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SUPPRESSION OF PENETRATIVE HYPHAE OF *RHIZOPUS OLIGOSPORUS* BY MEMBRANE FILTERS IN A MODEL SOLID-STATE FERMENTATION SYSTEM

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SUMMARY

Membrane filters overlaid on slabs of a model solid substrate enabled recovery of biomass of *Rhizopus oligosporus*. Although the presence of the membrane filter affects the growth of *Rhizopus oligosporus* it provides a useful tool for studying solid-state fermentation.

INTRODUCTION

A major obstacle to the study of fungal growth in solid-state fermentation (SSF) has been the inability to measure directly the biomass because of the close association between the mycelium and the substrate. Mitchell *et al.* (1986, 1988a) developed a model solid substrate consisting of cassava starch and other nutrients embedded in a kappa-carrageenan gel matrix. The model substrate could be removed by dissolving the gel matrix with heat and enzymatically digesting the starch, enabling the biomass to be recovered by filtration. However, the recovery procedure resulted in the loss of significant amounts of biomass (Mitchell *et al.*, 1988a).

The present paper describes the use of membrane filters to suppress penetrative hyphae during growth of *Rhizopus oligosporus* on the model solid substrate, enabling complete and rapid biomass recovery.

MATERIALS AND METHODS

Microorganism, maintenance and inoculum preparation: These were performed as described by Mitchell *et al.* (1986).

Membrane filter culture: The model substrate was the MZ-2N model substrate of Mitchell *et al.* (1988b) but the starch concentration was varied. After gelatinization the substrate was pressed into plastic dishes of 4 cm diameter and 5 mm depth. Once set, the substrate was overlaid with a wet 47 mm diameter polycarbonate Nucleopore membrane filter. A wire loop was used to spread 0.1 ml inoculum over the filter surface. The dishes were incubated at 37 ^{o}C, within a 9 l airtight plastic box to prevent excessive moisture loss. Triplicate dishes were removed for analysis. Samples of known area were cut, the membrane filter was discarded and the sample was weighed. Protein was determined by the Folin reaction and starch by digestion with amyloglucosidase, as described by Mitchell *et al.* (1986). Cross-sections of the slab were stained with iodine (0.3% iodine plus 3% potassium iodide) and observed microscopically at 100x magnification for the clearing of starch.

RESULTS AND DISCUSSION

Effect of membrane filter pore size

Low initial starch concentrations of 5 and 15 g starch per 100 ml water were used, compared with the concentration of 25 g starch per 100 ml water in the MZ-2N model substrate of Mitchell *et al.* (1988b). The aim was to make any limitations due to the membrane filters more obvious.

For both initial starch concentrations membrane filter pore sizes from 0.1 to 1.0 µm had no significant effect on either the depth to which starch was cleared or the fresh weight of biomass produced after 24 h of growth of *R. oligosporus*. However, in both cases the depth of clearing of starch was significantly greater in the absence of a membrane filter. Biomass production could not be compared because, without a membrane filter, the fresh biomass could not be separated from the substrate. Note that at the lower initial starch concentration some hyphae managed to penetrate through the 0.8 and 1.0 µm pores.

The presence of a membrane filter therefore decreases the starch-clearing activity of *R. oligosporus*. This is not due to restricted diffusion of the amylolytic enzymes through the membrane filter since pore size had no effect. Penetrative hyphae must be important. They might serve to decrease the distance over which the amylases must diffuse to reach the starch. Alternatively, firm anchorage of the mycelium by the penetrative hyphae may promote growth.

Since pore size did not affect starch-clearing activity, membrane filters of 0.2 µm pore size were used in the subsequent studies to ensure no hyphae could penetrate into the substrate.

Growth in membrane filter culture

During the growth of *R. oligosporus* in membrane filter culture the model substrate nearest the biomass became cloudy as opposed to the originally translucent appearance. Since this was obviously a consequence of the growth, the cloudy and translucent regions were partitioned into separate samples. The mycelium comprised a third sample. Whole samples containing all the substrate plus the mycelium were also analyzed.

Fig. 1 shows the fresh weights of the various samples during growth of *R. oligosporus* in membrane filter culture on the model substrate (MZ-2N with 25 g starch per 100 ml water). The decrease in the weight of the whole samples is due largely to conversion of the substrate into carbon dioxide. During growth the influence of the fungus on the substrate increased - the cloudy region comprised an increasing proportion of the model substrate.

Analysis of whole samples (Fig. 2) showed starch utilization was intially undetectable but later quite rapid. The glucose concentration increased slowly. A protein content of 9.9 mg per gram of sample was achieved by 38 h.

The partitioned samples gave an insight into the starch utilization. Not only did the cloudy region of the substrate increase in size as shown in Fig. 1 but also the starch concentration in this region decreased rapdidly (Fig. 3). This region contained significant glucose, a product of starch hydrolysis. The translucent region decreased in size (Fig. 1) but the starch concentration only began to fall at 38 h (Fig. 3). Therefore the translucent region consists of model substrate which the amylases excreted by the fungus have not yet reached. However, the change in substrate appearance from translucent to cloudy is not necessarily a direct consequence of amylase action. The slight decrease in starch concentration in the translucent region at 38 h indicates that

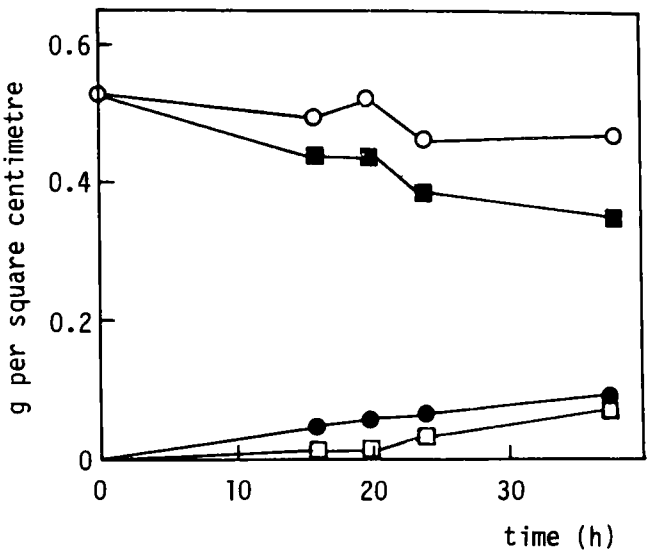

Fig. 1. Fresh weights of samples removed from membrane filter culture of *R. oligosporus*: (○) whole samples; (□) mycelium; (●) cloudy region of model substrate; (■) translucent region of model substrate.

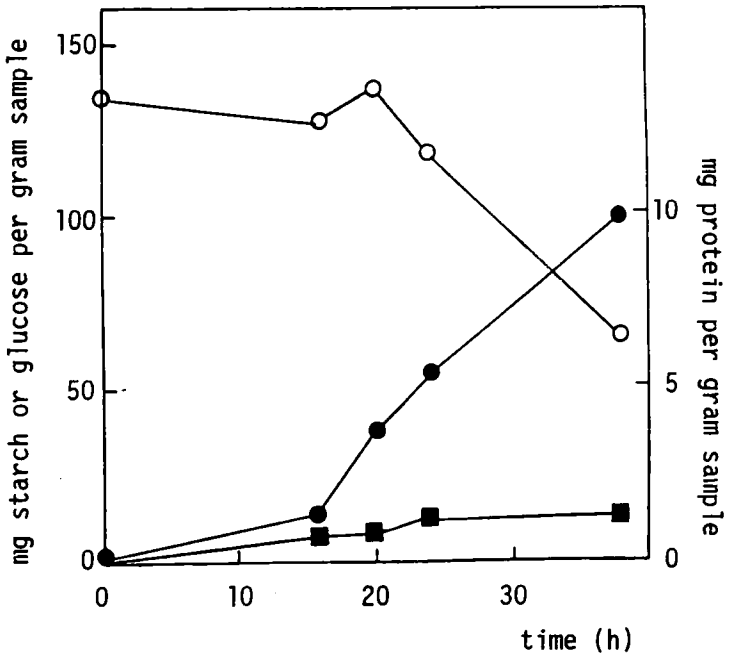

Fig. 2. Protein production and substrate utilization during membrane filter culture of *R. oligosporus*, as determined by analysis of whole samples: (○) starch; (■) glucose; (●) protein.

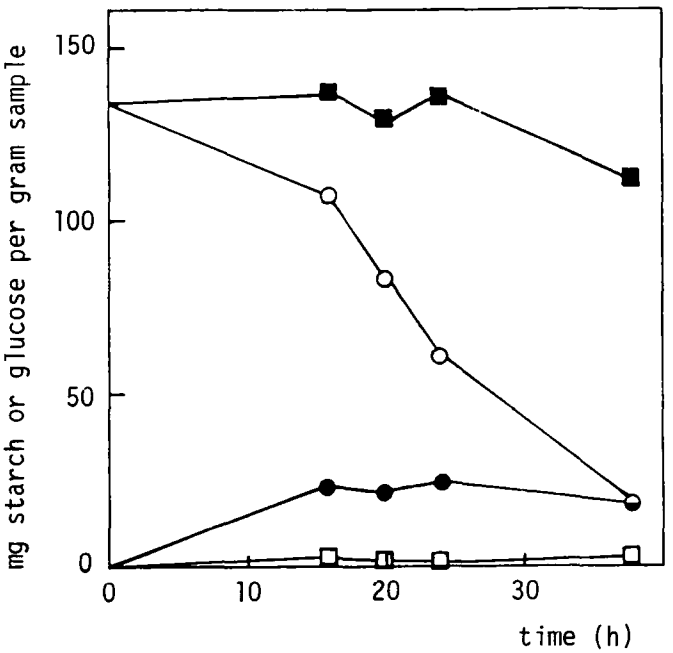

Fig. 3. Distribution of substrate utilization during membrane filter culture of *R. oligosporus*, as determined by analysis of partitioned samples: (O) starch in cloudy region of model substrate; (■) starch in translucent region; (●) glucose in cloudy region; (□) glucose in translucent region.

the boundary between the cloudy and translucent regions was becoming less sharp. Low glucose concentrations were detected in the translucent region. It must have diffused from the site of amylase action in the cloudy region. Note that most of the glucose released from the starch would diffuse to the surface where it would be metabolized by the mycelium.

Although the cloudy region was almost completely cleared of starch it accounted for only one fifth of the substrate at 38 h. To maximize the conversion of starch to protein much thinner substrate slabs must be used.

The protein content of the mycelium decreased as it aged, from 9.9% of the fresh mycelial weight at 16 h to 6.1% at 38 h. A decrease in mycelial protein content with age was also observed in an earlier study (Mitchell *et al.*, 1986).

Some protein was found in the model substrate and some starch and glucose in the mycelium, as shown in Fig. 4. The protein in the substrate was at the limit of the sensitivity of the assay. It would likely consist mostly of amylases. The starch in the mycelium is probably comprised of small oligosaccharides which have diffused from the substrate - the method of starch determination lumps together all the starch hydrolysis products except glucose. The presence of both glucose and oligosaccharides suggests *R. oligosporus* produces both glucoamylase and alpha-amylase, although this remains to be confirmed.

Implications of membrane filter culture in study of SSF

The idea of using membranes to suppress the penetrative hyphae of fungi growing on solid substrates is not new. Georgiou & Shuler (1986) proposed a model for fungal colony growth and differentiation on agar with glucose as the substrate. They suggested the use of a membrane

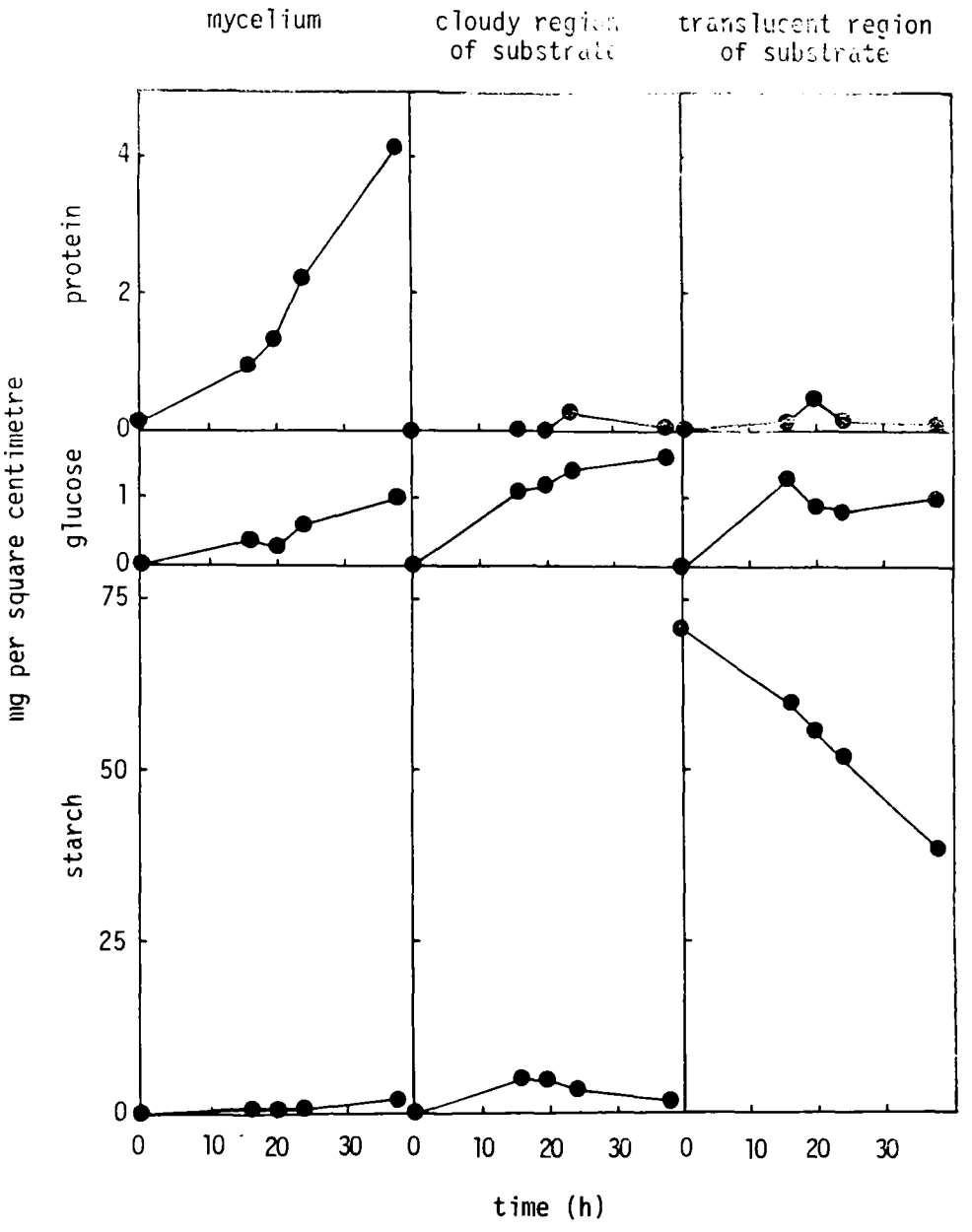

Fig. 4. Distribution of starch, glucose and protein during membrane filter culture of *R. oligosporus*.

filter to suppress penetration to simplify the model. They also briefly considered overculture which better represents the system in SSF where during inoculation a large number of spores are distributed across the substrate surface.

The present system is more complex than that of Georgiou & Shuler (1986) in that starch is not diffusible and therefore its utilization depends on the extra steps of amylase diffusion and action before the soluble sugars can diffuse back to the mycelium and be utilized.

The presence of the membrane filter limits the geometry of the solid substrate to a flat slab to ensure close contact between the membrane and the substrate surface. This does not represent the geometry of natural substrates, particles of which may not be flat and may have rough surfaces. However, extrapolation between systems is possible by use of the characteristic length of the particle (the ratio of volume to surface area). A further disadvantage of modelling SSF by membrane filter culture is that the membrane filter adversely affects growth.

Membrane filter culture has some advantages and presents an opportunity to study important aspects of SSF. The ability to recover the biomass rapidly and completely is a major advantage, since biomass estimation has been a significant problem in SSF. Tests can be performed on biomass which has changed minimally from its condition in the membrane filter culture - this is especially valuable in metabolic studies. Model substrates can be developed which nutritionally mimic natural substrates more closely, because components with insoluble residues can be incorporated. The original model substrate of Mitchell *et al.* (1988a) was limited to cassava starch rather than cassava flour because insoluble residues in the cassava flour interfered with the biomass recovery method (Mithell *et al.*, 1988b).

Complete removal of the mycelium from the substrate without either being destroyed allows the distribution of components to be determined. The biomass recovery method of Mitchell *et al.* (1986) not only destroyed the substrate but also leached soluble compounds from the biomass. In the present study the distribution of starch and protein were examined, but of more interest is identification and localization of the amylases present and the products of their action on starch. Metabolic byproducts excreted by the fungus can also be located. Purification of extracellular enzymes from membrane filter culture would be easy since the potentially contaminating proteins in the biomass are easily removed. With SSF of natural solid substrates the fungal biomass cannot be removed and harsh extraction procedures such as crushing or homogenizing the fermenting substrate would liberate intracellular proteins.

In conclusion, membrane filter culture provides a useful tool for studying fundamental aspects of fungal growth in SSF.

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