Growth of Anaerobic Rumen Fungi on Defined and Semi-defined Media Lacking Rumen Fluid

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Anaerobic fungi were isolated from rumen digesta of sheep and cattle and were purified using a plate culture technique. The isolates were successfully cultured on a semi-defined medium which lacked rumen fluid, and on a defined medium.

INTRODUCTION

Orpin isolated the obligate anaerobe Neocallimastix frontalis from the rumen of sheep and suggested that it was related to chytrid fungi (Orpin, 1975, 1977). N. frontalis was recently assigned to a new family (the Neocallimasticeae) in an order (the Spizellomycetales) of the Class Chytridomycetes (Heath et al., 1983).

At least three different methods have been used to isolate rumen fungi. Orpin (1975) isolated N. frontalis from sheep by overlaying sloppy agar medium, which contained antibacterial antibiotics, with a sample of rumen digesta. After incubation, the top layer of the culture was removed by aspiration and the lower layer, which contained zoospores, was overlaid onto fresh sloppy agar medium. The culture was then shaken gently to distribute the zoospores throughout the upper layer of the medium and was incubated again. Fungal biomass, formed in the top layer of these cultures, was transferred to fresh medium and the whole procedure was repeated until pure cultures were obtained. The isolation procedure of Joblin (1981) and Akin et al. (1983) involves straining rumen fluid through muslin, mixing the filtrate with molten agar medium plus antibiotics and preparing roll tubes (Hungate, 1969). Finally, Bauchop & Mountfort (1981) used strained rumen fluid to make enrichment cultures in sloppy agar medium containing antibiotics. After three subcultures in this medium, the culture was transferred to liquid medium and single colonies were picked up with a syringe and washed in buffer. Methanogenic bacteria, which were not eliminated by the first antibiotic treatment, were then removed using chloramphenicol.

The present paper outlines an isolation method which involves the use of plate cultures, and describes the development of semi-defined and defined media for growth of rumen fungi.

METHODS

Chemicals and solid substrates. All chemicals were of Analar grade and unless stated otherwise were obtained from BDH and made up in glass-distilled water. Except for biotin and 1,4-naphthoquinone (BDH) and neomycin (Pfizer) all vitamins and antibiotics were obtained from Sigma. Insoluble substrates included Avicel (Schleicher & Schüll), Whatman no. 1 filter paper and milled barley straw (cv. Sonja). Whatman no. 1 filter paper and milled barley straw were used for cellulose overlay plates and was treated with phosphoric acid (Wood, 1971) and then pebble milled for 4 d at 4 °C.

Preparation of media. Media were prepared, stored and inoculated using the aseptic, anaerobic techniques of Hungate (1950), Bryant (1972) and Miller & Wolin (1974). Except for the reducing agent solution (prepared and dispensed under oxygen-free N2), liquid media and solutions used to prepare media were dispensed under oxygen-
free CO₂. Two different types of media were used; medium A, which contained rumen fluid, and medium B, which did not. Solid media were prepared by adding Oxoid bacteriological agar no. 1 (final medium concentration, 18 g l⁻¹) to basal solution.

Medium A. Medium A contained, per litre: basal solution A, 830 ml; water (when insoluble substrates were used) or glucose solution (37.5 g l⁻¹), 100 ml; Na₂CO₃ solution (80 g l⁻¹), 50 ml; vitamin solution, 10 ml; and reducing agent solution, 10 ml. Sometimes 60 ml of the water was replaced with 50 ml antibiotic solution and 10 ml lysozyme solution.

Basal solution A contained: yeast extract, 2 g; Trypticase peptone, 2 g; clarified rumen fluid (Bryant & Robinson, 1961), 150 ml; mineral salts solution [as described by Leedle & Hespell (1980) except that the nitrogen source was 0.54 g NH₄Cl l⁻¹], 75 ml; K₂HPO₄ solution (6 g l⁻¹; Leedle & Hespell, 1980), 75 ml; haemin solution, 10 ml; fatty acid solution, 10 ml; resazurin solution (1 g l⁻¹), 1 ml. The pH of basal solution A was adjusted to 6.8 with 1 M-KOH and the volume was made up to 830 ml with water or a suspension of cellulose (final medium concentration 10 g l⁻¹) for the cellulose overlay plates. Haemin solution was prepared by dissolving 0.1 g haemin in 10 ml ethanol and adjusting the volume to 1 litre with 0.05 M-NaOH. The fatty acid solution was prepared by mixing 6.85 ml acetic acid, 3.0 ml propionic acid, 1.84 ml butyric acid, 0.55 ml 2-methylbutyric acid, 0.47 ml isobutyric acid, 0.55 ml valeric acid, and 0.55 ml isovaleric acid with 700 ml 0.2 M-NaOH. The pH of the fatty acid mixture was adjusted to 7.5 with 1 M-NaOH and its volume was adjusted to 1 litre with water.

Vitamin solution was prepared in 5 M-MOPS buffer and contained (g l⁻¹): 1,4-naphthoquinone, 0.25; calcium D-pantothenate, 0.2; nicotinamide, 0.2; riboflavin, 0.2; thiamin. HCl, 0.2; pyridoxine. HCl 0.2; biotin, 0.025; folic acid, 0.025; cyanocobalamin, 0.025; and p-aminobenzoic acid, 0.025. The reducing agent solution contained 2.5 g Na₂S, 9H₂O and 2.5 g L-cysteine. HCl in 100 ml water. The antibiotic solution contained (g l⁻¹): streptomycin sulphate, 2; penicillin G, 8; chloramphenicol, 6; oxytetracycline, 5; neomycin sulphate, 6. The lysozyme solution contained lysozyme (4 g l⁻¹) plus EDTA (disodium salt; 3 g l⁻¹).

Basal solution A, Na₂CO₃, glucose, water and the reducing agent solution were pre-reduced (boiled and gassed with oxygen-free CO₂) whilst basal agar A was steamed for 40 min and then pre-reduced.

Medium B. Medium B was prepared as described for medium A, except that 810 ml basal solution B, 10 ml KH₂PO₄ solution (68 g l⁻¹) and 10 ml yeast extract solution (50 g l⁻¹) replaced the 830 ml of basal solution A.

Basal solution B contained: KCl, 0.6 g; NaCl, 0.6 g; MgSO₄, 7H₂O, 0.5 g; CaCl₂, 2H₂O, 0.2 g; NH₄Cl, 0.54 g; Trypticase peptone, 1 g; PIPES buffer, 1.5 g; coenzyme M solution, 10 ml; fatty acid solution, 10 ml; trace element solution, 10 ml; haemin solution, 10 ml, resazurin solution (1 g l⁻¹), 1 ml. The pH of the solution was adjusted to 6.8 with 1 M-KOH and the volume was made up to 810 ml with water or with a suspension of cellulose. Coenzyme M solution was prepared by dissolving the sodium salt of 2-mercaptopoethane sulphonic acid in water to give a concentration of 4 g l⁻¹. The trace element solution was prepared in 0.2 M-HCl and contained (g l⁻¹): MnCl₂, 0.25; NiCl₂, 0.25; NaMoO₄, 2H₂O, 0.25; H₃BO₃, 0.25; FeSO₄. 7H₂O, 0.20; CoCl₂. 6H₂O, 0.05; SeO₂, 0.05; NaVO₃, 4H₂O, 0.05; ZnCl₂, 0.025; CuCl₂. 2H₂O, 0.025. All other solutions were the same as for basal solution A.

The KH₂PO₄ and yeast extract solutions were not pre-reduced prior to addition to the final medium.

Sterilization and preparation of liquid and solid media. The antibiotic, lysozyme and vitamin solutions were sterilized by membrane filtration (0.22 μm pore diameter). Glucose solutions were autoclaved at 115 °C for 10 min and all other solutions and agar media were autoclaved at 121 °C for 15 min.

Liquid media were dispensed in 10 ml volumes in thick-walled glass tubes or in 100 ml volumes in 125 ml serum bottles. For cultures grown on milled barley straw, 0.1 g substrate was added to each tube and was autoclaved at 121 °C for 15 min prior to the addition of 10 ml medium. Petri dish (4.7 cm diameter) cultures contained 2 ml agar medium in which water replaced the glucose solution, and this layer was overlaid with 2 ml cellulose agar medium. Plates were poured, dried and inoculated in an anaerobic chamber (Lab Line Instruments Inc.) under an atmosphere of N₂ (85%), CO₂ (10%) and H₂ (5%). Plate cultures were incubated in anaerobic jars at 39 °C under oxygen-free CO₂.

Rumen samples and enrichment culture. Samples of rumen digesta were collected by suction into CO₂-filled thermos flasks from fistulated sheep (fed on perennial ryegrass plus barley concentrate) and cattle (5-month-old steers fed on perennial ryegrass, and non-lactating dairy cows fed on perennial ryegrass silage plus barley concentrate) and were filtered through two layers of muslin prior to inoculation of liquid medium.

Fungal enrichment was achieved by incubating 1 ml rumen digesta with 0.1 g milled barley straw and 10 ml liquid medium B containing antibiotics and lysozyme. After 5 d incubation, cultures were transferred to the anaerobic chamber and colonized straw particles were used to inoculate plates of cellulose-medium B agar which contained antibiotics and lysozyme. Inoculated plates were inverted into anaerobic jars, removed from the chamber and incubated for 5 d. The jars were then returned to the chamber, the Petri dishes were removed and sterile Pasteur pipettes were used to transfer small plugs (about 1 mm diameter) from the margin of the colonies to liquid medium B, containing glucose but no antibiotics or lysozyme. One plug was used to inoculate each tube and five plugs were routinely taken from the margin of each fungal colony. Such colonies had a final diameter of about 2 cm and were up to 10 times larger than those which developed in roll tubes (Joblin, 1981). The liquid cultures...
were removed from the chamber, and were incubated for 5 d, at which time contaminated cultures were discarded and pure cultures were retained. This isolation procedure depends upon the ability of 'rhizoids' to grow radially outwards from the inoculum and from contaminating bacteria.

**Shake flask cultures.** Serum bottle cultures were incubated at 39 °C on a rotary shaker at 100 r.p.m. Dry weights were measured by harvesting the cultures on prewashed, dried and weighed Whatman no. 1 filter papers, washing the biomass with 200 ml distilled water and drying at 55 °C to constant weight.

**Analysis of fermentation products.** Culture supernatants were acidified (0.05 ml 3 M-H$_2$SO$_4$ per 2 ml culture supernatant) and volatile fatty acids and ethanol were determined from acidified extracts by GC (Hewlett Packard GC 5700A with flame ionization detector). The glass column (2 m × 2 mm internal diameter) was packed with Chromosorb 101, 60-80 mesh (Phase Separations); the oven temperature was 150 °C for determination of ethanol and 170 °C for determination of volatile fatty acids. Nitrogen was used as the carrier gas and quantification was achieved by comparison of peak times and peak areas with those of reference compounds. Formate and lactate were determined by the methods of Hopner & Knappe (1974) and Nanni & Baldini (1964) respectively.

**RESULTS AND DISCUSSION**

Various combinations of antibiotics and lysozyme (streptomycin, penicillin and chloramphenicol; tetracycline and chloramphenicol; tetracycline, chloramphenicol and lysozyme; ampicillin, penicillin and lysozyme; tetracycline, ampicillin and penicillin; tetracycline, neomycin, chloramphenicol and lysozyme) were tried in an attempt to isolate fungi free of bacteria, but the mixture described in Methods was the most successful in eliminating bacteria without inhibiting rumen fungi.

Pure cultures of anaerobic fungi were obtained from approximately 70% of all rumen samples. All isolates grew well on media A and B and microscopic observations suggested that they were similar to chytrid fungi, but, like *N. frontalis*, they produced polyflagellate zoospores. The method described above has also been used to purify cultures which became contaminated during culture maintenance. Once pure, fungal isolates were maintained on medium B with milled barley straw as the main carbon source. Routine culture maintenance involved subculturing at intervals of 5 d, and pure cultures have been kept in this way for up to 14 months.

Colonies formed on cellulose overlay plates (Fig. 1) were made up of regions of abundant 'mycelium' (rhizoids?) and relatively few zoosporangia which alternated with regions of

Fig. 1. Fungal colonies on a cellulose overlay plate. I, inoculum, Bar, 5 mm.
abundant zoosporangia and sparse 'mycelium'. Zoospores did not migrate over the surface of the medium but instead formed new zoosporangia close to their parent zoosporangia. These regions formed rings which radiated from the inoculum. Zoospores of Blastocladiella emersonii also fail to migrate over the surface of 'dry' agar medium (Lovett, 1967). Cellulose in the medium was completely cleared in advance of the colony margin (Fig. 1), suggesting that the isolates produce an extracellular cellulase.

One isolate has been studied further. Analysis of fermentation end products revealed that formate (1-35 g l⁻¹), acetate (1-0 g l⁻¹), lactate (0-6 g l⁻¹) and ethanol (0-4 g l⁻¹) were formed as end products when the fungus was grown for 5 d on medium B containing 10 g Avicel l⁻¹. No assays were made for CO₂ or H₂. These end products are similar to those reported by Bauchop & Mountfort (1981). In previous studies on rumen fungi, 'formic acid was calculated from the difference between total volatile fatty acids and acetic acid' (Howlett et al., 1976; Bauchop & Mountfort, 1981; Mountfort et al., 1982). In the present study, formate was determined enzymically. Like N. frontalis, the present isolate grew on medium with cellulose (Avicel or Whatman no. 1 filter paper) or milled barley straw as the main carbon source. However, there was only limited growth on barley straw that had not been milled. Cultures grown for 5 d on medium B with glucose, cellulose or milled barley straw as the main carbon source formed similar amounts of fermentation end products.

The isolate also grew on medium B from which Trypticase peptone and yeast extract had been omitted. Thus, not only can anaerobic fungi be grown on a medium which lacks rumen fluid, but it is possible to make this medium completely defined. When the isolate was grown in shaken liquid culture for 5 d at 39 °C on media containing 3-75 g glucose l⁻¹, a maximum biomass yield of 0-6 g l⁻¹ was obtained on medium B with Trypticase peptone and yeast extract and a yield of 0-8 g l⁻¹ was obtained when these components were omitted. Since the composition of rumen fluid varies from animal to animal and from the same animal at different times of the day, there is a considerable advantage to be gained in being able to cultivate anaerobic fungi on a medium which does not contain this ingredient.

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REFERENCES


