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Stimulation by potassium ions of the growth of *Rhizopus oligosporus* during liquid- and solid-substrate fermentations

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Soya beans and several other beans and cereals have been used as substrates for tempe fermentation with the fungus *Rhizopus oligosporus* Saito. Except for the presence of alkaloids, the chemical composition of lupins (*Lupinus mutabilis* Sweet) is similar to that of soya beans. Therefore the potential of lupins for tempe production in regions with a long tradition of lupin consumption is promising. The preparation of the fermentation substrate when using bitter lupins (which contain significant quantities of alkaloids) as starting material includes a debittering stage to remove the alkaloids. However, we found that the debittering process yielded lupins that did not support the mycelial growth required in the tempe fermentation. We discovered that potassium is preferentially leached out during the debittering process. The effect of potassium on fungal biomass formation was monitored using a computerized system that determines biomass accretion by measurement of the electrical capacitance at radio frequencies. The importance of potassium for the growth of *R. oligosporus* was confirmed in liquid cultures. A linear relationship was found between biomass yield and K^+ concentration in the range of 1 to 10 mg/l. The present report represents one of the few demonstrations of a mineral deficiency during the growth of a fungus on a natural, solid substrate.

(*continued on next page*)

Introduction

Tempe

Tempe is a well-known fermented food, produced by solid-substrate fermentation, generally of soya beans, by *Rhizopus oligosporus* Saito (Hesseltine *et al.* 1963; Ko & Hesseltine 1979; Steinkraus 1983). In addition to soya beans, other beans and cereals have been used to make tempe (Hesseltine *et al.* 1967; Kidby *et al.* 1977; Robinson & Kao 1977; Paredes-Lopez *et al.* 1987; Chavez & Peñaloza 1988; Robalino & Peñaloza 1988; Nout & Rombouts 1990).

The production of tempe, and especially of processed, storable products from tempe, such as crisp snacks, from the traditional produce of the different regions of the world may bring nutritional and economic benefits to those particular regions. For example, the debittered Andean lupin (*Lupinus mutabilis* Sweet) is a popular, but rather primitive snack in the highland markets of South America. However, it is a very perishable foodstuff. The development of lupin-based food products would therefore be of benefit to this region. Besides their high protein content, lupins are currently considered an almost exact substitute for soya beans (Hudson *et al.* 1976; Hill 1977; Aguilera & Trier 1978; Williams 1989).

Tempe produced either from 'sweet' lupin varieties (which do not contain significant amounts of alkaloids) or from debittered 'bitter' lupins has been found to be more palatable than soya bean tempe, both in Australia (Kidby *et al.* 1977) and in Ecuador (Chavez & Peñaloza 1988). However, the processing of bitter lupins for use in tempe fermentation must include a debittering stage to remove the alkaloids. Unfortunately, we found that Andean bitter lupins prepared for tempe fermentation in the UK consistently failed to yield a good quality tempe.

This study was undertaken to establish why the lupins that were subjected to debittering were unable to support as good a mycelial growth of R. *oligosporus* as reported in tempe made from other natural subtrates (Hesseltine *et al.* 1967, Kidby *et al.* 1977, Robin & Kao 1977, Paredes-Lopez *et al.* 1987, Blakeman *et al.* 1988, Chavez & Peñaloza 1988, Robalino & Peñaloza 1988). As described below, we established that, as well as the alkaloids, some nutrients and particularly potassium

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are leached away during the preparation of bitter lupins prior to fermentation, and that this deficiency can explain the poor growth of R. *oligosporus* on this substrate.

The Role of Potassium

Potassium is widely distributed in nature; moreover, it is the seventh most common element in the crust of the earth. Potassium is an essential element for plants and animals (Munson 1985). Lilly & Barnet (1951) pointed out, in a general way, that the elements required by fungi are the same as those required by bacteria, plants and animals. Potassium is, biologically, an essential element and no other element may completely replace it. Raulin in 1869 (cited by Lilly & Barnett, 1951), was apparently the first to demonstrate its essential nature by omitting potassium from the culture medium of microorganisms. In liquid culture, the relationships between the potassium concentration in the medium and the mycelium produced by various fungi were studied by a number of authors many years ago (Lilly & Barnett 1951). Nevertheless, as pointed out by Jennings (1987, 1988), much less attention has been paid to inorganic than to organic nutrients, particularly in the laboratory, where the use of agar and added nutrients is widespread, and media are often complex and thus ill-defined. Except for carbon and nitrogen, the other elements have generally been ignored, and assumed to be in excess (as 'impurities' in the complex nutrient sources). As long as all the required nutrients are in excess the exact composition, both qualitative and quantitative, is assumed to be relatively unimportant with respect to supporting the vegetative growth of saprotrophic fungi (Jennings 1988).

In solid-substrate fermentations, legume or cereal seeds, as well as agricultural wastes and lignocellulosic materials, are utilized as the nutrient source. Reviews of these fermentations indicate that the substrates have a K^+ concentration of about 1% (w/w) (Hill 1977; Munson 1985; van der Riet *et al.* 1987), and no deficiency of K^+ has ever apparently been detected. However, when trying to ferment debittered lupins with *Rhizopus oligosporus* to produce tempe, we identified a K^+ deficiency. The documentation of this unusual case, and its confirmation by growth studies in defined liquid cultures, is the subject of the present investigation.

Materials and Methods

Debittering of Lupins

Lupin seeds (*Lupinus mutabilis* Sweet) were debittered at the University College of Wales, Aberystwyth, UK, by an adaptation of the traditional Andean process, as follows: lupins were soaked overnight in three volumes of tap water at 25 to 30°C, then boiled at 100°C for 45 min and finally debittered in running tap water for at least 7 days, until the alkaloids, and consequent bitter taste, were removed from the beans.

Solid-substrate Fermentation of Lupins for Tempe Production

The debittered lupin seeds were processed for tempe production in a way similar to that used for soya beans, following the basic laboratory methods described in the literature (Steinkraus *et al.* 1960; Steinkraus 1983; Hesseltine *et al.* 1963). Briefly, the debittered lupins were dehulled and boiled at 100°C for 15 min in excess water with 10 ml of 5% (v/v) lactic acid solution per litre of cooking water. After cooking, the recovered cotyledons were superficially dried in a drying cabinet at 40°C to about 65% moisture. Then the cotyledons were chopped aseptically (in a laminar flow cabinet) to a particle size of 3 to 5 mm using a liquidizer (three 10 s bursts, with a shake in between). A freeze-dried starter of *Rhizopus oligosporus* (UCW-FF8001, prepared as described by Wang *et al.* (1975), was added as inoculum at about 3×10^5 c.f.u./g of wet beans. After mixing and

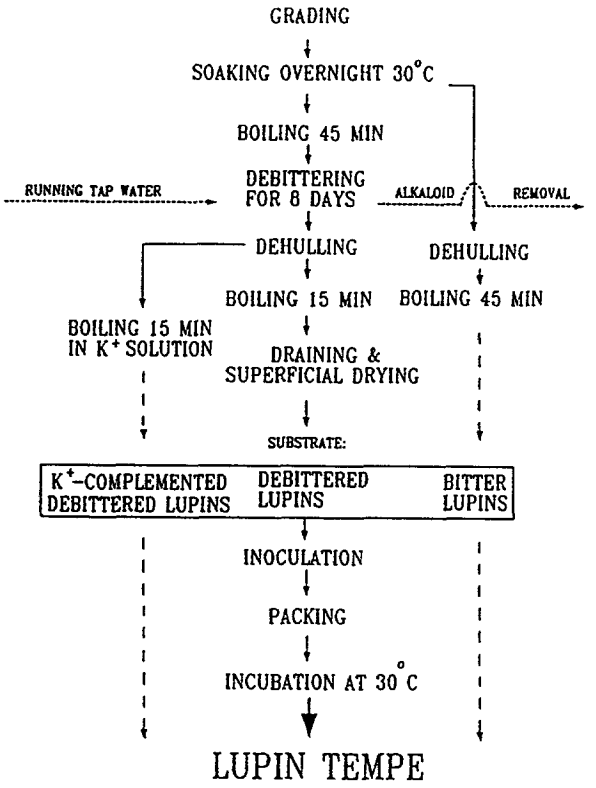

Figure 1. Flow diagram showing the variations in the preparation of bitter lupins (*Lupinus mutabilis* Sweet) for tempe, leading to three types of solid substrates.

packing into disposable, sterile petri dishes, incubation was at 31°C at a relative humidity of approximately 85%. The whole process of lupin preparation for tempe is summarized in Figure 1.

The colonization of the lupin beans by the mycelium of *R. oligosporus* was assessed by measuring the dielectric permittivity of the mycelium at a low radio frequency, i.e. 0.3 MHz. The theory of this new technique for biomass measurement in liquid fermentation systems has been described elsewhere (Harris *et al.* 1987; Pethig & Kell 1987; Davey *et al.* 1988; Stoicheva *et al.* 1989; Kell & Davey 1990; Kell *et al.* 1990; Markx & Kell 1990; Salter *et al.* 1990). The application of the same principle to solid-substrate fermentations is discussed in the accompanying paper (Davey *et al.* 1991), which also gives the details of the technique for fermentation monitoring using the Bugmeter (Aber Instruments, Science Park, Cefn Llan, Aberystwyth, Dyfed SY23 3DA, UK). The computerized system used in this study measures the capacitance at 0.3 MHz (in picofarads) as indicative of biomass, as well as the conductance and the pH, on-line and in real time during the incubation.

Identification of a Potassium Ion Deficiency in the Debittered Lupins

Approximately 0.15 ml of 1 M solutions of several mineral salts (some of which are shown in Figure 2) and of 1% (w/v) solutions of organic nutrients (such as glucose, yeast extract, etc.) were added to specific spots in plates containing inoculated debittered lupins. After 24 h of incubation at 31°C the extent of mycelial growth in the spots was assessed subjectively.

In addition, to obtain good tempe, the incorporation of K^+ into the debittered lupins was performed by adding 4 g of K_2SO_4 or 6.4 g of $KHSO_4$ to each litre of water in the final 15 min boiling of the dehulled, debittered lupins before inoculation.

Liquid Culture Fermentations

In general, the method of Graham *et al.* (1976) was followed to prepare the stock and working cultures of *R. oligosporus* NRRL 2710, UCW-FF8001 and UCW-

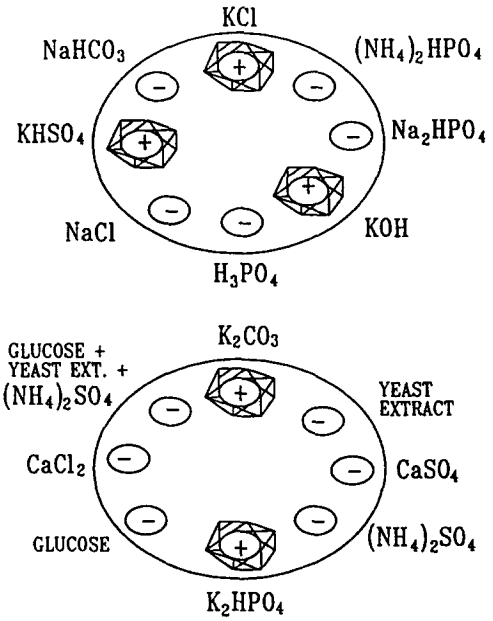

Figure 2. Mycelial growth of *Rhizopus oligosporus* after 24 h of incubation at 31°C on debittered lupins (*Lupinus mutabilis* Sweet) amended with drops of mineral salts and organic compounds. Incubations were carried out as described in the Materials and Methods section. (−) No growth; (+) good growth.

FF8002 on potato dextrose agar slants. The inocula were prepared by adding about 7 ml of sterile distilled water per sporulated slant culture and shaking for 30 s on a 'whirlimixer'. The spore suspension was centrifuged at 5000 × ***g*** for 5 min, the supernatant was discarded and the spores resuspended in sterile distilled water and again centrifuged. The process was repeated five times or more, under aseptic conditions, until the last supernatant was free of bits of mycelium. Analysis showed that residual K^+ from the agar was negligible. The spore concentration in the inoculum was determined by microscopic counting of spores in Mod-Fuchs Rosental chambers.

The composition of the basic liquid medium was as follows: glucose, 10 g; $(NH_4)_2SO_4$, 1.5 g; $MgSO_4.7H_2O$, 0.5 g; thiamine-HCl, 25 μg; mineral stock solution, 2 ml (giving a final concentration of 0.2 mg of each of the following mineral salts: $FeSO_4.7H_2O$, $MnSO_4.4H_2O$ and $ZnSO_4.7H_2O$); 400 ml of buffer pH 4 (a mixture of $Na_2HPO_4.12H_2O$, 0.2 M, and citric acid, 0.1 M); distilled water to make the total volume up to 1 litre. Salts such as K_2CO_3, K_2HPO_4 and KCl at various concentrations, as described in the Results section, were added to the medium as potassium sources. All chemicals used were of analytical reagent grade. The different liquid media were sterilized by filtration on 0.45 μm membrane filters (Millipore).

The sterile medium was inoculated at a ratio of 2×10^5 spores per ml of fresh medium. Then 100 ml of inoculated medium was aseptically distributed into 500 ml Erlenmeyer flasks. Incubation was at 37°C in a reciprocating shaker. The mycelium was recovered as described by Graham *et al.* (1976).

For the determination of potassium and other elements (phosphorus, nitrogen, calcium and magnesium) the samples were mixed with an acid-digestion solution in test tubes heated in aluminium blocks until the sample had been fully digested; after diluting the digest solution the analysis of the above elements was carried out simultaneously by using an Auto Analyzer (Faithfull 1971).

Results

The development of R. *oligosporus* mycelium on bitter lupins, i.e. those that had not been subjected to the debittering stage, and the appearance of the tempe cake were very good, and comparable to that found with soya beans.

The debittering stage using running tap water (pH about 8.3) at Aberystwyth

took 7 days or more, whereas in the Andes the alkaloids are traditionally removed from the bitter lupins in running water for about 3 days (Schoeneberger *et al.* 1987). A good quality tempe from lupins debittered at Aberystwyth was never obtained. The spores of *R. oligosporus* germinated well but mycelium growth was so poor that the debittered beans did not bind together into a cake, even after 48 h at 31°C. In contrast, an excellent lupin tempe was obtained with similar lupins debittered in the laboratory in Ecuador (Chavez & Peñaloza 1988).

After investigating most of the variables that are usually considered to cause trouble in making tempe, e.g. bacterial contamination [which has recently been found in soya bean tempe fermentations (Nout *et al.* 1985, 1987a,b; Tanaka *et al.* 1985; Samson *et al.* 1987; Molyowidarso *et al.* 1990)], the initial moisture content of the beans, the concentration of inoculum, the packing density of the substrate and so on, the last possibility, of some kind of nutrient deficiency due to the long period during which the lupins were soaked, was examined.

Various organic nutrients and inorganic salt solutions and combinations were added to specific spots in plates containing the inoculated debittered beans. Figure 2 shows that mycelial growth of *R. oligosporus* was abundant only around the spots containing added K^+. Other common nutrients, such as yeast extract, glucose, or nitrogen and phosphorus sources, did not improve mycelial growth. Although Na^+ and other cations have been claimed *partially* to replace K^+ (Steinberg 1946, cited by Lilly & Barnett 1951) they did not substitute for potassium ions in stimulating the growth of *R. oligosporus* mycelium. Indeed, provided that the plate contained at least one spot with a potassium salt, the full plate was colonized by mycelium after 48 to 72 h of incubation at 31°C.

The dramatic effect of K^+ in stimulating fungal biomass formation in solid-substrate fermentations is clearly shown in Figure 3. Biomass, measured as capacitance, varied considerably in the three types of substrate used: debittered lupins, potassium-supplemented debittered lupins and bitter lupins. Good mycelial development and a good-quality tempe cake were obtained only with the latter two lupins, in which the maximum change in capacitance was roughly five times higher than that of debittered lupins. This figure corresponded approximately to the difference in appearance of the final tempe obtained.

These findings strongly suggested that a potassium ion deficiency was the reason for the poor growth of *R. oligosporus* when debittered lupins were the substrate. Indeed, the potassium content of the substrate decreases during the preparation of lupins for human consumption as shown in Table 1, where it can be seen that potassium is gradually leached out in the different stages, from an initial content of 1.4% in dry seeds to a final concentration of 0.015% K^+ in the fully debittered lupins. Mg^{2+} was also leached out during this process, but its relative retention

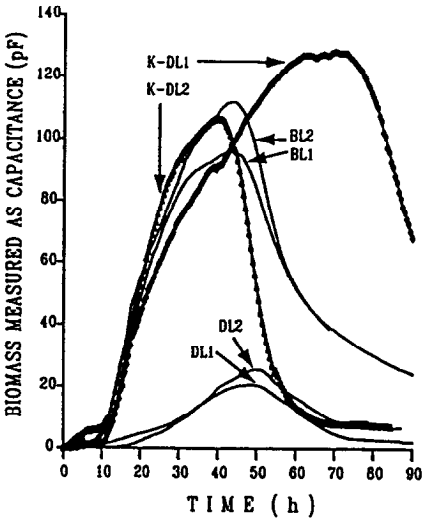

Figure 3. Time course of biomass formation of *Rhizopus oligosporus*, measured as the change in capacitance at 0.3 MHz during the solid-substrate fermentation of different lupin (*Lupinus mutabilis* Sweet) substrates. K-DL1 and K-DL2 are replicates of K^+-complemented debittered lupins; DL1 and DL2 are replicates of debittered lupins; BL1 and BL2 are replicates of bitter lupins processed for tempe as in Figure 1. Incubation was at 31°C and 85% relative humidity.

Table 1. Mineral composition of lupins (*Lupinus mutabilis* Sweet) during their processing (Figure 1) for human consumption and tempe fermentation with the fungus *Rhizopus oligosporus*.

Stage of lupin processing	Elemental composition (% w/w)				
	N	K	P	Ca	Mg
Raw (dry seeds)	6.8	1.4	0.78	0.14	0.33
Soaking	7.0	0.9	0.69	0.13	0.33
Cooking	7.4	0.6	0.63	0.13	0.23
Debittering	7.6	0.015	0.50	0.13	0.09
K^+-complemented lupins	7.6	0.5	0.49	0.13	0.09

Mineral contents were determined according to Faithfull (1971). K^+-complemented lupins refers to the debittered lupins, which were boiled at 100°C for 15 min in 4g/l K_2SO_4 solution, as described under Materials and Methods.

is much higher than potassium (about 30% of its initial value), phosphate suffers a minor loss, Ca^{2+} is stable while the nitrogen content is actually increased (in relative terms), from 6.8 to 7.6%. Hamman *et al.* (1986) showed that apart from carbohydrates, the ash content is reduced by 50% during the preparation of lupins in Egypt. Since the retention of phosphate and Ca^{2+} was nearly 100%, a K^+ loss is the most likely reason for the large reduction in ash content.

When a dilute K^+ solution was used instead of tap water for the final boiling of the debittered lupins, the K^+ content of the beans was restored to about 0.5% (Table 1), which is close to that of the original cooked (but still bitter) lupins. The appearance of the tempe obtained from both bitter lupins and K^+-supplemented, debittered lupins was similar, as can be also gauged from the data shown in Figure 3.

The effect of potassium salts and their concentration on the dry mycelium produced was then studied in shake-flask cultures. Table 2 shows that the addition of potassium at either 39 or 390 mg/l increases the dry mycelium produced to a similar level, the main difference being the potassium content of the dry mycelium. The three potassium salts tested gave similar results.

At lower concentrations of K^+ added to the liquid medium, a linear relationship between the original K^+ content of the medium and the dry mycelium produced was observed, within the range of 0 to 10 mg K^+/l. Higher K^+ concentrations did not increase the amount of dry mycelium produced, as shown in Figure 4. Thus in this case 10 mg K^+/l was the minimum to be added to the liquid medium. It corresponds to a basic requirement of 3 mg of K^+ per gram of dry mycelium to be produced. This requirement is substantially lower than those stated by Lilly & Barnett (1951): 150 and 40 mg/l for *Aspergillus niger* and *Penicillium chrysogenum*, respectively.

Although all the glassware for the liquid culture experiment was carefully rinsed at least three times with distilled water, the potassium in the impurities of the chemical constituents of the medium gave a residual K^+ concentration in the control medium of 0.5 mg/l (as measured according to the method described in the Materials and Methods section) which, together with the K^+ contained in the inoculum, would account for the slight mycelium growth in the control medium. There is also some evidence to indicate that the extent of accumulation of K^+ in the dry mycelium is increased as the K^+ concentration in the medium is raised (Figure 4).

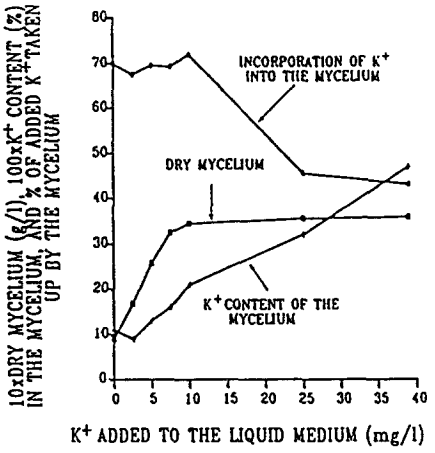

Figure 4. The effect of adding K^+ (as KCl) to the liquid medium on *Rhizopus oligosporus* UCW-FF8001 biomass and its K^+ content. The composition of the medium is given in the Materials and Methods section. A spore inoculum was provided at a level of 2×10^5 c.f.u./ml and incubation at 37°C for 48 h. The potassium concentrations are given on the basis of K^+.

Discussion

Potassium is generally believed to have a high mobility in living plants (Munson 1985), and it is possible that the high pH of running tap water during the

Table 2. Effect of the potassium source and its concentration on the amount of dry *Rhizopus oligosporus* mycelium produced in liquid shake-flask cultures at 37°C for 48 h. The potassium content (%) in the dry mycelium is also shown.

Potassium source	Concentration (mg/l)	Strain: NRRL2710		Strain: UCW-FF8002	
		Dry mycelium (g/l)	K^+ (%)	Dry mycelium (g/l)	K^+ (%)
Blank	—	0.81	0.06	0.69	0.06
K_2CO_3	39	3.24	0.42	2.95	0.41
	390	3.41	0.81	3.30	1.00
KCl	39	3.13	0.35	3.40	0.39
	390	3.08	0.48	3.33	0.88
K_2HPO_4	39	2.98	0.39	3.08	0.39
	390	3.06	0.77	3.06	0.84

debittering stage of lupins led either to a preferential leaching of K^+ (Table 1) or simply that at high pH (8.3 of tap water in Aberystwyth) the alkaloids (being bases) are removed more slowly than in South America, and that consequently the potassium required to support the fungal growth is depleted due to the longer period of soaking. In fact, Lucisano *et al.* (1984) observed that the extraction of alkaloids from *L. mutabilis* was improved when hydrochloric acid was added to the solvents.

In green plants potassium has many functions; one that is well established is turgor-pressure-dependent growth (Munson 1985; Glaser & Donath 1988). A similar situation is thought to exist in fungi (Lysek 1984; Jennings 1979). The concentration of K^+ along the fungal hypha is not uniform, the lowest being found at the apex. It is believed that K^+, after being absorbed into the hypha, is driven to the tip. The net consequence of this is a flow of water and vesicles along the hypha to the tip to support the extension and growth of the youngest compartments of the hyphae (Jennings 1979). Since the exact organization and dynamics of the hyphal cytoplasm in the apical region have not been completely elucidated (Bracker & Lopez-Franco 1990), it is difficult to establish the precise role of potassium in the mechanics of hyphal extension and growth. It is very plausible that the uptake and efflux of potassium ions, protons and calcium ions are important for the control of apical growth (Gow 1990). Under conditions of a very low concentration of K^+ in the medium, the relevant transport processes, and hence growth, can not proceed. This in turn could provide a mechanistic explanation of the poor mycelial development of *R. oligosporus* in both potassium-depleted lupin (debittered lupin) and in liquid cultures with less than 10 mg K^+/l.

In the solid substrate, an overall K^+ content of about 0.5% was necessary for complete mycelium development. Since *R. oligosporus* does not penetrate deeply into the cotyledons (Jurus & Sundberg 1976), sufficient potassium must be at or near the colonizing surface, which in turn may explain the relatively high K^+ requirement of 0.5% in the beans compared with 10 mg K^+/l for liquid cultures. It is also probable that in the solid substrate much of the potassium is simply unavailable, since, given that it takes several days to deplete the lupins of potassium, it may be assumed that there are many binding sites for this element in the substrate. All of these points should be taken into account when using materials whose preparation may lead to a preferential leaching of K^+.

Finally, as shown in the accompanying paper (Davey *et al.* 1991) and in Figure 3, the on-line measurement of fungal biomass (through capacitance readings) proved to be extremely useful in establishing the effect of K^+ on mycelium growth of *R. oligosporus* in solid-substrate fermentations. It is evident that the same technique could usefully be applied to further studies of fungal physiology.

Acknowledgements

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