Short Communication

Growth of *Rhizopus arrhizus* in fermentation media

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SUMMARY

Rhizopus arrhizus biomass attached itself to fermentor walls, baffles and impellers when grown in casein/ glucose media. In shake flasks, dispersed filamentous growth was produced in media containing certain concentrations of glucose and soya flour. Other media tested produced pelleted or clumpy growth. Medium initial pH did not affect morphology type. Dispersed growth could not be obtained by addition of detergents, oils and polymers to a clear glucose/soya peptone medium. Addition of maize solids to this medium resulted in dispersed growth which occurred even in the presence of calcium, which in most media caused pellet formation. Mycelia appeared to bind to the maize particles and use these as growth centres, thereby preventing pellet or clump formation. Mycelial pellets appeared to originate either from a single spore or by interaction of branched hyphae from different spores. Medium composition and macro-morphology type correlate with differences in hyphal structures.

INTRODUCTION

Species of *Rhizopus* produce commercially interesting alkaloids [24], enzymes [13], fumaric acid [20,21], sterols [28] and terpenoids [29] and can also catalyze the biotransformation of many steroids [23], cyclic hydrocarbons [24], pesticides [16,25] and herbicides [11,26]. Traditionally, *Rhizopus* species

have been grown in surface culture fermentations on steamed soya bean media for production of tempeh [1]. In submerged culture, *R. arrhizus* may grow in different morphological forms, depending on environmental conditions [2]. Product formation in fungal fermentations may be influenced by growth morphology. Filamentous growth is preferred for production of fumaric acid by *R. arrhizus* [17], for pectic enzyme production by *Aspergillus niger* [5] and single cell protein (SCP) formation by *Fusarium graminearum* [22]. Higher specific growth rates are

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achieved with filamentous growth as fungal growth in pellets is normally confined to the external region [14,18,27]. Both filamentous and pelleted growth have been used for penicillin production [15,17] whereas pelleted growth is preferred for production of itaconic acid by *A. terreus* and citric acid production by *A. niger* [30]. We have previously shown that the physical properties of pellets may be controlled [3] and that anionic polymers promote dispersed growth [2]. In this paper we describe morphologies of *R. arrhizus* obtained in laboratory fermentors and in shake flasks containing a variety of media and some relationships between culture media, morphological appearance and hyphal structures.

MATERIALS AND METHODS

Inoculum preparation and culture conditions

R. arrhizus ATCC 10260 was cultured on yeast-malt extract agar (yeast extract, 4 g/l; malt extract,

10 g/l; glucose, 4 g/l; Oxoid Agar No. 3, 20 g/l; pH 7.0), 20 ml in a 250 ml Erlenmeyer flask, for 4 days at 30°C. Spores were washed from the culture with 20 ml of Triton X-100 (0.1 ml/l) under sterile conditions, counted according to standard procedures [6], and adjusted to give $1-3 \times 10^7$ spore/ml. Shake flask experiments were carried out in 250 ml Erlenmeyer flasks, containing 100 ml culture medium. Medium formulations are given in Table 1. Cultures were incubated at 30°C on an orbital shaker-table at 150 rpm (50 mm displacement). Laboratory fermentor experiments were carried out in 7.5 liter Laboferm fermentors (New Brunswick Scientific) containing 5 liters of media. Aeration rate was 0.5 v/v/m and temperature was 30°C. Stirred fermentors contained three impellers (diameter 5.0 cm) and four baffles (width, 4.0 cm). Erlenmeyer flask and fermentor media were sterilised by autoclaving at 15 p.s.i. (121°C) for 20 min and 35 min respectively.

Analytical methods

The method for biomass determination was de-

Table 1

Effect of medium constituents on culture morphology

| Medium No. | Constituents (g/l) | | | | | | | pH | | Growth morphology[a] |
	glucose	hydrolysed casein	yeast extract	soya peptone	soya flour	corn steep solids	CaCO$_3$	initial	final	
1	45	13	–	–	–	–	–	6.1	4.1	4
2	10	5	–	–	–	–	–	6.4	6.7	4
3	10	13	–	–	–	–	–	6.4	6.8	4
4	80	13	–	–	–	–	–	6.0	3.9	3
5	80	5	–	–	–	–	–	6.0	3.6	1
6	10	–	5	–	–	–	–	6.0	3.1	2
7	10	–	–	5	–	–	–	6.1	3.3	4
8	20	–	–	5	–	–	–	5.9	3.2	4
9	10	–	–	–	–	10	–	6.2	3.9	4
10	10	–	–	–	10	–	–	6.3	4.1	4
11	20	–	–	–	10	–	–	6.0	3.7	4
12	30	–	–	–	10	–	–	6.0	3.7	D[b]
13	45	–	–	–	10	–	–	6.0	3.6	D
14	45	–	–	–	10	–	10	7.3	5.7	2

[a] 1 – 4 are morphology types illustrated in Fig. 2.
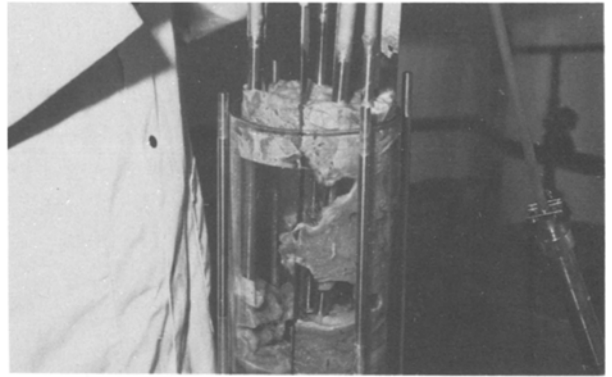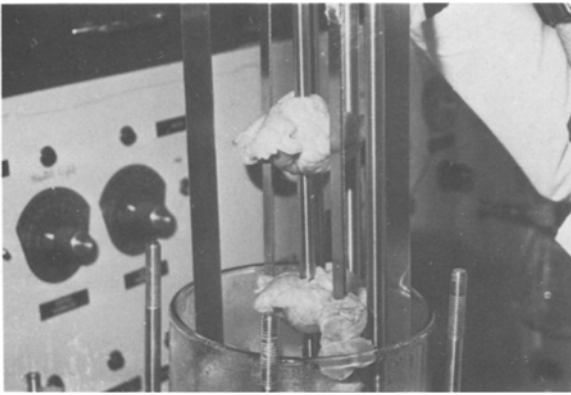[b] D = dispersed growth.

Fig. 1. Growth of *R. arrhizus* attaching to walls, baffles and impellers in Laboferm fermentors.

scribed previously [2,4]. Fungal morphology was observed in glass petri dishes. Growth was taken to be fully dispersed when dilution of a culture sample in ten times its volume produced a relatively homogeneous filamentous suspension. Microscopic observations and photomicrographs were carried out on wet-mounted slides using a Nikon Optiphot phase contrast microscope. Hyphal measurements were made using a micrometer attached to the microscope at 100 × magnification and by determining the average values from at least 20 representative hyphae.
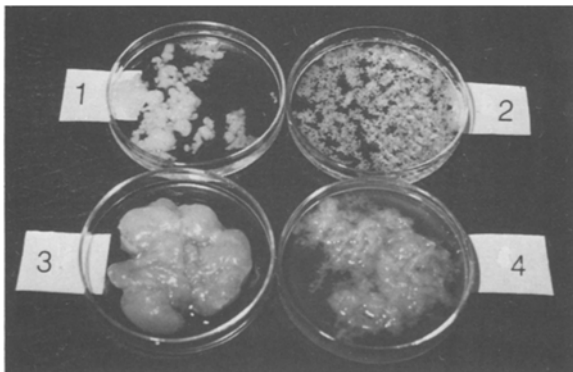

Fig. 2. Growth morphologies of *R. arrhizus* in shake-flasks using a range of media. Growth media are described in Table 1. 1, pelleted growth; 2, discrete pellets; 3, clumped, coalesced growth; 4, clumpy growth.

RESULTS

Laboratory fermentors containing medium 1 were inoculated with a 10% dispersed filamentous vegetative inoculum or a spore inoculum (4×10^3 spore/ml of culture). In all instances, growth attached itself to walls, baffles and impellers after 24 h (Fig. 1). When baffles and impellers were removed and aeration/agitation supplied using a ring sparger (6 cm diameter with 12 perforations), clumped growth was observed but growth remained in the medium without adhesion to fermentor surfaces. Because of the operational problems associated with clumped or surface-bound mycelium, the effect of some medium constituents on culture morphology was investigated. Shake flasks were inoculated with 2×10^5 spore/ml of culture and incubated for 24 h. The main morphology types observed, other than dispersed homogeneous growth, are illustrated in Fig. 2 and the effect of medium constituents on morphology are shown in Table 1. Dispersed filamentous growth was only produced in media containing soya flour (1%) plus high levels of glucose (3 or 4.5%). All other media gave pelleted or clumped morphology. While the final pH of these cultures varied, we have also shown that altering the initial pH in the range 3.4–7.8 does not affect morphology type.

The effect of detergents, oils and polymers on mycelial growth and morphology was determined

Table 2

Effect of medium supplements on growth morphology of *R. arrhizus*

Supplement	Content per litre	pH		Dry weight	Growth morphology[a]
		initial	final		
Control		5.9	3.2	180	4
Triton X-100	1.0 ml	6.0	4.2	158	2
Span-20	1.0 ml	5.9	3.5	157	3
Span-20	4.0 ml	5.9	3.6	179	1/4
Sodium deoxycholate	0.1 g	6.0	3.2	191	3
Sodium deoxycholate	0.6 g	5.8	3.8	157	2
Oleic acid	1.0 ml	5.9	3.3	216	3
Soya bean oil	20.0 ml	5.9	3.2	n/m	3
Peanut oil	20.0 ml	5.9	3.2	n/m	3
Polyethylene glycol	3.0 g	5.9	3.3	214	3
Polyvinyl alcohol	3.0 g	5.9	3.3	178	3
Methyl-cellulose	20.0 g	6.3	2.7	n/m	1/4
Dextran	20.0 g	5.8	3.0	187	3

[a] 1 – 4 are morphology types illustrated in Fig. 2. 1/4 is a clumped-pelleted morphology intermediate between types 1 and 4.

after a 24 h incubation using a clear medium (No. 8) to facilitate biomass measurement (Table 2). All supplements tended to increase pelleting or coalescence. Addition of maize solids to medium 8 resulted in dispersed growth of *R. arrhizus*, even in the presence of calcium ions, which in most media cause pellet formation. On microscopic examination, mycelia appear to bind to the maize particles and use these as growth centres, thus appearing to prevent formation of pellets or clumps.

The sequence of events leading to pellet formation was followed microscopically in a variety of media inoculated with 2×10^5 spores/ml. Germination of spores occurred in most media after a 6 h incubation. Hyphae became elongated and branched, forming mycelial conglomerates from 9 h
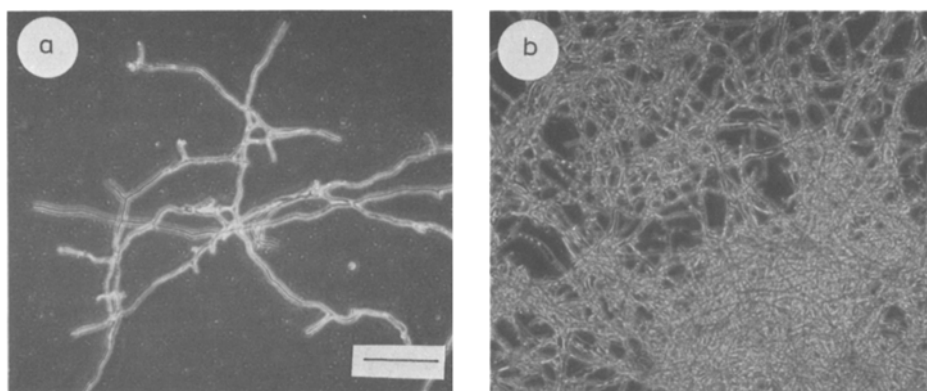

Fig. 3. Photomicrographs of hyphae grown from spores in medium 1. (a) After 6 h. (b) During pellet formation. Bar = 50 μm.

Table 3

Hyphal structures formed by *R. arrhizus* in different media

Medium	Supplement and growth morphology (t = 24 h)	Hyphae, t = 12 h					Hyphae, t = 24 h				
		total length (μm)	primary branches		secondary branches		total length (μm)	primary branches		secondary branches	
			No.	length (μm)	No.	length (μm)		No.	length (μm)	No.	length (μm)
8	0.1% (v/v) Triton X-100, discrete pellets	Spores germinating					173.3	6 (few free hyphae)	55.0	2	25.0
8	1.25 mM MgSO$_4$, discrete pellets	400.0	5	50.0	–	–		(no free hyphae)			
8	– clumpy growth	650.0	5	179.0	2	50.0	387.5	6	71.0	2	26.6
8	Na-alginate 20 g/l, dispersed filamentous growth	830.0	6	212.7	6	113.6	725.0	9	201.7	2	50.0
8	CM-cellulose 40 g/l, dispersed filamentous growth	790.0	7	234.3	8	25.0	800.0	7	350.0	4	230.0
11	– dispersed filamentous growth	480.0	6	56.5	1	25.0	837.5	14	54.0	7	12.5

onwards. Photomicrographs illustrating hyphal appearance in medium 1 after 6 h and later during pellet formation are presented in Fig. 3. Hyphal structures in different culture media were characterised after 12 and 24 h (Table 3). The results indicate that *R. arrhizus* hyphae grew much longer in media supplemented with polymers. Shorter, less branched hyphae were observed mainly in media leading to pelleted growth. Hyphae in polymer-supplemented media appeared broader under the microscope. Hyphae in media giving pelleted growth appeared to be thinner. After a 12 h incubation, hyphae formed in soya media were shorter compared with hyphae from media supplemented with polymers. After a 24 h incubation, however, longer hyphae were observed in soya media with a higher frequency of branching.

DISCUSSION

The results reported above illustrate some of the problems encountered in achieving relatively homogeneous dispersed growth of *R. arrhizus* in submerged culture. While many filamentous fungi produce a ring of mycelial growth at the top edge of the medium in shake flasks or fermentors [5], the fermentor and impeller wall growth observed with *Rhizopus* was much more severe and tended to draw most of the biomass from the medium. In general,

pellet formation is undesirable in cultures to be used for inoculum development, since growth rates are reduced and unwanted growth lags may be encountered [5]. Use of relatively insoluble medium constituents appears to counteract pellet formation. In the production fermentor, it is essential to establish optimal fungal morphology for product formation. Pelleted morphology can be advantageous in reducing medium viscosity in large-scale fermentors [17].

Our results suggest that in *R. arrhizus* cultures, pellet formation appears to originate either from a single spore or by interaction of branched hyphae arising from different spores. The high capacity of *Rhizopus* hyphae to bind metals [7] suggests that surface charge may also play a role in pellet formation. In the case of *Aspergillus,* there is evidence to suggest that pellet formation arises from the association of a number of spores prior to germination and that this process is pH-dependent [10,2]. pH also influences pellet formation in *Penicillium chrysogenum* [19]. In contrast, pH does not appear to have a major influence on *Rhizopus* morphology.

Medium composition and macro-morphology type also reflect differences in hyphal structures. Pellet formation by *P. chrysogenum* was linked with more extreme aberrant hyphal forms such as swollen, distorted, short hyphae [8,9,19].

A greater understanding of the phenomena influencing fungal morphology in submerged culture and the relationship between morphology and fungal productivity should significantly benefit fermentation research and development. We are now proceeding to further characterise *Rhizopus* morphologies by electron microscopy and cell wall analysis.

ACKNOWLEDGEMENT

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