.

Materials and methods

Fungus

The culture used was *Laccaria laccata* (Scop. ex Fr.) Berk. & Br. E439 from the culture collection of the Commonwealth Scientific and Industrial Research Organisation's Division of Forestry, Perth, W. Australia. In plate culture, the solid medium used was modified Melin-Norkrans agar (Marx 1969) and incubation was at 25°C.

Growth medium

The initial medium used in shake flask culture was the same as one previously used for ectomycorrhizal fungi (Litchfield and Arthur 1983) except for the amount of glucose used. It comprised (g l^{-1}), peptone (Difco), 10.0; yeast extract (Difco), 2.0; NH_4NO_3, 3.0; KH_2PO_4, 2.38; K_2HPO_4, 5.65; $MgSO_4.7H_2O$, 1.0; $CuSO_4.5H_2O$, 0.0064; $FeSO_4.7H_2O$, 0.0011; $MnCl.4H_2O$, 0.0019, $ZnSO_4.7H_2O$, 0.0015. Glucose at desired concentrations was added prior to autoclaving. Variations of this formulation were tested as indicated in Table 1. In the case of the phosphate salts, reductions where mentioned, were made equally of both.

Sterilization

Media and apparata were autoclaved at 121°C for 15 min.

Production of inoculum for shake flask culture proper.

Colonies were established on agar plates. On each 9 cm modified Melin-Norkrans agar plate, 3 colonies of the fungus were established until they were 2 - 3 cm in diameter. From three plates, agar plugs (approximately 0.5 by 0.5 cm) were removed from the edges of all the colonies and placed in one 250 mL screw-capped Erlenmeyer flask containing 50 mL of Growth Medium with the same glucose concentration as that in the following steps. The loosely capped flasks were incubated for 7 days in a gyratory incubator (New Brunswick Scientific, Innova 4330) at 25°C and 200 r.p.m. The contents of each flask, singly or in combination, were homogenized for 10 seconds in a sterile, sealed homogenizer (Sorvall Omnimix).

Table 1 The sequence of examination of the effects of some physicochemical parameters on shake flask culture of *Laccaria laccata* E439.

Sequence	Medium			Inoculum size	Incubation	
(Run Number)	Added PO_4 salts	Non-PO_4 ingredients	Glucose (g l^{-1})	(% v/v)	Shaking speed (rpm)	Temperature (°C)
1	1	1	10	4	200	25
2	1/9	1/1	10	4	200	25
3	1/9	1/1	10	4	100	25
4	1/9	1/1	20	4	100	25
5	1/9	1/1	20	10	100	25
6	1/9	1/1	20	4	100	20, 30
7	1/9	1/3	20	4	100	25
8	1/9	1/6	20	4	100	25
9	1/9	1/9	20	4	100	25

Shake flask culture

Homogenate from the previous step was used to inoculate new 250 mL screw-capped Erlenmeyer flasks at the rate of 2 mL of inoculum to 48 mL of Growth Medium (2% v/v inoculum) except where indicated (5% v/v inoculum). These flasks were incubated as described above at the temperature and shaking speed indicated in Table 1. At each sample time, the entire contents of 3 replicate flasks were filtered (Whatman No. 1 paper). The pH and concentration of residual glucose (using Sigma Glucose Test Kit No. 510-A) in the filtered culture liquor was then determined. The recovered biomass was washed with 3 volumes of D.I. water and then dried at 80°C until constant weight was achieved.
to be suitable in that glucose was completely consumed and

Results

The cultures were characterized by the measurement of residual glucose, biomass yield and pH through the course of the fermentation. The initial medium formulation used proved

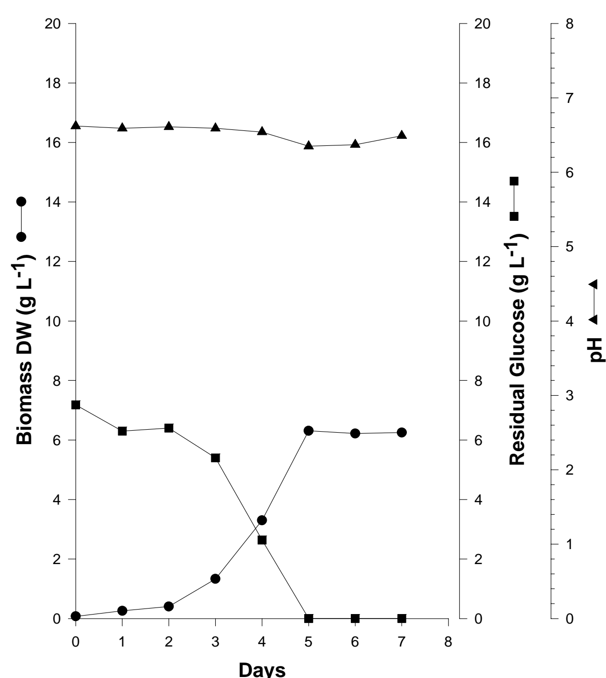
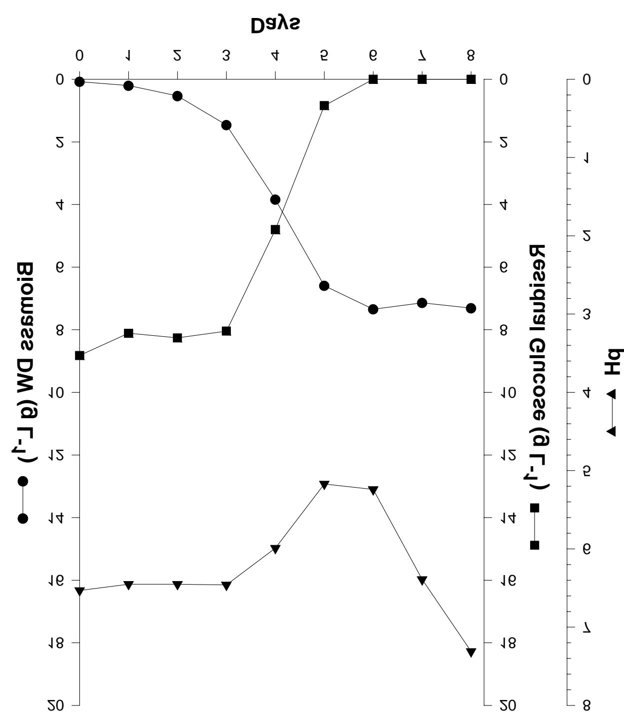


Fig. 1 Run 1. The initial indicator profiles for a shake flask culture of *Laccaria laccata* E439 using a complex medium. Conditions: PO_4 salts at original concentration; Non-PO_4 nutrients at original concentrations; glucose 10 g l^{-1}; inoculum size 4% (v/v); shaking speed 200 rpm; temperature 25°C.

to be suitable in that glucose was completely consumed and culture pH held steady (Fig. 1).

The biomass accumulation curve was a reciprocal of the glucose curve. Mycelium grew in both filamentous and pellet form, and the color of the culture became darker as the culture progressed. In the following run, the added phosphate content of the medium was reduced to 1/9th of the original. The reduction in phosphate concentration did not significantly affect the glucose consumption and biomass accumulation profiles (Fig. 2). However, the medium had a reduced buffering capacity and a reduction in pH of about 1.25 units accompanied glucose consumption. After exhaustion of glucose, culture pH rose by about two units even though the culture was already in the plateau phase of growth. Subsequent reduction of shaking speed from 200 to 100 r.p.m. did not appear to alter the glucose consumption, biomass accumulation and pH profiles of the culture (Fig. 3). At the lower shaking speed, when the amount of glucose supplied was doubled to 20 g l^{-1}, again the shape of the glucose consumption and biomass accumulation profiles were



not significantly altered (Fig. 4). However, the final yield of biomass was increased by about 1.7

Fig. 2 Run 2. A repeat of Run 1 with the concentration of added phosphate in the medium reduced to 1/9th of the original. Other conditions: Non-PO_4 nutrients at original concentrations; glucose 10 g l^{-1}; inoculum size 4% (v/v); shaking speed 200 rpm; temperature 25°C.

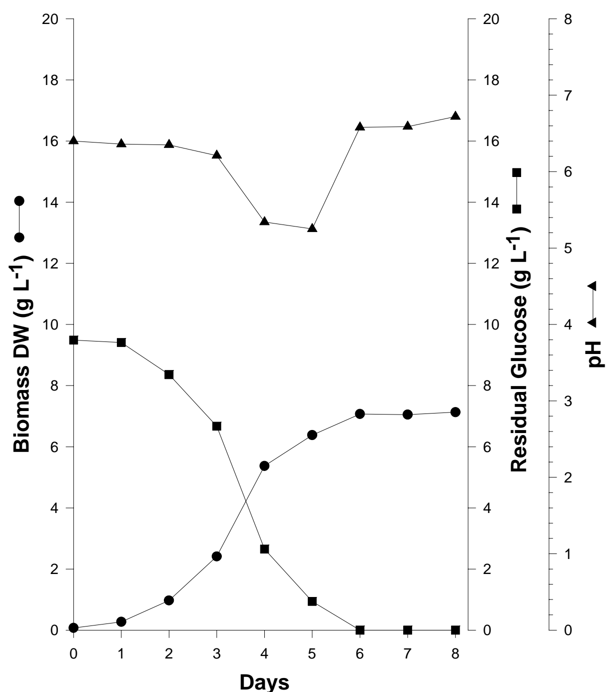


Fig. 3 Run 3. A repeat of Run 2 with shaking speed reduced from 200 to 100 r.p.m. Other conditions: 1/9th PO_4 salts; Non-PO_4 nutrients at original concentrations; glucose 10 g l^{-1}; inoculum size 4% (v/v); temperature 25°C

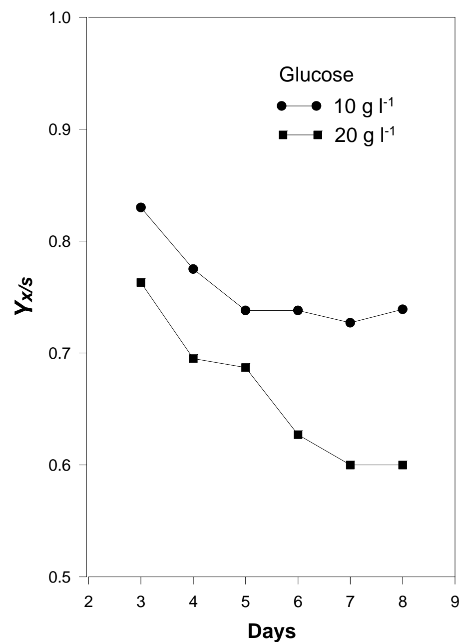


Fig. 5 A comparison of the yield coefficients ($Y_{X/S}$) in shake flask culture of *Laccaria laccata* started with 10 g l^{-1} (Run 3) or 20 g l^{-1} (Run 4) of glucose.

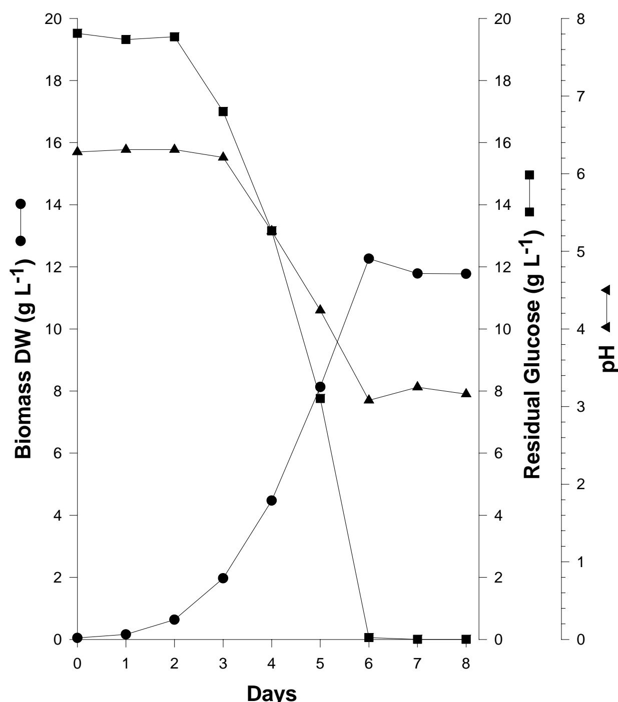


Fig. 4 Run 4. A repeat of Run 3 with the glucose concentration of the medium increased from 1 to 20 g l^{-1}. Other conditions: 1/9th PO_4 salts; Non-PO_4 nutrients at original concentrations; glucose 20 g l^{-1}; inoculum size 4% (v/v); shaking speed 100 r.p.m.; temperature 25°C.

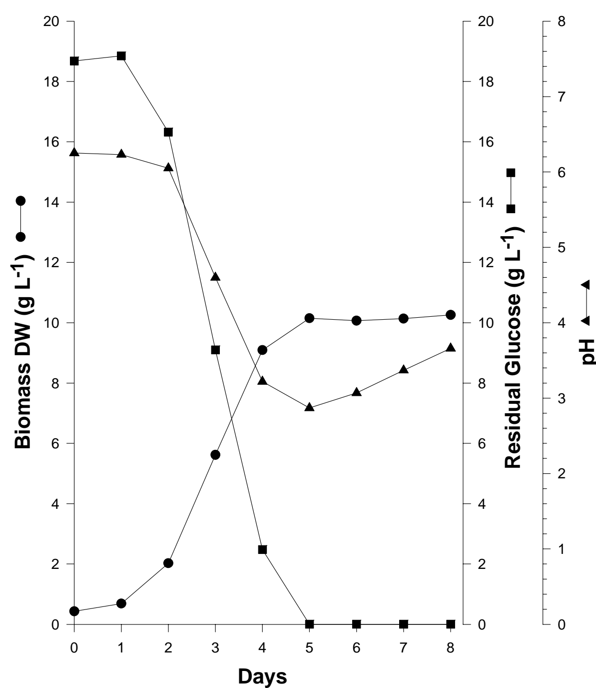


Fig. 6 Run 5. A repeat of Run 4 with the inoculum size increased from 4 to 10% (v/v). Other conditions: 1/9th PO_4 salts; Non-PO_4 nutrients at original concentrations; glucose 20 g l^{-1}; shaking speed 100 r.p.m.; temperature 25°C.

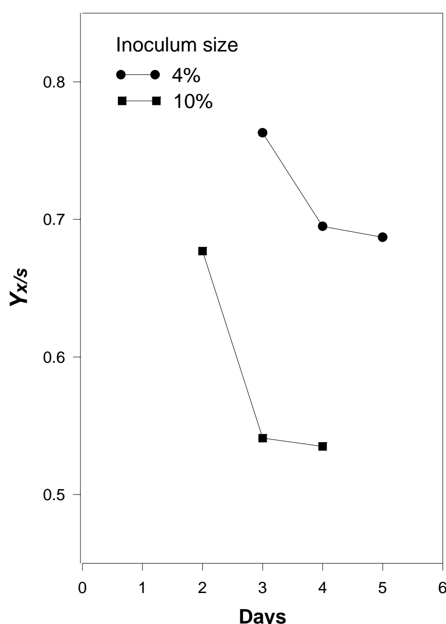


Fig. 7 A comparison of the yield coefficients ($Y_{x/s}$) in the exponential phases of shake flask cultures of *Laccaria laccata* started with 4 or 10% (v/v) inocula

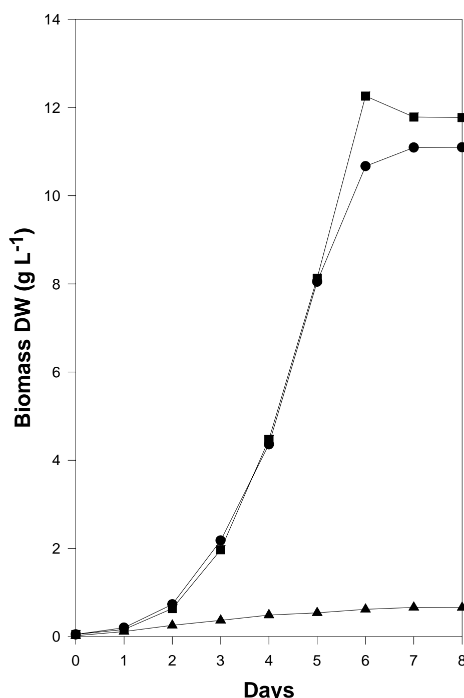


Fig. 8 Run 6. Repeats of Run 4 at 20° and 30°C. Other conditions: 1/9th PO_4 salts; Non-PO_4 nutrients at original concentrations; glucose 20 g l^{-1}; shaking speed 100 rpm

times (compared to the previous run) without affecting the time to attainment of maximum biomass. A comparison of the yield coefficient (biomass produced to glucose consumed, $Y_{x/s}$) showed that although biomass

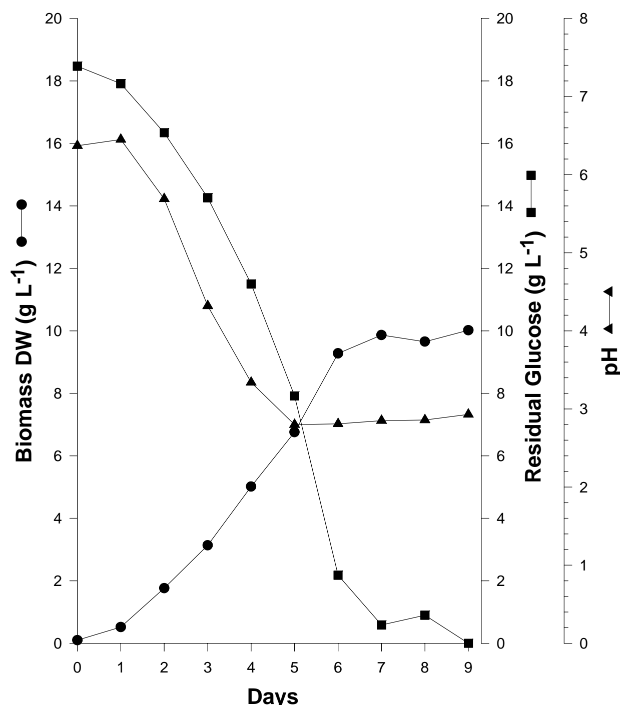


Fig. 9. Run 7 A repeat of Run 4 with the concentration of non-phosphate nutrients in the medium reduced to 1/3rd of the original. Other conditions: 1/9th PO_4 salts; glucose 20 g l^{-1}; shaking speed 100 r.p.m.; temperature 25°C.

yield was higher when 20 g l^{-1} of glucose was supplied, $Y_{x/s}$ was smaller at all stages of the culture (Fig. 5).

An increase in inoculum size from 4 to 10% resulted in a shift of the glucose consumption, biomass accumulation and pH profiles to the left on the X axis by about one day *i.e.* the culture was completed earlier (Fig. 6). The use of the larger inoculum size resulted in a 15% decrease in the final biomass yield. A comparison of the yield coefficients in the exponential phases of the 4% and 10% inoculated cultures revealed consistently lower values when 10% inoculum was used (Fig. 7). Biomass accumulation profiles at incubation temperatures of 20°C and 25°C were similar (Fig. 8). However, at 30°C, little growth resulted. The reduction in non-phosphate nutrients to 1/3 and 1/6th levels resulted in reductions in final yields by about 15 and 25% respectively compared to the 1/9th level (Fig. 9 and 10 compared with Fig. 4). Further , the lag phase appeared to be shorter with use less non-phosphate ingredients. Reduction of the non-phosphate nutrients to 1/9th level resulted in the loss of the characteristic biomass accumulation and glucose consumption profiles (Fig. 11). When the pH profiles of all the runs are viewed collectively, *Laccaria laccata* appears to be capable of growing in a pH range of 2.8 - 6.5.

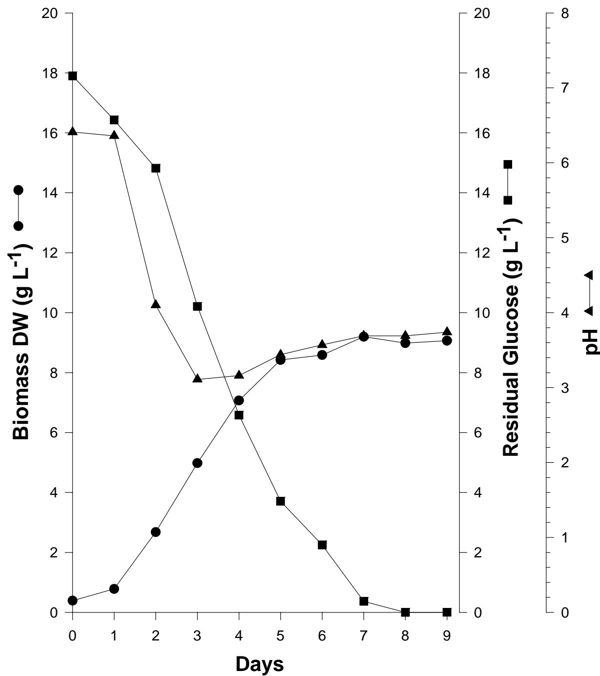


Fig. 10. Run 8. A repeat of Run 4 with the concentration of non-phosphate nutrients in the medium reduced to 1/6th of the original. Other conditions: 1/9th PO_4 salts; glucose 20 g l^{-1}; shaking speed 100 rpm; temperature 25°C.

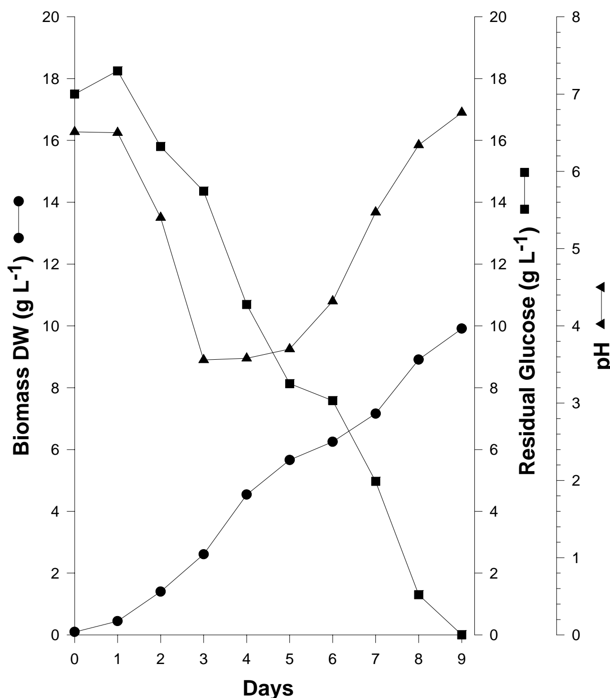


Fig. 11. Run 9. A repeat of Run 4 with the concentration of non-phosphate nutrients in the medium reduced to 1/9th of the original. Other conditions: 1/9th PO_4 salts; glucose 20 g l^{-1}; shaking speed 100 rpm; temperature 25°C.

Discussion

The initial medium used proved satisfactory for the shake flask culture of *Laccaria laccata.* The complete consumption of glucose must have meant that the other medium substrates were adequate in composition and quantity. The buffering capacity of the medium was proved to be good by later data which showed pH swings in contrast to the stability found in the first run. The first manipulation made in the culture conditions was the reduction in the concentration of added phosphate. This was desired because of the requirement in later work to culture the fungi as immobilized mycelia in calcium alginate. The presence of high concentrations of phosphates can dissolve calcium alginate and is thus a situation to avoid. Further, other studies have found that the formation of ectomycorrhiza and their functioning are diminished at high concentrations of soil phosphate (Beckford *et al.* 1985; Shaw *et al.* 1987; Bougher *et al.*1990). Therefore, ectomycorrhizal fungi should be cultured at the lowest possible concentration of phosphate so that they are well adapted to the lower soil phosphate concentrations that will apply in the use of mycorrhizal technology. The lowering of amount of phosphate salts to 1/9th the original was made on the basis of a previous study where that amount of phosphate (5.56 mM) was found to be compatible with growth of both free (Kuek and Armitage 1985) and alginate-immobilized fungal mycelia (Kuek 1991). The reduction of phosphate to 1/9th level not appear to significantly affect biomass production although there appeared to be a 10% increase in yield with reduced phosphate concentration when 10 g l^{-1} glucose was supplied. This may indicate a sensitivity of the culture to inorganic phosphate. *Laccaria laccata* appears capable of growing in the range 5.56 - 50 mM phosphate (the actual range will be higher than this when the phosphates which would be in the other ingredients peptone and yeast extract are accounted for). The reduction in phosphate concentration resulted in a loss of buffering capacity in the medium (compare Fig. 1 with Fig. 2 *et seq.*). This loss of buffering capacity was not significant because it did not affect the cultures' ability to completely exhaust glucose. With the depletion of glucose, culture pH rose back up past the initial values probably due to the use of alternative substrates by the biomass for maintenance energy which may have resulted in either the removal of the acids produced earlier and/or the production of basic compounds. It is clear from Run 4 that 1/9th the original of added phosphate was in excess of requirements in the runs with 10 g l^{-1} glucose. This is because in Run 4 reciprocal production of biomass accompanied the complete exhaustion of the 20 g l^{-1} of glucose supplied (Fig. 4). Thus, phosphate was not the

limiting nutrient it would be if 1/9th concentration was inadequate.

Since the reduction of the added phosphate concentration to 1/9th level proved possible without affecting biomass productivity and yield, it should follow that the non-phosphate nutrients can also be reduced accordingly. The finding that the non-phosphate nutrients cannot be reduced by the same proportion as the phosphate salts says that the original medium formulation (Litchfield and Arthur 1983) was unbalanced for *Laccaria laccata.* Further optimization of the fermentation using 1/3rd and 1/6th levels may enable the retention of the gain in shortened lag phase but improve on the final yield which was lower than when the 1/9th level was used. The shortened lag suggests that the fungus prefers lower levels of nutrients in the initial phases of the culture. As evident in the reductions chosen in this work, lower levels of nutrients can result in lowered final biomass yields. This conflict between yield and requirement for lower concentrations of medium components in the early phases of a culture can be solved by using a fed-batch mode of culture.

Growth of *Laccaria laccata* was relatively unresponsive to shaking speed since the indicator profiles of the 100 and 200 rpm cultures supplied 10 g l^{-1} were similar. This probably means that the fungus is not demanding of oxygen. Similar biomass productivities were found in the exponential phase of Run 2 (Fig. 2) and Run 4 (Fig. 4), despite the doubling of the starting glucose concentration and the halving of the shaking speed in the latter *i.e.* the reduction in mixing and oxygenation had little effect on biomass productivity. No further increases in glucose concentration was studied because it was apparent that whilst biomass yields might be higher, the yield coefficients would be poorer (Fig. 5). Correspondingly, the doubling in glucose supplied did not result in a doubling of the biomass yield (*cf* Fig. 3 with Fig 4). This observation is probably related to the ratio of glucose consumed for production of new cells versus consumption for other cellular needs such as maintenance and production of metabolites, and/or the possibility that the highest yield obtained was approaching the aeration limit of the shake flask at 100 rpm. Both of the latter possibilities are affected by the degree of oxygenation of the culture. Thus, future studies could investigate the use of $\geq$ 20 g l^{-1} glucose, in combination with increased shaking speed to see if $Y_{X/S}$. can be improved.

Laccaria laccata is more sensitive to the higher than the lower side of the temperature optimum. The higher limit was found to be between 25° and 30°C. This temperature profile reflects the origin of the fungus in soil and forest litter where cooler temperatures are more likely than high.

As expected, the effect of increasing the inoculum size from 4 to 10% resulted in earlier completion of the culture as indicated by the biomass and glucose profiles. This remains a route for further optimization of the culture with respect to time considering that 20% inocula are routinely used in fermentations. The lower yield resulting from the use of 10% inoculum (15% reduction) may be explained by $Y_{X/S}$ values in the growth phase which were lower than those found with 4% inoculum. Thus, some of the glucose in the 10% inoculum run was inefficiently fermented to non-biomass products resulting in a lower final biomass yield. Depending on whether fermentation time or $Y_{X/S}$ might be the higher imperative, the use of higher inoculum sizes may require further work to optimize $Y_{X/S}$.

Notwithstanding the differences due to medium formulation, fungal species and strain, the biomass yields found in this study were of the order of those found for shake flask culture of *Laccaria laccata* (13.5 g l^{-1} DW; Sasek 1989) and *Pisolithus tinctorius* (13.5 g l^{-1} DW; Litchfield and Arthur 1983) but higher than those for *Pisolithus tinctorius* (4.9 g l^{-1} DW; Pradella *et al.* 1990), *Lyophyllum shimeji* (7 g l^{-1} DW; Ohta 1990), and *Hebeloma cylindrosporum* (4 g l^{-1} DW; Le Tacon *et al.* 1985). This study has shown that *Laccaria laccata* E439 can be satisfactorily shake flask cultured using the original medium (Litchfield and Arthur 1983) modified to contain 1/9th and 1/3rd of the original specification for the phosphate salts and non-phosphate nutrients respectively; 20 g l^{-1} of glucose; an inoculum size of 4%, a shaking speed of 100 rpm; and an incubation temperature of 25°C. Further optimization of the system is possible.

Acknowledgment

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