GROWTH AND PRODUCTION OF LIPASE BY *RHIZOPUS ARRHIZUS* IN SUBMERGED CULTURE

Thesis

presented for the degree of

DOCTOR OF PHILOSOPHY

by

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CONTENTS

1. INTRODUCTION.

1.1. SUMMARY OF THE PROJECT.

In this project the physiology and growth of Rhizopus arrhizus was investigated in detail with a view to developing an industrial fermentation process to produce a lipase enzyme. Spore and vegetative inocula were examined and biomass production was optimized in flasks and fermenters. Pelleting of growth was controlled by developing suitable inoculum procedures and media. Storage conditions for stock cultures were optimized also as part of this work. Factors affecting lipase production were investigated initially in shake-flasks, and later in stirred laboratory fermenters. The inoculum development and production fermentations were then integrated and scaled-up to plant-scale (3,000 litres). Economic yields of lipase were obtained at this scale. Other experiments were conducted to determine the biochemical characteristics of several lipase enzymes and to develop a rapid and inexpensive lipase assay. Finally, growth morphology of R. arrhizus was examined in a range of media to investigate some possible causes and mechanisms associated with pelleting of growth.

This project was funded by the National Board for Science and Technology under the Higher Education-Industry Co-operation Scheme. Additional financial and technical support was obtained from Biocon

Ltd., Cork, Ireland. All of the research work, with the exception of pilot-and plant-scale fermentations, was conducted at the National Institute for Higher Education, Dublin. The large-scale fermentations were conducted at Biocon Ltd., Cork, Ireland.

1.2. THE GENUS RHIZOPUS.

Fungi are a diverse group of micro-organisms ranging from yeasts and rusts to mushrooms, and include many organisms of industrial amd medical importance. They are constructed as cylindrical, branching filaments or hyphae (although some are single-celled) and have well defined cell walls. (Burnett, 1976). They are eucaryotic micro-organisms with an associated structural and organizational complexity (Carlille, 1980). All fungi are aerobic and heterotrophic organisms growing on a wide variety of nutrient sources. In nature, fungi take up a variety of structural forms during sexual or asexual reproductive cycles.

Filamentous fungi of the genus Rhizopus belong taxonomically to the class Zygomycetes, order Mucorales and family Mucoraceae. They are terrestrial phycomycetes commonly found in soil and as food spoilage organisms (Alexopoulos & Mims, 1979; Bessey, 1950). Growth on solid substrata occurs as coencytic stolons which penetrate surfaces with rhizoids over which sporangiophores are erected bearing asexual

sporangiospores. Ultrastructural studies of Rhizopus hyphae have shown that metabolic activity occurs mainly at the tips with older portions of the hyphae becoming vacuolated (Syrop, 1973).

Several species of the genus Rhizopus have found many applications in industrial processes. These include food, organic acid and enzyme production, and the bioconversion of steroids (Arima, 1964; Blain, 1975; Favéro et al., 1977; Hanish et al., 1980; Laboureur & Labrousse, 1966; Liu & Luh, 1978; Rhodes et al., 1959, 1962; Rose, 1980; Schneider, 1974; Wood & Min, 1975).

1.3. MICROBIAL LIPASES.

Extracellular lipases have been shown to be produced by a wide range of micro-organisms (Alford et al., 1964). They are classified by the Enzyme Commission as E.C. 3.1.1.3. (Florkin & Stotz, 1964). Lipases are hydrolytic enzymes which cleave the carboxyl-ester bonds joining glycerol to fatty-acids in triglyceride substrates (Desnuelle, 1972). They are distinguished from the esterases on the basis that lipases will act only on emulsified substrates, that is, at an oil-water interface. In contrast, esterases will act only on water-soluble substrates (Desnuelle, 1972; Desnuelle & Savary, 1966). Microbial lipases act on a variety of substrates but differ in positional specificity of reaction against triglycerides. Some enzymes may cleave only the 1,3

fatty-acids, others may react at all three positions (Alford et al., 1964).

Lipase enzymes are assayed using emulsified triglycerides as substrates. The most commonly used method is the potentiometric titration of olive oil emulsions (Desnuelle et al., 1955; Näher, 1974). In this assay olive oil is hydrolysed by lipase releasing fatty-acids which reduce the pH. The addition of sodium hydroxide solution is used to titrate the amount of fatty-acids released during incubation at a constant pH (Desnuelle, 1972). A turbidometric lipase assay has been described (Verduin et al., 1973). Other assays in the literature include the use of radio-labelled triglycerides and techniques using copper-salt titrations to quantify fatty-acids released (Boyer et al., 1970; Desnuelle, 1972; Schotz et al., 1970).

Microbial lipases have found many industrial applications in food processing, detergents and pharmaceutical preparations (Beckhorn et al., 1965; Macrae, 1983; Seitz, 1974; Tokiwa & Suzuki, 1977).

1.3.1. Lipases from the genus Rhizopus.

Rhizopus lipases have been widely studied in many species of this genus (Aisaka & Terada, 1979, 1981; Fukumoto et al., 1964; Laboureur & Labrousse, 1966; Tatsuoka et al., 1959). Lipases produced by different

species of Rhizopus differ in their activity and stability over a range of pHs and temperatures. This may suggest that the lipases produced are different. These apparent differences may be partly due to the different assay methods employed in these studies (Godfrey, 1983).

The lipase of R. arrhizus has been particularly well studied (Benzonana, 1973). It has been shown to have an optimum temperature of 37°C and to have two pH-activity optima at pH 3.5 and 7.0. The enzyme is stable on storage at up to 37°C and between pH 2 and 7 (Laboureur & Labrousse, 1964, 1966). Various substrates have been shown to be hydrolysed including olive oil, tributyrin, triacetin and polyester molecules (Sémériva & Dufour, 1972; Tokiwa & Suzuki; 1977). R. arrhizus lipase hydrolyses fatty-acids from the 1,3-positions of triglycerides (Sémériva et al., 1967 b). Calcium and sodium ions have been shown to activate the enzyme, whereas iron ions and E.D.T.A. inhibit activity (Laboureur & Labrousse, 1966; Sémériva & Dufour, 1972). The enzyme is a glycoprotein with a molecular weight of 43k daltons (Sémériva et al., 1969). Two active forms of the enzyme have been identified (Sémériva et al., 1967 a). The molecular conformation and active site of R. arrhizus lipase have been studied also (Tombs & Blake, 1982).

1.4. PRODUCTION OF INDUSTRIAL ENZYMES BY FUNGI.

Microbial enzymes have found many applications in industry (Beckhorn et al., 1965; Blain, 1975; Godfrey & Reichelt, 1983). In terms of bulk production proteases and carbohydrases are the most important enzymes. Most of these enzymes are produced by fungi (Beckhorn et al., 1965; Godfrey & Reichelt, 1983). Lipases represent only a small proportion of total enzyme production.

Production of fungal enzymes involves a cultivation or fermentation stage, followed by a suitable product extraction stage (Aunstrup et al., 1979; Blain, 1975; Lambert, 1983). The fermentation may be conducted by either solid or submerged culture. Solid culture is conducted using sterilized, moistened, solid substrates on trays, which are then inoculated and incubated at constant temperature and humidity. The organism grows aerially over the medium and generally sporulates. This often allows a blend of enzymes to be produced and the technique is still used to produce Rhizopus amyloglucosidase and Aspergillus acid protease (Blain, 1975). Submerged culture techniques were originally developed for fumaric acid production by Rhizopus, and later for penicillin production by Penicillium (Miall, 1975). The technique involves cultivation in stirred, liquid culture media which are forcibly aerated (Gaden, 1981). Generally, only vegetative growth occurs under these conditions and enzyme production may be directed

more specifically. This is the most commonly used technique for enzyme production.

Production of enzymes by microorganisms is subject to a number of biochemical control mechanisms. The most important of these are: enzyme induction, feed-back repression and catabolite repression by carbon or other nutrients (Demain, 1982; Paigen & Williams, 1970; Wang et al., 1977). Genetic constraints on enzyme production may be modified by strain mutation and selection (Calam, 1972; Johnston, 1975). The molecular basis of enzyme production, control and secretion have recently been reviewed (Priest, 1984; Rose, 1980). Other factors affecting microbial growth also affect enzyme production. These include levels of nutrients, temperature and aeration-agitation in fermenters. To avoid unwanted by-products, enzyme fermentations are invariably run aseptically (Gaden, 1981).

1.4.1. Production of lipase by Rhizopus species.

Production of lipase by Rhizopus has been studied in several species of this genus (Aisaka & Terada, 1979; Fukumoto et al., 1966; Laboureur & Labrousse, 1966; Tatsuoka et al., 1959). These studies have all used submerged culture techniques. Lipase is produced extracellularly mainly after growth has ceased and glucose has been depleted from the medium (Aisaka & Terada, 1979; Akhtar et al., 1974; Laboureur &

Labrousse, 1966; Macrae, 1983). The presence of glucose represses lipase production.

Although lipids have been shown to be required to induce lipase production in Candida paralipolytica, Rhizopus cultures do not have such an absolute requirement, but lipids do stimulate production in some species (Aisaka & Terada, 1979; Ota et al., 1968; Volkova & Lebedeva, 1979). Lipase production by R. arrhizus has been shown to be inhibited by addition of olive oil to the culture (Akhtar et al., 1974). R. oryzae, when grown in solid culture on soya beans, was found to produce high yields of lipase (Steinkraus et al., 1960; Wagenknecht et al., 1961). The lipid components of the soya beans were considered to have stimulated production. Sorenson and Hesseltine (1966) have shown that R. oligosporus ultilizes mainly the fatty components of soya flour as most of the component sugars are not metabolizable by this species. Soya flour also contains a considerable nitrogenous component. R. cohnii cultures produced higher levels of lipase in the presence of full-fat soya flour, but much less with defatted soya flour (Volkova & Lebedeva, 1979).

Various complex nitrogen sources have been used for lipase production by Rhizopus. These include peptones, yeast hydrolysates, casein and corn steep solids (Aisaka & Terada, 1979; Zubenko et al, 1978). Different optimum medium formulations have been devised for different

Rhizopus species. Surfactants have been shown to stimulate enzyme secretion in fungi but no studies have been conducted using these in lipase production (Reese & Maguire, 1969). Cultivation temperatures have usually been around 30°C, which allows good growth of the mould. Oxygen levels in submerged fermentation have been shown to be critical to good lipase production (Giuseppin, 1984). Strain mutation and selection have been used to improve yields also (Sultanova & Zakirov, 1979).

Studies on Rhizopus physiology and growth are also relevant when media are being designed for a given purpose. Mineral requirements and substrate ulitization range have been investigated in R. oligosporus and R. stolonifer (Fothergill & Yeoman, 1957; Sorenson & Hesseltine, 1966). Zinc ions and other heavy metals have been shown to strongly affect Rhizopus growth and physiology (Foster & Waksman, 1938; Foster, 1939; Wegener & Romano, 1963). R. oryzae and R. arrhizus have been shown to be potent producers of lactic and fumaric acids (Lockwood & Ward, 1936; Rhodes et al., 1959, 1962). Mineral nutrition and metabolism of sporulation and germination have been studied in R. arrhizus (Ekundayo & Carlille, 1964; Ekundayo, 1966; Lawler & Weber, 1980). Studies on mineral nutrition allow the determination of the minimum levels of nutrients required for growth and whether a given substrate is utilizable by the organism being studied.

1.5. FERMENTER CULTIVATION OF FUNGI.

Submerged fermenter cultivation is the most widely used technique for aerobic production fermentations (Blain, 1975; Gaden, 1981). It allows greater control of temperature, pH, aeration and broth homogeneity, compared with solid cultures (Gaden, 1981). The equipment usually consists of a baffled sterilizable tank, fitted with rotary impellers for agitation and piping for forced aeration (Gaden, 1981; Rhodes & Fletcher, 1966; Solomons, 1969). Some fermenter designs have no impellers, but use aeration alone to provide mixing and oxygenation of broth (Barker & Worgan, 1981; Greenshields & Smith, 1971).

Fungi present a number of problems when grown in submerged culture in fermenters. These include the provision of suitable inoculum, prevention of fouling of baffles and tubing with growth, and the dispersion of growth in the medium (Solomons, 1975; Rowley & Bull, 1973). These problems may be particularly acute in laboratory fermenters. The provision of adequate aeration and agitation is often problematic in fungal fermentations (Wang & Fewkes, 1977; Wang et al., 1979 b). These problems arise mainly due to the form or morphology of fungal growth. This morphology may be filamentous or pelleted (Metz & Kossen, 1977; Whitaker & Long, 1973). The morphology may be affected by strain, inoculum, medium and conditions of growth. The

literature on fungal pelleting is discussed more fully in Section 1.6.

Growth morphology affects mass transfer of nutrients, oxygen and heat within a fermentation broth (Atkinson & Daoud, 1976; Wang & Fewkes, 1977). Filamentous growth reduces mass transfer to the bulk fermentation fluid and its increased viscosity requires a higher power input to maintain adequate mixing (Blakebrough & Hamer, 1963); Solomons & Weston, 1961). Such fermentation broths may be non-newtonian in character (Wang & Fewkes, 1977). Aeration of the bulk fluid is more easily effected in pelleted than in mycelial fermentations (Blakebrough & Hamer, 1963; Brierley & Steel, 1959; König et al., 1982; Phillips, 1966; Wang & Fewkes, 1977). However, pellets have a resistance to oxygen diffusion, such that pellet interiors are anaerobic (Huang & Bungay, 1973; Phillips, 1966). Aeration of bulk fermentation fluids may be enhanced by increasing aeration rate, vessel pressure, agitation speed or impeller diameter (Asai & Kono, 1982; Elsworth et al., 1957). In dense mycelial fermentations these parameters interact in a complex manner due to the non-newtonian nature of such fluids (Wang & Fewkes, 1977). Filamentous fungi are often susceptible to damage by shear from impellers (Clark & Lentz, 1963; Dion et al., 1954, 1959). R. nigricans has been shown to be sensitive to shear (Hanisch et al., 1980). This limits the extent to which agitation may be increased to obtain higher aeration rates. If an organism is especially

sensitive to shear this can be reduced by removing the baffles from the fermenter (Oldshue, 1983).

Air-lift and tower fermenters have lower shear rates as mixing is provided by forced aeration alone (Malfait et al., 1981; Smith & Greenshields, 1974). Power input is considerably lower in these fermenters compared with stirred tank reactors (König et al., 1982; Schügerl et al., 1983). Different designs for aerated fermenters have been proposed such as the air-lift with internal or external circulation of broth, and tower fermentation systems (Greenshields & Smith, 1971; Kiese et al., 1980; König et al., 1982). These systems have successfully been used to cultivate fungi at laboratory and pilot-plant scales (Barker & Worgen 1981; Kiese et al., 1980; König et al., 1982; Morris et al., 1973; Smith & Greenshields, 1974). Aeration in tower fermenters has been shown to be adequate for growing a variety of aerobic micro-organisms (Morris et al., 1973; Smith & Greenshields, 1974).

1.6. FACTORS AFFECTING FUNGAL GROWTH MORPHOLOGY.

The phenomenon whereby fungal filaments aggregate to grow as clumps is termed fungal pelleting. The structure and form of such clumps or pellets varies widely. They range from loose, fluffy conglomerates of hyphae to larger pellets with a tissue-like structure and an autolyzed central cavity (Burkholder & Sinnott, 1945;

Clark, 1962). In larger pellets it has been found that only the outer hyphae are metabolically active (Yanagita & Kogane, 1963). The morphology of fungal cultures may be controlled by varying strain, inoculum, growth medium and conditions (Burkholder & Sinnott, 1945; Cocker & Greenshields, 1977; Metz & Kossen, 1977; Whitaker & Long, 1973). Generally it has been observed that different fungal species react differently to a given set of growth parameters. The level of spore inoculum used in flask cultures has been shown to affect morphology. Low spore inocula led to pelleted growth, whereas levels above a critical number gave dispersed, filamentous growth in Penicillium chrysogenum and Aspergillus niger (Camici & Sermonti, 1952; Steel et al., 1954; Testi-Campasano, 1959).

Growth media have been shown to affect fungal growth morphology. P. chrysogenum has been shown to grow as filaments in complex media at a pH of 6.0, but as pellets in media with a mineral nitrogen source at the same pH (Pirt & Callow, 1959). Growth morphology of A. niger is affected by pH, ferrocyanide levels, surface active agents and lipids (Baig et al., 1972; Galbraith & Smith, 1969; Steel et al., 1954; Takahashi et al., 1965). High pHs (8.0), the presence of Tween-20 (0.05% v/v) or ferrocyanide (0.45 g/l) gave pelleted growth, whereas low pH (3.0) and the presence of Span-20 (0.05% v/v) resulted in filamentous growth of A. niger. Levels of metal ions and chelating agents have been shown to

affect A. niger growth in defined media (Choudhary & Pirt, 1965). Mortierella vinacea has been shown to form pellets in media of pH 5, but filamentous growth at pH 8 (Kobayashi & Suzuki, 1972 a). Surface-active agents did not affect this mould but oleic acid reduced the pellet sizes (Kobayashi & Suzuki, 1972 b). The presence of solid materials in the form of ground corn has been shown to disperse growth of A. candidus (Smiley et al., 1967). Increasing the medium viscosity by addition of sodium alginate, carboxymethyl cellulose or dextran disperses growth of P. chrysogenum and A. niger (Takahashi et al., 1960 a, b). Conversely, viscosity has been shown to cause pelleting in M. vinaceae (Kobayashi & Suzuki, 1972 b). A number of non-viscous polymers have been shown to affect the morphology of A. niger (Elmayergi et al., 1973). Anionic polymers were most effective at dispersing growth. One of these, Carbopol-934, a carboxypolymethylene polymer, was shown to affect growth rate, respiration and potassium and oxygen uptake (Elmayergi et al., 1973; Elmayergi & Moo-Young, 1973; Moo-Young et al., 1969). These effects were considered to be partly due to the dispersion of fungal growth.

Physical parameters such as agitation have been shown to affect growth morphology. Higher shaker speeds reduce the pellet sizes of M. vinaceae in flask cultures (Kobayashi & Suzuki, 1972 a). Agitation in stirred fermenters also affects mould morphology (Dion &

Kaushal, 1959; Suijdam & Metz, 1981). High levels of agitation reduced the lengths of hyphae in P. chrysogenum cultures, although a similar effect was observed at lower agitation levels when aeration was increased (Dion et al., 1954). A. flavus has been shown to be dispersed by agitation,whereas intense agitation resulted in pellet formation in Rhizopus and Mucor species (Campasano et al., 1959; Dion & Kaushal, 1959).

The sequence of events leading to pellet formation include spore-spore, spore-mycelial and inter-mycelial interactions (Cocker & Greenshields, 1977; Galbraith & Smith, 1969; Metz & Kossen, 1977; Whitaker & Long, 1973). These interactions would be affected by the surface properties of both mycelia and spores (Atkinson & Daoud, 1976; Galbraith & Smith, 1969).

1.7. FUNGAL FERMENTATION PROCESS DEVELOPMENT.

In developing a fungal fermentation process a number of important considerations need to be borne in mind. A productive stock culture of the producing organism needs to be maintained (Rhodes & Fletcher, 1966). This may be achieved by subculturing regularly on suitable agar media and by lyophilization when no culture-collection source is available (Collins & Lyne, 1979). Industrial strains may be screened for from natural sources and subsequently improved by mutation procedures (Calam, 1972; Johnston, 1975). These stock cultures need to be

kept free of strain variation and contamination. (Gaden, 1981; Rhodes & Fletcher, 1966).

Inoculum development procedures must be standardized as these affect subsequent growth and production capabilities (Calam, 1976; Meyrath & Suchanek, 1972). Fungal metabolism in particular is affected by the inoculum conditions due to senescence of mycelia (McIntosh & Meyrath, 1963; Meyrath & McIntosh, 1963; Taber, 1957; Ward & Coletelo, 1960). Both spore and vegetative inoculation procedures need to be standardized. Spore inocula are generally standardized by counting in suitable chambers and by using fresh spore suspensions obtained from agar cultures (Collins & Lyne, 1979; Meyrath & Suchanek, 1972). Vegetative inocula may be standardized using culture age, depletion of nutrients and dry weight analysis (Calam, 1969, 1972). The physical conditions and media used may be optimized using these procedures to develop reproducible inocula.

Developing a production medium involves selection of nutrients which will allow good growth and production. A number of the factors affecting enzyme production have already been discussed (Sections 1.4 and 1.4.1). If some nutrients are found to inhibit product accumulation when present above a critical concentration, fed-batch methods may be used to limit such inhibition (Gaden, 1981). Media ingredients for industrial fermentations

need to be cheap, so a wide range of nutrient by-products from agriculture are used (Calam, 1967; Rhodes & Fletcher, 1966; Solomons, 1969). Production media may differ from inoculum media (Rhodes & Fletcher, 1966).

Fungal growth morphology may be filamentous or pelleted depending on strain and conditions used (Metz & Kossen, 1977; Calam, 1976; Whitaker & Long, 1973). Different fermentations require different growth morphologies for optimum product yields. Morphology may be affected by inoculum development procedures used. Filamentous growth is generally preferred for penicillin production from P .chrysogenum,although pelleted growth has also been tried (Calam, 1976; König & Schügerl, 1982). Citric acid production by A. niger requires pellets of a particular size for optimum production (Sodeck et al., 1981; Steel et al., 1954). For itaconic acid production by A. terreus,pelleted growth is preferred (Nelson et al., 1952). Higher yields of fumaric acid were obtained from R. arrhizus when it was grown in a filamentous form (Rhodes et al., 1959, 1962). Pectic enzyme production by A. niger has been reported to be enhanced when growth occurred as filaments (Tuttobello & Mill, 1961). Therefore,in developing a new fungal process the optimum growth morphology must be determined.

Much developmental work, such as screening for strains, media optimization and inoculum development, may be

conveniently carried out using shake-flask methods (Kuenzi & Auden, 1983; Rhodes & Fletcher, 1966; Solomons, 1969). Laboratory fermenters may then be used to determine the optimum conditions of agitation and aeration for product accumulation. The final scale of production is determined by assessing the economics of the overall process. (Stowell & Bateson, 1983; Wang et al.,1979 b). The aim of scale-up of fermentation processes is to maintain optimum conditions at each scale (Wang et al., 1979 b). Conditions most often examined include power input, impeller tip-speed, oxygen transfer rates and mixing patterns in fermenters (Einsele, 1978; Lilly, 1983; Oldshue, 1983; Wang et al., 1979). In fungal fermentations adequate mixing must be provided, while avoiding mechanical damage to mycelia. Aeration may be affected by the presence of a heavy mycelial suspension (Wang & Fewkes, 1977). Alternative fermenter designs to the stirred tank reactor may be considered, such as the air-lift and tower fermenter reactors (Barker & Worgan, 1981; Greenshields & Smith, 1971).

Extraction of extracellular enzymes from fermentation media is usually developed in parallel with production media development. This is because media constituents may affect subsequent filtration and extraction of the enzyme (Atkinson, 1973; Aunstrup et al., 1979; Blain, 1975; Lambert, 1983). For an industrial fermentation to remain competitive it must be continually improved

(Küenzi & Auden, 1983; Stowell & Bateson, 1983). This may be achieved both by routine laboratory selection of media and strains, and by continually monitoring performance in plant-scale equipment.

2. MATERIALS AND METHODS.

2.1. CHEMICALS.

Chemicals used in enzyme assays and other biochemical analyses were of Analar or analytical grade, unless otherwise stated. Those used for laboratory cultures were of General Purpose Reagent grade. Materials used for 16, 250 and 3,000-litre fermenters were of industrial or food grade and were obtained from Biocon Ltd., Ireland. The source of laboratory chemicals was either B.D.H. Ltd., England or Reidel-de-Haen AG, Germany, depending on availability. Biochemicals were obtained from Sigma London Ltd., England. Routine microbiological agar and media were of Oxoid brand. Commercial enzyme preparations were obtained from Biocon Ltd., Ireland. Carbopol-934 was obtained from B.F. Goodrich Ltd., U.S.A., as a gift. Sodium taurocholate (F.I.P. controlled) was obtained from Biocon Ltd., Ireland. Water used for media preparation and enzyme assays was distilled and deionized. Batching of 250 and 3,000-litre fermenters used tap water.

2.2. FUNGAL CULTURE.

2.2.1. Source of strain.

The culture was obtained from the American Type Culture Collection, U.S.A. (A.T.C.C.) number 10260 (also listed

as 12732), and stated as being the equivalent of Central Bureau de Schimellcultures, Holland, (C.B.S.) 329.47; and Northern Regional Research Laboratory, U.S.A. (N.R.R.L.) 1526. The organism was named Rhizopus arrhizus Fischer by A.T.C.C., but as R. oryzae by N.R.R.L.

2.2.2. Culture maintenance.

Stock cultures were maintained on yeast-malt extract agar slopes, subcultured each month and stored at 4°C. Cultures took 4-5 days to grow and sporulate at 30°C. The growth had a fluffy appearance which filled the universal container until growth pressed against the glass. White mycelia formed initially, with black sporangia appearing later. Yeast-malt extract agar contained: yeast extract, 4 g/l; malt extract, 10 g/l; glucose, 4 g/l; Oxoid Agar No.3, 20 g/l; pH 7.0. The materials used were of food grade

2.2.3. Detection of contamination.

Bacterial contamination of stock cultures, spore suspensions or fermentation samples was detected by plating samples onto Nutrient Agar (Oxoid) containing 0.1 mg/l cycloheximide. Incubation was conducted at 30°C overnight. This procedure was used routinely but contamination was encountered only rarely.

2.2.4. Lipase productivity test.

Stock cultures were routinely checked for lipase productivity by inoculating 250ml conical flasks containing 100 ml Medium E with a standard spore inoculum ($1x10^4$/ml) and incubating on an orbital shaker under standard conditions (Sections 2.3 and 2.4). Lipase activity was then assayed after 72 hours growth (Section 2.8.2). Under these conditions up to 100 u/ml were obtained from good stock cultures.

2.3. SPORE INOCULUM PREPARATION.

R. arrhizus was cultured on yeast-malt extract agar (Section 2.2.2), 20 ml in a 250 ml conical flask, for 4 days at 30°C. Spores were washed from the culture with 20 ml of Triton-X 100 (0.01% v/v) under sterile conditions. This produced a dense, black spore suspension free of mycelia. Spores were counted using a phase-contrast microscope and a Hawksley counting chamber. Suspensions were counted in triplicate according to standard procedures (Collins & Lyne, 1979). The suspension was adjusted to give $1-3x10^7$ spores/ml. Suspensions were found to be unaffected by storage at 4°C for 1 month. If stored for longer periods, however, poorer growth and enzyme yields resulted.

2.4. SHAKE-FLASK CULTIVATION.

Throughout the experimental work shake-flask cultures were incubated at 30°C on an LH Engineering 2-tier orbital shaker (model MK II/III) at 150 r.p.m. with a displacement of 50 mm. The table had accomodation for all conical flask sizes used from 250 ml to 2-litre. Unless otherwise stated, 250 ml conical flasks containing 100 ml of medium were used.

2.5. BIOMASS DETERMINATION USING DRY WEIGHT ANALYSIS.

Dry weight is a convenient method for measuring fungal biomass but is subject to a number of limitations (Calam, 1969). It is used to measure levels of vegetative growth in soluble media.

The procedure developed was as follows: filter papers (Whatman type 1, 9 cm diameter) were dried at 110°C to constant weight in glass petri plates. These were then transferred to a dessicator and cooled under vacuum. The papers were then weighed on a balance weighing grams to 4 decimal places. Biomass was harvested from flask cultures using the filter papers on a Buchner funnel. The mycelia were then washed with three culture volumes of distilled water. The filters were then dried, cooled and weighed as before. Dry weight was determined by difference. Controls consisted of water or media, but generally gave only ~2 mg. Determinations in which

controls exceeded 10% (w/w) of samples were discarded. Good reproduction was obtained among triplicates.

2.6. FERMENTER CULTIVATION.

Fermenters used on laboratory-scale were of 7.5-litre (Laboferm, New Brunswick Scientific) and 16-litre (Microferm, New Brunswick Scientific) gross capacities. These were run with 5 and 10 litres of media, respectively. Inoculation and sampling of fermenters was carried out using sterile techniques. Laboferm fermenters were sterilized at 15 p.s.i. and 121°C for 35 minutes. Microferm fermenters were sterilized in-place with steam and maintained at 15 p.s.i. and 121°C for one hour or longer, depending on media being used. Insoluble medium components, such as maize or starch, required longer sterilization periods.

Pilot and plant-scale fermenters, of 250- and 3,000-litres gross capacity respectively, were also used. These were of stainless steel construction and were run with 200 and 2,000 litres of media. They were sterilized by steam also, as above. The dimensions and specifications of the fermenters used are shown in Table 2.1.

All fermenters, except Laboferm (7.5-litre) were run at 5 p.s.i. back-pressure to control foaming and to reduce the risk of contamination. Levels of aeration and agitation used were as indicated with individual

Table 2.1. Dimensions of fermenters used.

(Volumes measured in litres, lengths in cm).

Fermenter	A.	B.	C.	D.
Total volume	7.5	16.0	250.0	3,000.0
Medium volume	5.0	10.0	200.0	2,000.0
Vessel diameter	14.0	22.0	50.0	122.0
Impeller number	3	3	1	2
Impeller diameter (Di)	5.0	7.0	20.0	45.0
Impeller spacing	9.5	9.5	-	77.0
Impeller speed r.p.m. (N)	450	200	230	72/104
Impeller-tip speed (π N Di) (a) cm.s^{-1}	117.8	73.3	240.9	169.6/245.0
Impeller shear ($N^{\frac{1}{2}}Di^{\frac{1}{2}}$) (b) x10^{3}	1.4	0.54	5.9	2.9/6.1
Number of baffles	4	4	4	4
Baffle width	1.5	2.0	6	15
Distance baffle-wall	0.5	1.5	1.0	3.5
Distance impeller tip-wall	2.8	4.0	8.0	20.0
Aeration-maximum level (v/v/m)	2.0	2.0	2.0	2.0

References: (a) Wang et al., 1979 b.

(b) Wang & Fewkes, 1977.

experiments in Results. All fermenters were run at 30°C. Foaming was controlled by addition of Kg-Antifoam (Biocon, Ireland) as required at ~3 ml/l.

2.7. FERMENTATION MEDIA.

2.7.1. Medium A. Casein inoculum medium.

Casein hydrolysate, 13 g/l; glucose 45 g/l; pH 6.0.

Casein (food grade) was hydrolysed as a 200 g/l suspension by adding 6 g/l Fungal Protease (2.7×10^6 H.U.T. units, Biocon, Ireland) and incubating at 50-55°C with vigorous agitation for 4 hours. The assay used for quantifying the enzyme is given in Appendix 1. Glucose used was of food grade also.

2.7.2. Medium B(1/2). Peptone inoculum media.

(1) Soya peptone, 13 g/l; glucose, 45 g/l; at pH 6.0.
(2) Soya peptone, 5 g/l; glucose, 20 g/l; at pH 6.0.

Materials used were of food grade. Soya peptone was obtained from Biocon Ltd., Ireland.

2.7.3. Medium C. Soya inoculum medium.

Soya flour (full-fat), 10g/l; glucose, 45 g/l; at pH 6.0.

Food grade materials were used.

2.7.4. Medium D. Lipase production medium

Ground maize (hydrolysed), 45 g/l; casein hydrolysate, 13 g/l; $(NH_4)_2SO_4$, 13.3 g/l; $MgSO_4$. 7 H_2O, 8.8 g/l; $CaCl_2$, 28 g/l; $CaCo_3$, 20 g/l; at pH 6.50.

Maize was hydrolysed enzymatically by suspending in a solution containing 2 mM NaCl and 2 mM $CaCl_2$ at pH 6.0. Canalpha was added at 450 u/ml and incubation conducted at 75°C for 30 minutes. The pH of the suspension was adjusted to 4.5 and 2 u/ml of Amylo added. Incubation was continued for a further 30 minutes at 60°C. Canalpha is an α-amylase preparation which partially liquefies the maize. Amylo is an amyloglucosidase preparation which saccharifies starch. These products were obtained from Biocon Ltd., Ireland, and the method used to assay them is given in Appendix 1.

Casein hydrolysate was prepared for this medium in the same way as for Medium A. Grades of chemicals used were as indicated in Results and Section 2.1.

2.7.5. Medium E. lipase productivity medium.

Ground maize (hydrolysed), 45 g/l; Bacteriological Peptone (Oxoid), 12.7 g/l; $CaCO_3$, 20 g/l; $MgSO_4 . 7 H_2O$, 8.8 g/l; KNO_3, 5.7 g/l; $(NH_4)_2 H PO_4$, 3.7 g/l; $CaCl_2$, 0.01 g/l; at pH 6.5.

Ground maize hydrolysed as for Medium D, but only Canalpha hydrolysis applied. Chemicals of General Purpose Grade were used.

2.7.6. MEDIUM F. DEFINED MEDIUM.

Component solutions:	Final concentration:
1. Glucose	10g/l (unless otherwise stated)
2. KH_2PO_4-K_2HPO_4 buffer, pH 2.2	0.01 M
3. $(NH_4)_2SO_4$	0.10 M
4. $MgSO_4 . 7H_2O$	1.25 mM
$ZnSO_4 . 7H_2O$	0.03
$MnSO_4 . 4H_2O$	0.20
5. $FeCl_3 . 6H_2O$	0.09 mM
$CuSO_4 . 5H_2O$	0.03

These solutions were sterilized separately and recombined to the final concentrations shown. All materials used were of Analar grade.

2.7.7. Source of media formulations.

Media A, D and E were developed at Biocon Ltd., Ireland. Media B and C were developed in the course of this project. The defined medium was based on that of Sorenson and Hesseltine (1966), the only modification being that we used 0.03 mM $ZnSO_4$ with no E.D.T.A. in the standard formulation.

2.7.8. Sterilization procedures.

Media were sterilized in flasks at 121°C and 15 p.s.i. for an appropriate period. Insoluble ingredients required longer autoclaving periods, whereas soluble media with glucose tended to carmelize if sterilized for long periods. Large fermenters were sterilized by steam injection. Defined medium components were sterilized either by autoclaving or by Milli-pore filtration.

2.8. ANALYTICAL PROCEDURES.

2.8.1. Estimation of reducing-sugars.

Reducing-sugars were estimated using the D.N.S. method (Miller, 1959). Results were expressed as reducing

equivalents g/l, using glucose as standards.

D.N.S. reagent·

3,5-dinitrosalycylic acid, 10 g/l; potassium sodium tartarate, 300 g/l; sodium hydroxide, 16 g/l were dissolved in 600 ml water by heating, without boiling. The solution was cooled and diluted to 1 litre.

Procedure:

Samples and standards (glucose) were diluted to 0.3-1.5 g/l reducing sugar equivalents. 1 ml samples were added to 1 ml water and 2 ml D.N.S reagent. The tubes were placed in boiling water for 10 minutes and then cooled. 10 ml water was then added to each tube, the contents mixed by inversion and absorbance at 540 n.m read on a Pye Unicam SP6-550 Spectrophotometer. The instrument was set at zero using a substrate blank, made by adding 2 ml water to 2 ml D.N.S reagent and boiling and diluting as for test samples. Reducing - sugars in unknown samples were determined using a standard curve of glucose 0.1-1.5g/l plotted against absorbance at 540 n.m.

2.8.2. Lipase assays.

Two assays were used: one using olive oil as substrate based on Naher (1974); and another using tributyrin as substrate was developed in the course of this project

(Section 3.5). Units were expressed according to the Federation Internationale Pharmaceutique or F.I.P. units (Näher, 1974).

2.8.2.1. Olive oil assay.

Principle:

Lipase enzymes react with an olive oil emulsion, at pH 7.0 and 37 °C with constant stirring, releasing fatty-acids from triglycerides. These lower the pH when ionized, but pH is held constant by continuous titration with sodium hydroxide solution. The number of moles of NaOH required to maintain pH is equivalent to number of moles fatty-acids released. Some fatty-acids released have pK_a above 7.0, so the reaction mixture is titrated, after holding at pH 7.0 for 10 minutes, to pH 9.0. The number of moles of fatty-acids released per minute, per ml of enzyme, is defined as one F.I.P. unit. Sodium taurocholate added acts as a fatty-acid acceptor, preventing enzyme inhibition and enabling linear kinetics to be obtained. Calcium ions aid ionization of long-chain fatty-acids and gum arabic stabilizes the emulsion (Desnuelle, 1972).

1. Olive oil neutralization:

Free fatty-acids in olive oil (Sigma London Ltd.) were neutralized by adding 500 ml to 200 ml of an aqueous

slurry containing 150g sodium carbonate. The mixture was stirred gently for 30 minutes at 45°C, and left overnight in a separatory funnel. The oil was then decanted and centrifuged to remove excess sodium carbonate.

2. Gum arabic solution:

110 g gum arabic (Sigma London Ltd) and 12.5g $CaCl_2 . 2H_2O$ were dissolved by stirring in water. The solution was diluted to 1 litre, centrifuged, and stored at 4°C.

3. Substrate emulsion:

400 ml gum arabic solution and 130 ml neutralized olive oil were blended at high speed for 15 minutes at 4°C. A Sorvall Omni-Mixer was used.

4. Sodium hydroxide solution:

10 mM NaOH solution was made immediately prior to use with boiled distilled water. A burette (50 ± 0.1 ml) was filled with this solution.

5. Sodium taurocholate solution:

0.25 g of F.I.P. controlled sodium taurocholate was diluted to 50 ml with water.

6. Enzyme diluent:

A solution containing NaCl (10 g/l) was used to dilute samples to 2-4 F.I.P. units/ml.

Procedure:

100 ml glass beakers were used as reaction vessels and were maintained at 37°C in a water bath, stirring the contents continuously with a magnetic stirrer. The following reaction mixture was added to each vessel: 24 ml substrate emulsion, 9 ml water and 2 ml sodium taurocholate solution. The temperature was equilibrated to 37°C and pH adjusted to 7.0. The enzyme sample (5 ml) was then added and pH maintained at 7.0 for 10 minutes, by titration with 10 mM NaOH. After 10 minutes the pH was brought rapidly to 9.0. The volume of NaOH used was read from the burette (V_1). An enzyme blank was run for each sample by adding 5 ml of enzyme dilution to a reaction mixture and bringing the pH to 9.0 immediately (V_2).

Calculation:

Since 1 ml of 10 mM NaOH neutralizes 10 μmoles of fatty acids, and 5 ml of enzyme dilution was reacted for 10 minutes, enzyme activity may be calculated from:

$$\frac{(V_1 - V_2) \times 10}{10 \times 5} \times \text{Dilution} = \text{F.I.P. units/ml.}$$

2.8.2.2. Tributyrin assay.

Principle.

This assay was developed during the project (Section 3.5), based on suggestions in the literature (Desnuelle, 1972; Sémériva, 1972). The assay uses a tributyrin emulsion as substrate instead of olive oil. It differs from olive oil assay in that the emulsion is maintained only by mechanical agitation. Bile salts were found to inhibit enzyme activity completely in the assay.

Reagents:

1. Tributyrin emulsion:

Tributyrin (grade II, Sigma London Ltd.) 5 ml, was added to 100 ml diluent containing 0.1 M NaCl and $CaCl_2$. The mixture was then agitated vigorously on a magnetic stirrer using a 2 cm magnetic bar.

2. Sodium hydroxide solution:

5 mM NaOH was prepared using boiled distilled water. A 10 ml ± 0.02 ml burette was filled with the solution.

3. Enzyme diluent:

A solution containing 10 g/l NaCl was used to dilute sample to 10-20 F.I.P. units/ml.

Procedure:

100 ml glass beakers were used as reaction vessels and maintained at 37°C using a thermostatic water bath. 30 ml of the tributyrin emulsion were dispensed into these beakers and vigorous stirring maintained during the assay. The temperature was held at 37°C and pH adjusted to 7.0. 1 ml enzyme dilution was added at zero time and pH held constant for 5 minutes. The volume of NaOH required was measured (V). No titration to a higher pH was required as butyric acid has a pKa of 4.5.

Calculation:

Since 1 ml of 5 mM NaOH neutralizes 5 μmoles of butyric acid, and 1 ml of enzyme was reacted for 5 minutes, activity may be calculated by:

$$\text{Tributyrinase units/ml} = \frac{V \times 5 \times \text{dilution}}{5}$$

Lipase activity on tributyrin was consistently found to be a factor of 5-times less than activity on olive oil. The calculation was modified to give:

$$\text{F.I.P. units/ml} = V \times \text{dilution} \times 5$$

2.8.3. Assay for divalent cations.

The method was the Solochrome-Black titration method used for determining water-hardness (Vogel, 1978).

Principle:

Solochrome-Black when complexed with divalent ions is red in colour. If all these ions are removed by E.D.T.A. the colour changes to blue (Vogel, 1978). Magnesium ions added sharpen the colour change.

Solutions:

1. Buffer pH 10.0: 142 ml concentrated ammonia (sp. gr. 0.88-0.90) was mixed with 175g NH_4Cl, the pH adjusted to 10.0 and the volume made up to 250 ml.

2. Indicator: Solochrome-Black 0.3 g/l and 29.7 g/l KNO_3 were added to distilled water as an insoluble

suspension.

3. Mg-EDTA: solution containing 0.2M EDTA and $MgSO_4$

4. Standards: 0.1-1.0 mM $MgSO_4$

Procedure:

50 ml of sample or dilution was added to a 250 ml flask. 1 ml buffer, 1 ml indicator and 0.1 ml Mg-E.D.T.A. were then added and the colour changed to a pure red. The mixture was titrated with 0.01 M E.D.T.A., swirling constantly, until the colour changed to a pure blue.

Calculation:

Titre x 0.01 x 20 = mM divalent cations.

2.9. FUNGAL GROWTH MORPHOLOGY.

Samples of growth were taken, where possible, using a 10 ml pipette, either inverted or upright. This was diluted in 100 ml water and shaken to see how well it dispersed. Reference was then made to a key drawn up in the course of the project (Section 3.7).

For macro-photography, samples of growth were placed in petri-plates before being photographed with a standard Olympus Om-10 camera with ASA 50 Ilford film, under

flash-gun illumination.

For micro-photography, samples of growth were wet-mounted onto slides and photographed under a Nikon Optiphot phase-contrast microscope. The camera used was the same as above, except an extension shutter release cable was used.

Magnifications used ranged from 400 to 100 times. Micrometry was conducted using an eye-piece micrometer graduated using a standard graduated slide for each magnification (Collins & Lyne, 1979).

2.10. ROUTINE MEASUREMENTS AND INSTRUMENTATION

pH was measured using an Orion Research Ionalyser Model 501. Temperature could also be measured using this instrument. Viscosity was determined using a Brookfield Digital Viscometer (Model LVTD), fitted with a UL-Adaptor.

Spectrophotometric measuremts were conducted on a Pye Unicam SP6-550 u.v./vis. Spectrophotometer with a 1 cm light path. Balances used routinely included an Oertling Top Pan balance (1.6 Kg $\pm$ 0.1g); Sartorious 1219 MP electronic balance (600g $\pm$ 0.01g) and Precisa 80A electronic balance (30g $\pm$ 0.0001g). Centrifugation was carried out using a bench-top Heraeus Christ Model 600 capable of up to 5,000 r.p.m.

Microbiological techniques were generally carried out according to Collins and Lyne (1979).

2.11. EXPERIMENTATION AND ANALYSIS

Experiments were carried out in duplicate and repeated at least twice. Reproducibility of experiments was generally good. Results or findings which could not be repeated were discarded and accordingly are not reported in this thesis.

3. RESULTS.

3.1. CHARACTERIZATION OF FLASK INOCULUM.

Many authors have emphasized the importance of inoculum standardization for experiments with fungi (Calam, 1976; Ward & Coletelo, 1960). Similarly, for reproducible results from industrial fermentations a suitable inoculum needs to be provided (Meyrath & Suchanek, 1972; Rhodes & Fletcher, 1966). Growth may be affected by inoculum type, age and levels, culture medium and conditions (Calam, 1976; Solomons, 1975; Whitaker & Long, 1973). For cultivation of fungi two types of inoculum may be used: spores and vegetative growth. Spore suspensions may conveniently be used as an initial standardized inoculum for developing vegetative growth in shake-flasks (Solomons, 1975). The levels used can be standardized by counting and dilution (Section 2.3). The following exeriments were conducted to characterize vegetative inocula for flasks and fermenters.

3.1.1. Age of stock cultures and spore suspensions.

The age of stock cultures of R. arrhizus was found to affect the organisims' ability to grow on agar and to sporulate. From exerience accumulated during the project a standard procedure was devised for subculturing (Section 2.2.2). It was found to be essential to subculture at monthly intervals, otherwise strain

degeneration occurred.

Similarly, the storage period of spore suspensions at 4 ^{o}C affected growth and enzyme production. A standard procedure was devised for preparing spore suspensions (Section 2.3). If suspensions were stored for longer then 4 weeks, degeneration occurred and growth and enzyme yields were lower. This experience is consistent with previous findings related to maintaining fungal cultures for enzyme fermentations (Ward O.P., personal communication).

3.1.2. Vegetative growth in shake-flasks.

Levels of spore inocula were varied in replicate 250 ml conical flasks containing 100 ml Medium A (Sections 2.3 and 2.7.1). Incubation was conducted for 18 hours, under standard conditions, and vegetative biomass yields determined by dry weight (Sections 2.4 and 2.5). The results are shown in Table 3.1.

As the spore inoculum was increased biomass yield after 18 hours also increased. The morphology of this growth was reasonably dispersed giving good growth throughout the medium (Figure 3.1). At levels of inoculum below those shown in Table 3.1, growth was poor, occurring as a few, discrete round marbles up to 1 cm diameter.

A spore inoculum level of $1x10^{4}$/ml culture was used,

under the same conditions, to determine the effect of incubation time on biomass yield. Replicate flasks were given the same spore inoculum and three replicates were harvested for biomass analysis after different incubation periods. The results are shown in Figure 3.2.

Table 3.1. Effect of spore inoculum level on biomass production by R. arrhizus

Spore inoculum level (No./ml culture)	Biomass (mg/100 ml)	Final pH
5×10^3	168.6	3.53
1×10^4	206.1	3.40
2×10^4	246.1	3.03
5×10^4	340.5	2.84

Cultures were grown in 100 ml Medium A in 250 ml conical flasks at 30°C and 150 r.p.m. and given different inoculum levels. Replicate flasks, at least 3 each, were harvested after 18 hours incubation for biomass analysis.

Biomass increased at an approximately linear rate between 15 and 28 hours, after which biomass remained constant. It was shown that the growth rate was not exponential by the non-linear plot of logarithms of dry weight against time (Choudhary & Pirt, 1965). The biomass remained dispersed (i.e. filling medium) up to

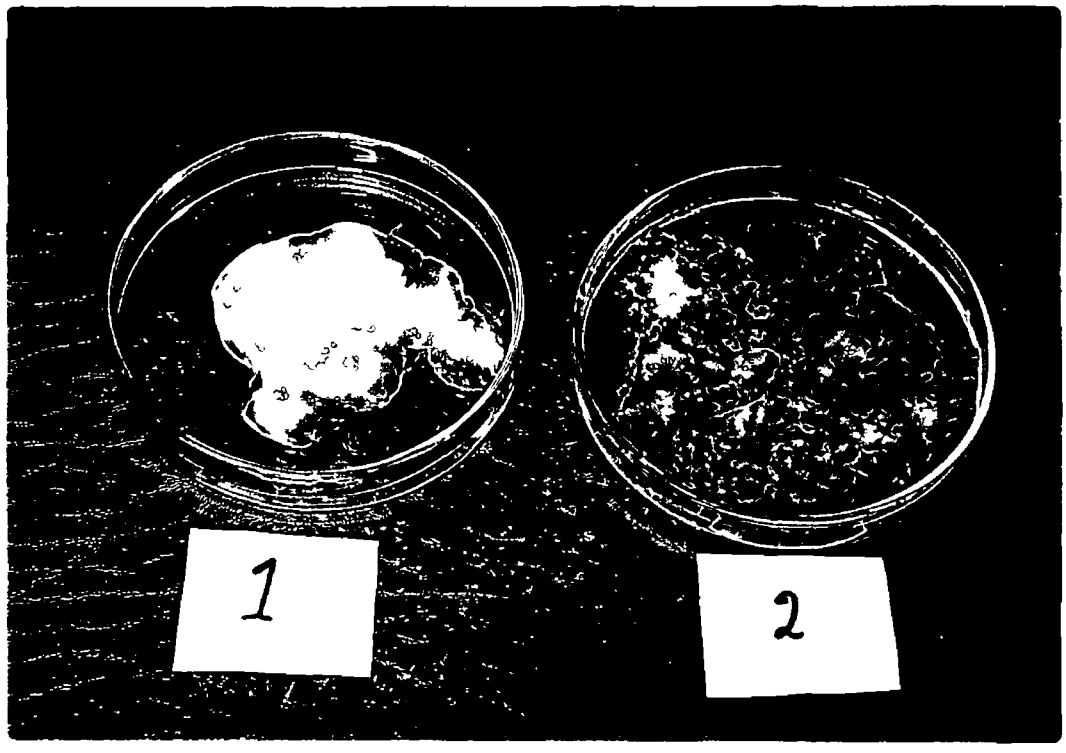

Figure 3 1 Samples of R.arrhizus biomass removed from shake-flask cultures in Medium A.

1. Clumped, coalesced growth after 24 hours incubation

2 More dispersed growth after 18 hours

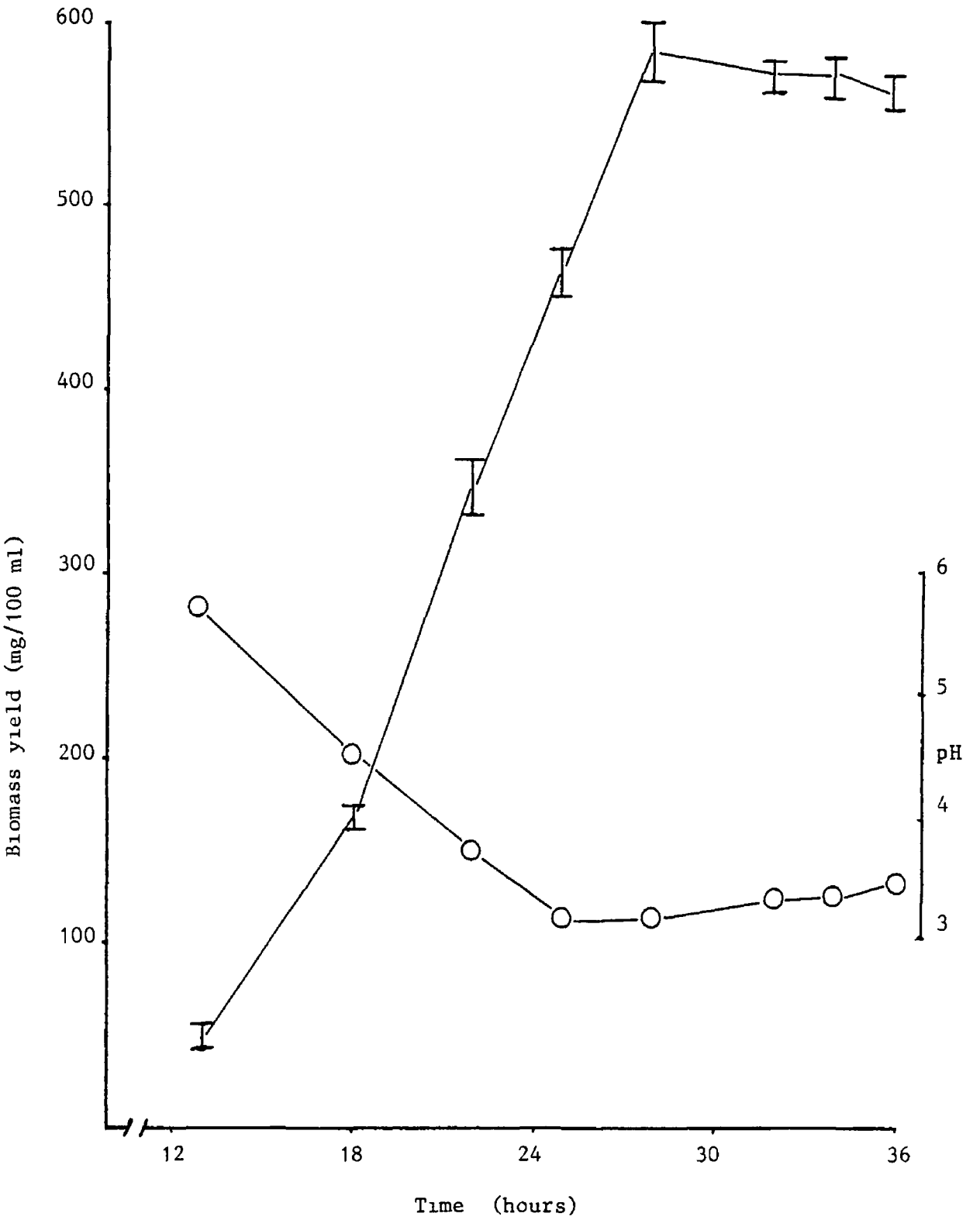

Figure 3 2 Effect of incubation time on biomass yield (I) and pH (0) of R arrhizus cultures in Medium A (100 ml) in 250 ml conical flasks, with spore inoculum 1 x 10^4/ml, and incubation conducted at 30°C and 150 r p m

20 hours incubation, after which it coalesced to form one single clump or fungal mass. This was especially marked for incubation periods greater then 24 hours, when the vegetative growth was difficult to remove from the culture flasks. These growth morphologies are illustrated in Figure 3.1. This coalescence of growth was considered undesirable for an inoculum as it dispersed poorly in inoculated media. It should be noted also that pH dropped from 6.0 to 3.2, due to fumarate production (Rhodes et al., 1959).

3.1.3. Preliminary experiments on growth morphology.

Media constituents have been reported to affect growth and morphology of fungi in submerged culture (Metz & Kossen, 1977; Whitaker & Long, 1973). In cultures of other fungi, peptone derived from soya beans has been shown to induce dispersed growth (Biocon Ltd., personal communication).

Limited experiments were conducted at this stage to try to prevent coalescence of growth occurring in inoculum flasks. Comparative cultures were grown in Medium A and Medium B(1), in which casein hydrolysate was replaced by soya peptone (Sections 2.7.1 and 2.7.2). Media (500 ml) were dispensed into 1-litre conical flasks, inoculated with 1×10^4 spores/ml culture, and incubated for 18 hours under standard conditions, when biomass was determined. It was found necessary to use larger flasks because

wall-growth occurred when using Medium B(1) in 250 ml conical flasks (Section 2.7.2). Typical results are presented in Table 3.2.

Table 3.2. Comparison of growth R. arrhizus in casein and peptone media

Medium (g/l):			Biomass:	pH	
Glucose	Casein	Peptone(Medium)	Dry weight (mg/100ml)	Initial	Final
45	13	- (A)	93.1	6.12	3.90
45	-	13 (B.1)	114.2	6.00	3.78

Although biomass levels in Medium B(1) were higher than in Medium A, the growth was not dispersed.

Surface-active agents, such as Span-20, have been reported to disperse growth of fungi (Takahashi et al., 1965). An experiment was conducted in which Medium A and B(1) were supplemented with Span-20 and the effect on growth examined. Replicate flasks were inoculated with $1x10^4$ spores/ml culture, and incubated under standard conditions (Sections 2.3, 2.4, 2.5 and 2.7). After 18 hours, growth morphology was examined and biomass levels determined. The results are presented in Table 3.3. The level of biomass was reduced when Span-20 was added to Medium A or B(1). Higher levels of Span-20 reduced the yield of biomass further. At 0.4% (v/v) Span-20, growth

remained dispersed up to 30 hours incubation, after which coalescence occurred. Medium A with Span-20 was later evaluated for use as an inoculum for production flasks (Section 3.2.2).

Table 3.3. Effect of Span-20 on growth of R. arrhizus on peptone and casein media

Medium	Span-20 % (v/v)	Biomass (mg/100ml)
Casein (A)	-	191.2
	0.01	213.6
	0.1	144.9
	0.4	102.3
Peptone (B 1)	-	NM
	0.01	165.3
	0.1	138.6
	0.4	120.2

Levels of nitrogeneous materials in culture media have been shown to affect growth morphology and pH of fungal fermentations (Fukumoto et al., 1974; Solomons, 1969; Whitaker & Long, 1973). An experiment was conducted in which levels of casein hydrolysate and glucose in Medium A were varied and the effect on growth morphology, biomass and pH determined. Because replicate flasks were

required to accurately measure biomass yields, the number of combinations of glucose and casein which could be tested were limited. Cultures were grown under standard conditions for 18 hours, from a spore inoculum of $1x10^4$ spores/ml culture. Growth morphology, biomass yield and pH were then determined (Sections 2.3, 2.4, 2.5 and 2.9). The results of two such experiments are presented in Table 3.4. Increasing glucose levels from 20 g/l to 100 g/l progressively reduced biomass, while affecting pH and growth morphology very little. Higher levels of casein hydrolysate (e.g. 30 g/l) reduced biomass yield,while final pHs were higher. However, growth coalesced in these cultures even earlier than in Medium A. Other combinations tested affected biomass yield or pH, or both, without significantly affecting growth morphology.

3.1.4.

This work allowed reproducible vegetative inocula to be developed for subculturing into further media. The standard Medium A was adopted because vegetative growth remained dispersed up to 20 hours incubation, under standard conditions. This was found to disperse into inoculated media better then coalesced growth. In the next section vegetative growth of known age, biomass and pH was used to inoculate production media in shake-flasks to evaluate the effect of different factors on lipase production.

Table 3.4. Effect of varying Medium A components on growth of R. arrhizus

Medium (g/l)			Biomass: Dry weight	pH	
Casein	Glucose		(mg/100ml)	Initial	Final
A. 13	20		184.6	6.30	3.45
13	45	(Medium A)	174.9	5.95	3.47
13	70		151.6	5.92	3.57
13	100		124.9	5.85	3.76
30	45		175.1	5.89	4.11
60	45		114.8	5.75	4.76
100	45		73.1	5.50	4.71
B. 13	45	(Medium A)	136.7	5.84	4.79
20	20		149.1	6.17	5.10
30	10		161.9	6.25	5.45
30	20		133.5	6.12	5.59
30	30		130.9	5.91	5.43

3.2. STUDIES ON LIPASE PRODUCTION IN SHAKE-FLASKS.

In the development of fermentation processes a thorough study of growth and production media is required. Often relatively small changes in media can result in large gains in product yield. For preliminary studies, shake-flask cultures provide a suitable experimental system (Kuenzi & Auden, 1983; Solomons, 1969; Wang et al., 1979). Many factors have been shown to affect enzyme production including, carbohydrate and nitrogen levels, pH, aeration and the presence of inducing substances (Demain, 1979; Wang et al., 1979). Some of the factors reported to affect lipase production by Rhizopus species have already been discussed (Section 1.4.1). The effect of different parameters on lipase production by R. arrhizus were studied in this section in shake-flasks. Preliminary work by Biocon Ltd., led to the formulation of a production medium (Biocon Ltd, personal communication). Variations in this medium and culture conditions were evaluated for lipase production with a view to maximizing yields. The shake-flasks used were 2-litre conical flasks containing 1 litre of Medium D, unless otherwise stated. Incubation conditions were as standard (Section 2.4). Vegetative inocula (10% v/v) were developed as described in Section 3.1.3. It was decided to work on this scale as it was found to be easier to aseptically transfer whole flask cultures (100 ml), rather than attempt aseptic division of flask

contents. In many cases the growth morphology would have prevented such division. Lipase and sugar determinations were conducted using procedures described (Sections 2.8.1. and 2.8.2). Production results were rated using the Greek-Latin-Square method (Auden et al., 1967).

3.2.1. Time-course of lipase production in flasks.

A typical shake-flask production fermentation consisted of growing vegetative inoculum from spores in 250 ml inocula flasks and transferring the contents (100 ml) aseptically into 2-litre flasks containing 1 litre of production media. This was incubated and sampled aseptically (10 ml) for pH, sugar and lipase analysis. A typical time-course is shown in Figure 3.3. Lipase was produced approximately linearly up to 96 hours after which the rate decreased. Reducing-sugars were utilized rapidly in the first 24 to 48 hours, after which a constant level remained. pH dropped to 5.70 during the first 24 hours, but then rose above 6.0 and remained stable for the rest of the incubation. Morphology of growth varied in these flasks with time of incubation. On transfer of inoculum, and for the first 24 hours incubation, large marbles were formed. These were variable in shape and size, approximately 1 cm in length. After further incubation' the growth dispersed to give a heavy, porridge-like consistency to the medium. This morphology was found to be associated with high lipase productivity. During later experiments incubation

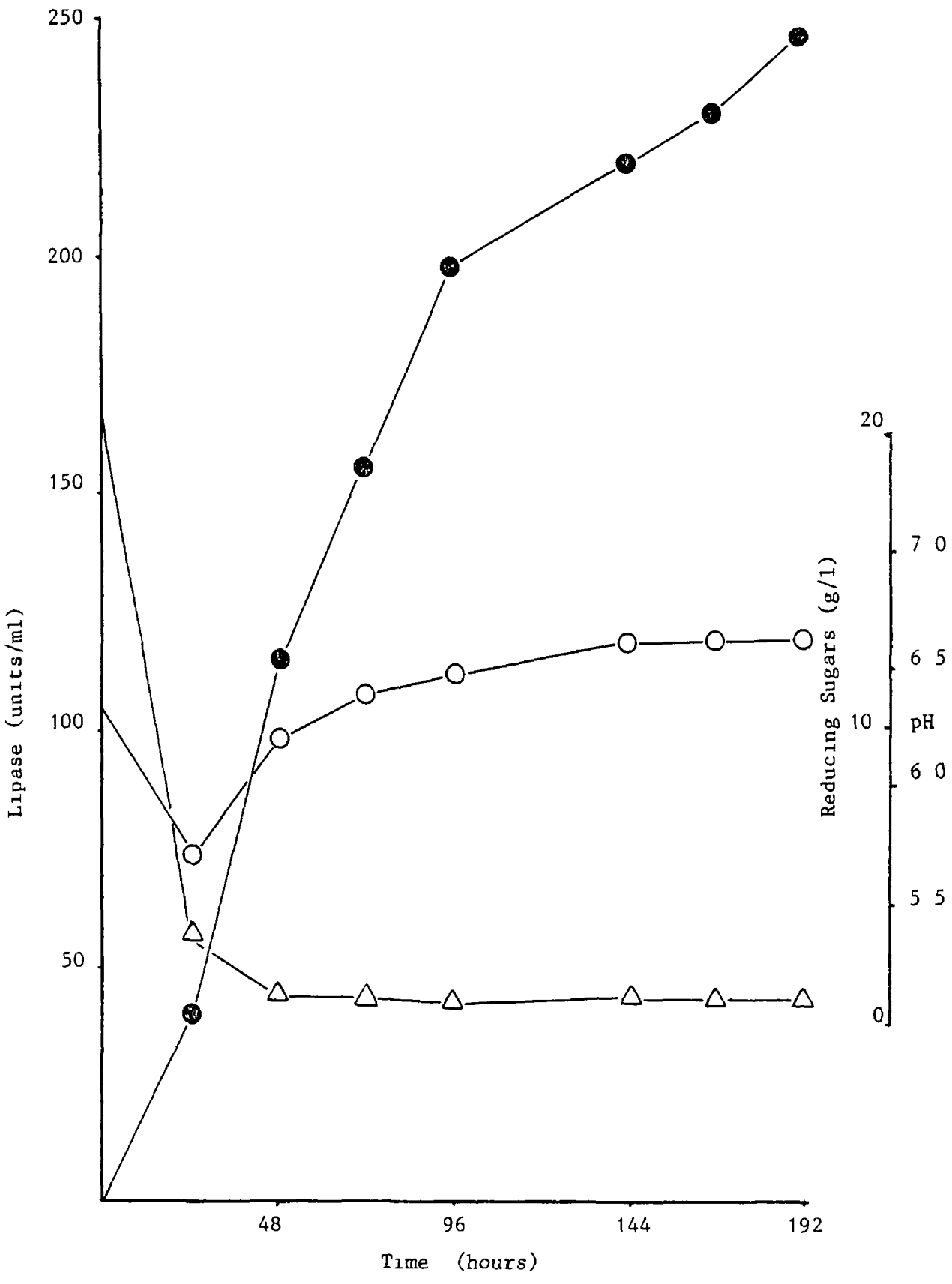

Figure 3 3 Lipase production (●), reducing sugars (Δ) and pH (○) of R arrhizus cultures in Medium D (1 l) in 2-l conical flasks incubated at 30°C and 150 r p.m Vegetative inoculum was grown in 100 ml Medium A in 250 ml conical flasks, from 1 x 10^4 spores/ml, incubated as above for 19 hours

periods were reduced and fewer samples were assayed for sugars and lipase. This was to allow a larger number of flasks and conditions to be examined in any given experiment.

3.2.2. Effect of inoculum on lipase production.

Several experiments were carried out in which inocula of different incubation periods were transferred to production media and evaluated for lipase production. The dry weights of inocula were determined in replicate flasks. Typical results are shown in Figure 3.4. These results show that production was affected very little by age or dry weight of inocula. Somewhat higher yields were obtained from the 24-hour inoculum, even though this had coalesced prior to transfer. pH patterns were all similar in that a drop to pH ~5.35 was followed by a slow rise to 6.0 during the incubation. Other experiments were done and it was shown that 15-hour inocula, or earlier, with biomass levels of ~100 mg/ 100 ml, produced less enzyme and utilized reducing-sugars more slowly. Section 3.1.3. described how Medium A, when supplemented with Span-20, produced more dispersed growth even after 30 hours incubation. Flask cultures were developed as described and used as inocula for production media. Incubation under standard conditions was continued for 144 hours before sampling for lipase analysis. Sugar utilization in the first 24 hours was also determined. pH patterns were found to be similar in

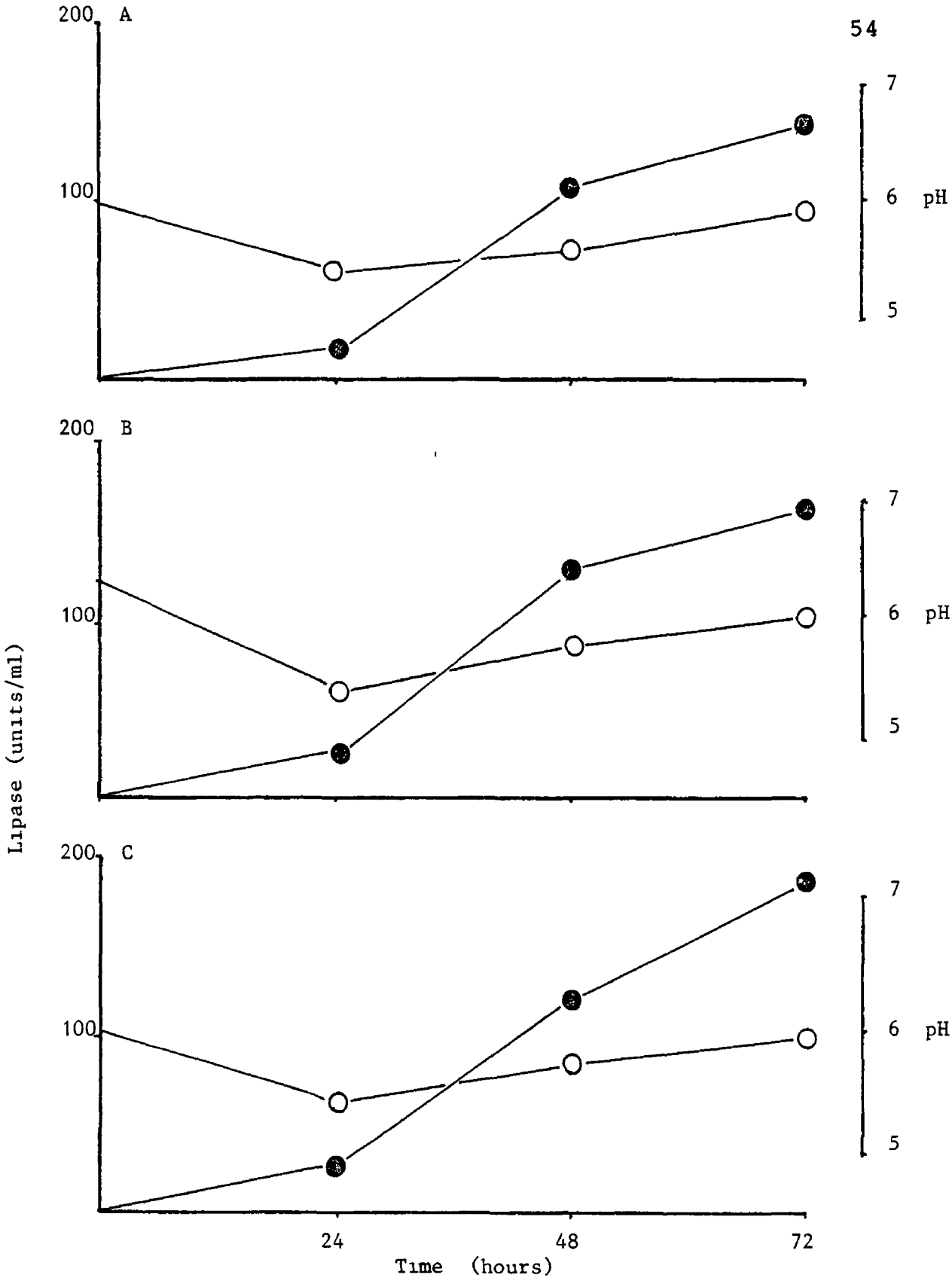

Figure 3 4 Effect of inoculum age on lipase production (●) and pH (○) of R arrhizus cultures in Medium D (1 1) in 2-l conical flasks, incubated at 30°C and 150 r p m Vegetative inocula were grown in Medium A (100 ml) in 250 ml conical flasks from 1 x 10^4 spores/ml, incubated as above for the following periods A 18 hours (140 9 mg/100 ml), B 20 hours (272 mg/100 ml), C 24 hours (426 1 mg/100 ml)

each flask fermentation. The results are presented in Table 3.5.

It should be noted that biomass levels varied in media containing Span-20, due to different incubation periods used. Addition of Span-20 to the inoculum medium did not enhance enzyme yields in the producion medium. When the inoculum medium contained 0.1% (v/v) Span-20, enzyme production appeared to be inhibited even though biomass was transferred at a relatively high level. Sugar utilization by these cultures was slower compared with the standard inoculum transferred to production media. It was difficult to explain why 0.1% (v/v) Span-20 had a more inhibitory effect then did 0.2% (v/v) Span-20. However, since the standard inoculum produced more enzyme in production flasks further experiments with Span-20 were not conducted.

In the lipase production experiments that follow a standard inoculum procedure was used: 100 ml Medium A in 250 ml conical flasks, inoculated with $1x10^4$ spores/ml medium, and incubated under standard conditions for 19 hours. Replicate flasks have been shown to produce comparable amounts of biomass in each culture, so these were used to inoculate experimental production media. It should be noted that biomass levels (dry weight) could not be determined in production media due to the insoluble nature of many of its components.

Table 3.5. Effect of varying inoculum on lipase production

Medium	Inoculum: Age (hours)	Biomass (mg/100 ml)	pH	Production: Lipase (u/ml) t=144 hours
A	19.5	405.3	3.63	195.0
A + 0.1% (v/v) Span-20	19.5	306.2	3.71	75.0
A + 0.1% Span-20	30.0	521.6	3.71	65.0
A + 0.1% Span-20	19.5	276.5	3.69	198.0
A + 0.1% Span-20	30.0	478.1	3.76	170.0

Inocula were developed in 100 ml Medium A, 250 ml conical flasks incubated at 30°C and 150 r.p.m., for specified periods. They were then transferred to 2-l conical flasks containing 1 l of Medium D and incubated as above.

3.2.3. Effect of aeration on lipase production.

Aeration in shake-flasks is provided by continuous shaking of media in flasks. This process is impaired if larger volumes of media are used in the flasks. This is due to the lower surface-area to volume ratio. It was considered that 1 litre of production medium in a 2-litre conical flask might result in poor aeration of the culture. Aeration has been reported to be of importance for lipase production by R. delemar (Giuseppin, 1984). A number of experiments were carried out in which 500 ml, instead of 1 litre, of Medium D was used in 2-litre conical flasks. A 10% (v/v) vegetative inoculum was used and production compared with standard conditions. Typical results are shown in Figure 3.5. The results indicate that the fermentation pattern was not affected by reducing the volume of media in flasks (i.e. increasing the aeration). This implies that aeration levels in 2-litre shake-flasks with 1 litre of medium is adequate for growth and lipase production.

3.2.4. Effect of varying carbohydrates on the the lipase fermentation.

Levels and types of carbohydrate can affect enzyme fermentations due to catabolite repression (Wang et al., 1979). This occurs when high levels of rapidly utilizable sugars repress enzyme production (Paigen & Williams, 1970). It has been reported that for lipase

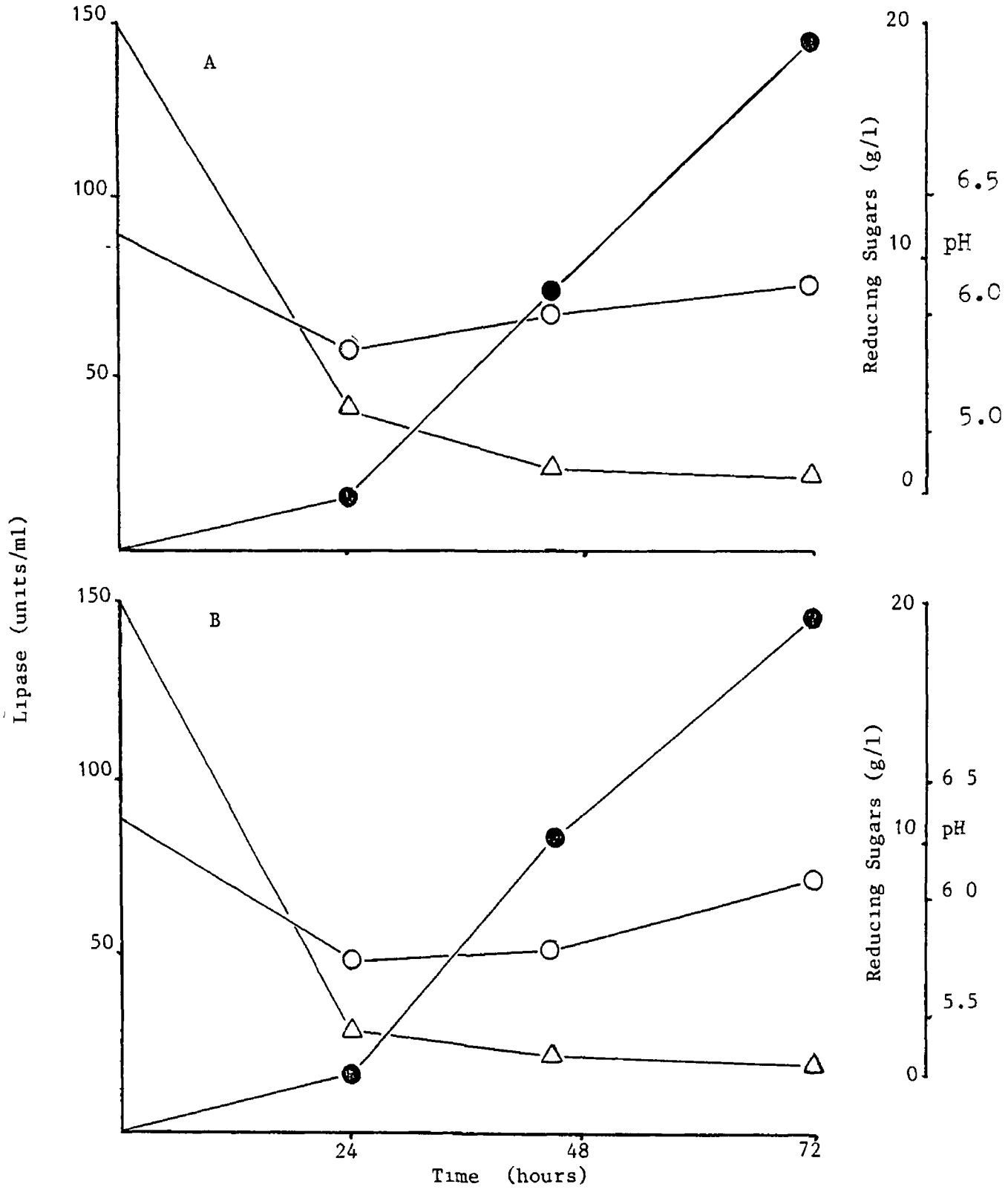

Figure 3 5. Effect of aeration on lipase production (●), reducing sugars (Δ) and pH (O) in cultures of R arrhizus in Medium D in 2-1 conical flasks incubated at 30°C and 150 r p m Medium D volumes were A. 500 ml, B 1 litre. Vegetative inocula (10% v/v) were grown as standard (Section 3 2 1)

production by Rhizopus species, glucose levels need to drop below a critical level (Macrae, 1983). This problem may be solved by using slowly metabolizable carbohydrates, such as starch or lactose (Demain, 1982). Fed-batch processes are now increasingly being considered as an alternative, especially for controlling supply of carbohydrate and nitrogen sources (Kuenzi & Auden, 1983). The following experiments were conducted to characterize and optimize lipase production by varying the carbohydrate feed in different ways.

3.2.4.1. Effect of varying maize hydrolysate levels on the fermentation.

Maize levels in Medium D were varied over a range from 35 to 85 g/l after standard enzymatic hydrolysis (Section 2.7.4). Fermentations were conducted under standard incubation conditions. The results are presented in Table 3.6. Higher levels of maize hydrolysate produced higher reducing-sugar levels in the medium. At levels above 45 g/l maize, these sugars appeared, to progressively reduce lipase yields. At 85 g/l maize the sugars were utilized very slowly with no lipase being detected.

3.2.4.2. Use of alternative carbohydrate sources for the fermentation.

Several commercial carbohydrate materials were tried,

55			65			85		
pH	Reducing sugars (g/l)	Lipase (u/ml)	pH	Reducing sugars (g/l)	Lipase (u/ml)	pH	Reducing sugars (g/l)	Lipase (u/ml)
6.86	30.6	-	6.89	42.3	-	6.10	36.3	-
5.59	5.6	14.0	5.67	23.8	15.8	5.58	36.3	0
5.80	2.4	85.5	5.20	2.9	60.0	5.50	35.9	0
6.39	2.8	102.4	6.23	7.3	84.0	5.54	22.5	0

with 1 litre medium D, modified as above.

incubation conducted at 30 °C and 150 r.p.m.

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4. Dextrin MD01 45 g/l			5. Hydrolyzed Maize 45 g/l + Glucose (10 g/l)			6. Hydrolyzed Maize 25 g/l + Glucose (10 g/l)			7. Glucose 25 g/l		
pH	Reducing Sugars (g/l)	Lipase (u/ml)	pH	Reducing Sugars (g/l)	Lipase (u/ml)	pH	Reducing Sugars (g/l)	Lipase (u/ml)	pH	Reducing Sugars (g/l)	Lipase (u/ml)
6.52	12.7	—	6.37	35.0	—	6.40	23.1	—	6.50	26.6	—
5.60	20.4	53.0	5.83	23.5	17.1	5.80	11.0	41.4	5.80	13.0	49.5
6.10	1.5	44.0	6.18	2.4	59.5	6.21	1.4	109.0	6.12	1.0	82.5
6.04	1.3	95.0	6.26	1.8	109.0	6.36	1.1	176.0	6.16	1.2	110.0
6.08	1.0	104.0	6.41	1.7	118.0	6.45	1.2	180.0	6.25	1.2	127.0
6.30	1.0	131.0	6.45	1.5	147.0	6.52	1.2	223.0	6.39	1.1	158.0
6.35	1.0	141.0	6.38	1.5	156.0	6.49	1.1	239.0	6.40	1.1	155.0

Cultures in 2-l conical flasks with 1 l Medium D, modified as above,

150 r.p.m.

either alone or in combination with maize, to see if they were suitable for fermentation. The results of one such set of fermentations is presented in Table 3.7. The only medium which gave comparable yields to maize at 45 g/l contained maize at 25 g/l with a 10 g/l glucose supplement. Corn starch (hydrolysed) gave a higher level of reducing-sugars and a lower enzyme yield. When glucose was used alone, at levels comparable to those in Medium D, a reduced enzyme yield was observed. In general, carbohydrate sources yielding higher levels of reducing-sugars gave lower lipase yields. In other experiments, sucrose and lactose were shown not to affect the fermentation. These sugars have been reported not to be utilized by Rhizopus species (Rhodes et al., 1959; Sorenson & Hesseltine, 1966).

3.2.4.3. Carbohydrate feeding during the fermentation.

Feeding carbohydrates to a fermentation may overcome the inhibitory effect of adding it all in one batch. This strategey has been used for the penicillin fermentation instead of using lactose (Aharonowitz & Cohen, 1981; Duckworth & Harris, 1949). Some experiments were conducted in which lipase flask fermentations were dosed with different levels of dextrin (a breakdown product of starch) after initial reducing-sugars were depleted. Dextrin MD-05 was obtained from Roquette, France. Typical results are presented in Figure 3.6.

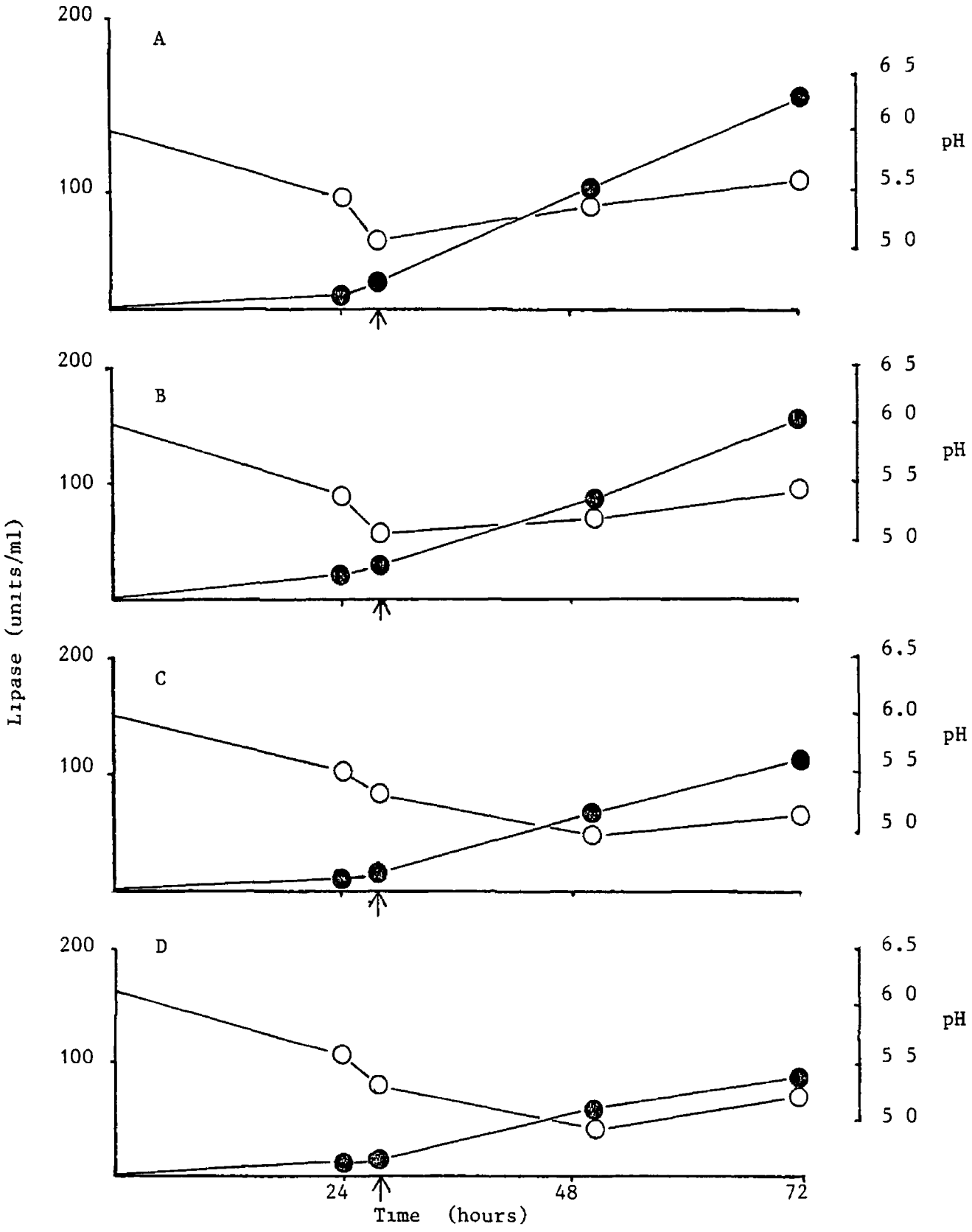

Figure 3 6. Effect of dose-feeding dextrin on lipase production (●) and pH (○) of R.arrhizus cultures in Medium D (1-1) in 2-1 conical flasks incubated at 30°C and 150 r.p m. Dextrin dose-fed at 28 hours A 0(control), B 5 g/l, C 10 g/l, D 15 g/l.

Vegetative inocula (10% v/v) were grown as standard (Section 3 2.1)

These show that carbohydrate fed to the fermentation reduced the lipase yields. It is significant that the effect was proportional to the dose of carbohydrate given. Reducing-sugars were not monitored closely, but all t=50 hour samples had comparable levels of sugars indicating that the added sugars had been utilized. Another indication that the sugars were used came from the fact that final pHs were reduced when the cultures were dose-fed. This may be, in part, the reason yields were lower. However, closer examination of pH values shows that in flask C the pH was lower than in flask D, even though flask C gave higher enzyme yields. It would appear that the principal reason yields were reduced was the higher carbohydrate levels present.

A novel approach to slow-feeding of sugars to the fermentation was then tried. It consisted of adding cellulose to Medium D as Solka-floc (BW-40) and adding a commercial preparation of cellulase, Bioglucanase, both obtained from Biocon Ltd, Ireland. The enzyme preparation was confirmed as having 600 C.M.-cellulase units/ml (Appendix I). Fermentations were conducted using Medium D supplemented with 20 g/l Solka-floc. Different doses of sterile Bioglucanase were added to the flasks aseptically after inoculation. Typical results are presented in Figure 3.7. Higher doses of Bioglucanase generated more reducing-sugars and this was reflected in the progressivly higher levels found at

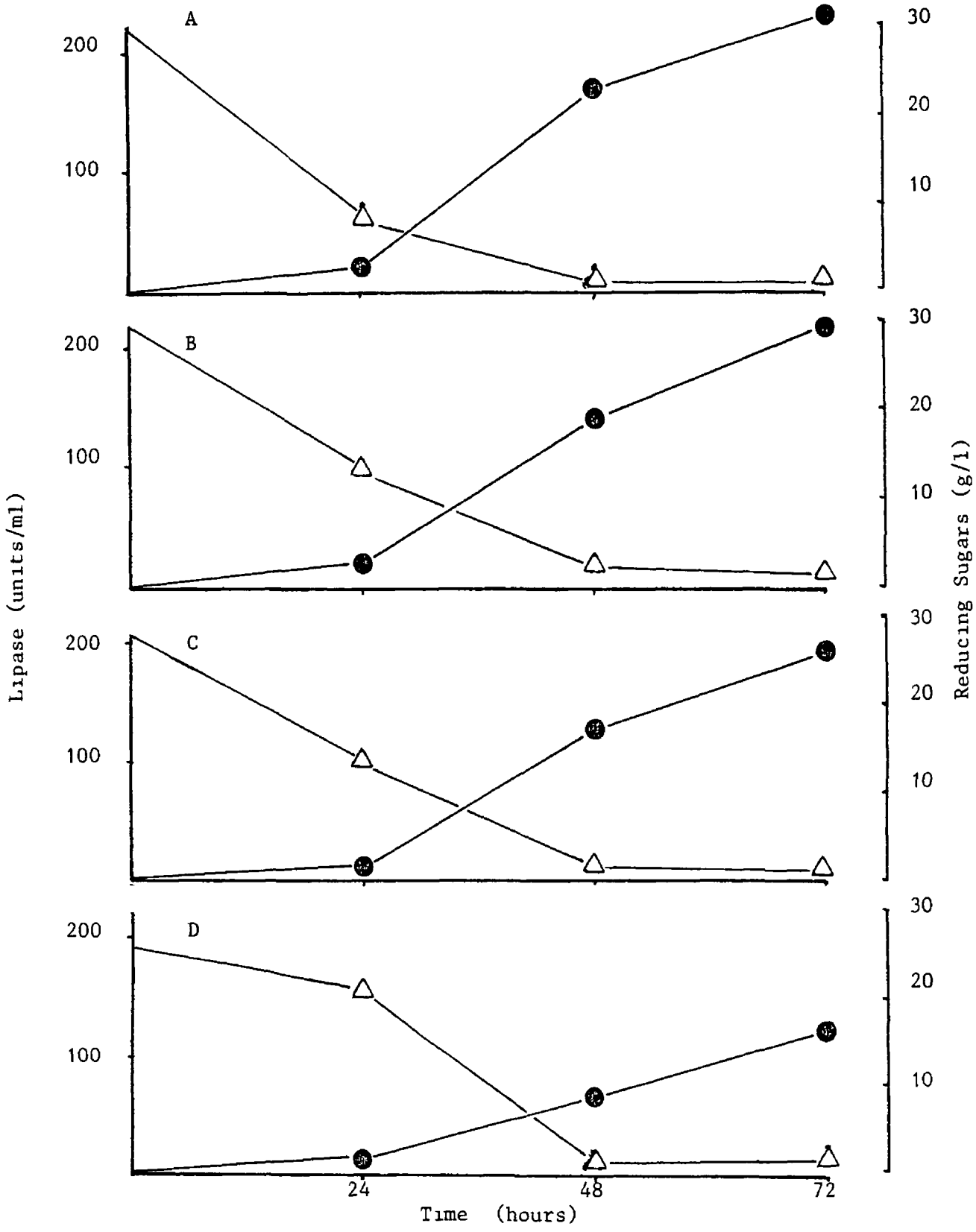

Figure 3 7 Effect of actively hydrolysing cellulose on lipase production (●) and reducing sugars (Δ) in R arrhizus cultures in Medium D (supplemented with 20 g/l Solka-floc) 1 l in 2-l conical flasks, incubated at 30°C and 150 r p m Sterile, active Bioglucanase added at t=0. A. 0 (control), B. 3 units/ml, C. 15 units/ml, D 30 units/ml Vegetative inocula (10% v/v) were grown as standard (Section 3 2 1)

t=24. Lipase yields were progressively reduced also. The pH patterns in each flask were similar to the control, with no depression. Cellulose was hydrolysed to release free glucose units and these appear to have repressed enzyme production.

3.2.5. Nitrogen sources and their effect on the lipase fermentation.

Complex organic nitrogen sources have been reported to affect lipase production by *Rhizopus* species (Zubenko et al., 1979). Many sources have been suggested in the literature and these include peptones, yeast extract, casein and corn steep solids (Aisaka & Terada, 1979; Mauvernay et al., 1970). It has also been shown that relative amounts of carbohydrate and nitrogen sources can affect production (Fukumoto et al., 1966). The following experiments were conducted to investigate the effect of different nitrogen sources on lipase production by *R. arrhizus* in Medium D.

3.2.5.1. Effect of varying maize and casein levels on lipase yields.

Maize hydrolysate and casein hydrolysate levels were varied over a limited range to evaluate their effect on lipase production. The results of one such experiment are presented in Table 3.8.

Table 3.8. Effect of varying maize and casein levels on lipase yields

Production Medium:				Lipase (t=48 hours)	
Maize (g/l)	Casein (g/l)	pH_i	Reducing Sugars (g/l)	yield (units/ml)	pH_f
45	13	5.86	30.3	110.0	5.60
45	20	5.95	28.9	119.0	5.80
45	30	5.86	25.1	77.3	5.70
30	13	6.10	15.6	120.0	6.00
30	30	6.18	15.0	74.0	5.90
20	13	6.35	12.1	112.8	6.15
20	30	6.30	12.2	73.0	6.00

R. arrhizus cultures were grown in 2-l conical flasks with 1 l Medium D, modified as above, from a standard inoculum 10% (v/v) vegetative growth (Section 3.7.1) and incubated at 30°C and 150 r.p.m.

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Casein at 30 g/l gave reduced lipase yields, whereas at 20 g/l slightly higher yields were obtained. The increase was not sufficient to justify the extra medium cost, however. Reducing-sugar levels varied with the level of maize added, but pH patterns were shown to be affected very little. Slightly higher yields were obtained at t=48 hours when lower levels of maize were used. This may be explained by the earlier achievement of low reducing-sugar levels in these fermentations, facilitating earlier lipase yields. Subsequent experience has shown that higher overall yields were eventually attained using maize at 45 g/l, probably due to higher biomass levels being produced.

3.2.5.2. Effect of addition of supplementary nitrogen sources on the lipase fermentation.

Suggestions were obtained from the literature regarding levels and types of organic nitrogen sources which might enhance lipase yields from *Rhizopus* species. Medium D was supplemented with these and the fermentations conducted in standard shake-flasks. The results are shown in Table 3.9. The results show that, under the conditions used, no significant benefit was derived for lipase production by using supplementary nitrogen sources. Production was repressed in some fermentations (eg flasks 3 and 5), and this may be due to another form of catabolie repression by the nitrogen source (Demain, 1982).

Table 3.9. Effect of addition of supplementary nitrogen sources on the lipase fermentation

Medium D	1		2		3		4		5		6		7	
Supplementary organic nitrogen source:	Control		Corn steep 10 g/l		Corn steep 30 g/l		Yeast extract 3 g/l		Yeast extract 10 g/l		Soya flour 20 g/l		Soya flour 30 g/l	
Time (hours)	pH	Lipase (u/ml)	pH	Lipase (u/ml)	pH	Lipase (u/ml)	pH	Lipase (u/ml)	pH	Lipase (u/ml)	pH	Lipase (u/ml)	pH	Lipase (u/ml)
0	6.42	-	6.41	-	6.47	-	6.47	-	6.46	-	6.31	-	6.26	-
72	6.18	176.0	6.30	166.0	6.42	0	6.30	147.0	6.32	84	6.05	84.5	6.26	49.0
145	6.42	280.0	6.48	231.0	7.19	0	6.42	244.0	6.43	154	6.20	280.0	6.30	206.0

R. arrhizus cultures were grown in 2-litre conical flasks with 1-litre Medium D, modified as above, standard inoculum 10% (v/v) vegetative growth (Section 3.2.1); incubation was conducted at 30 C and 150 r.p.m.

3.2.6. Effect of trace metals on the fermentation.

During preliminary experiments in shake-flasks it was observed that different grades of chemicals affected the fermentation pattern. Medium D was prepared with distilled/deionized water and its formulation required high levels of salts, especially $CaCO_3$ and $CaCl_2$. It was noted that use of Analar, or highly purified chemicals, resulted in different lipase production patterns compared with cultures made using General Purpose Reagent or cruder grade chemicals.

The literature on fungal physiology has reported that fungal metabolism is affected by trace heavy-metal ions (McHargue & Calfee, 1931). Rhizopus species have been shown to be profoundly influenced by zinc ions in particular, which affect glucose utilization, growth and organic acid production (Foster & Waksman, 1938; Rhodes et al., 1959; Waksman & Foster, 1938; Wegener & Romano, 1963). Experiments were carried out in which lipase production fermentations using A.R. or G.P.R. chemical ingredients were supplemented with zinc ions to test if enzyme yields were affected. The results are presented in Figure 3.8. These show that addition of zinc to the medium containg A.R. chemicals significantly improved yields of the enzyme. This effect was not observed in media containing G.P.R. chemicals, perhaps because levels of trace metals were already sufficiently high, and so masked any additional effect.

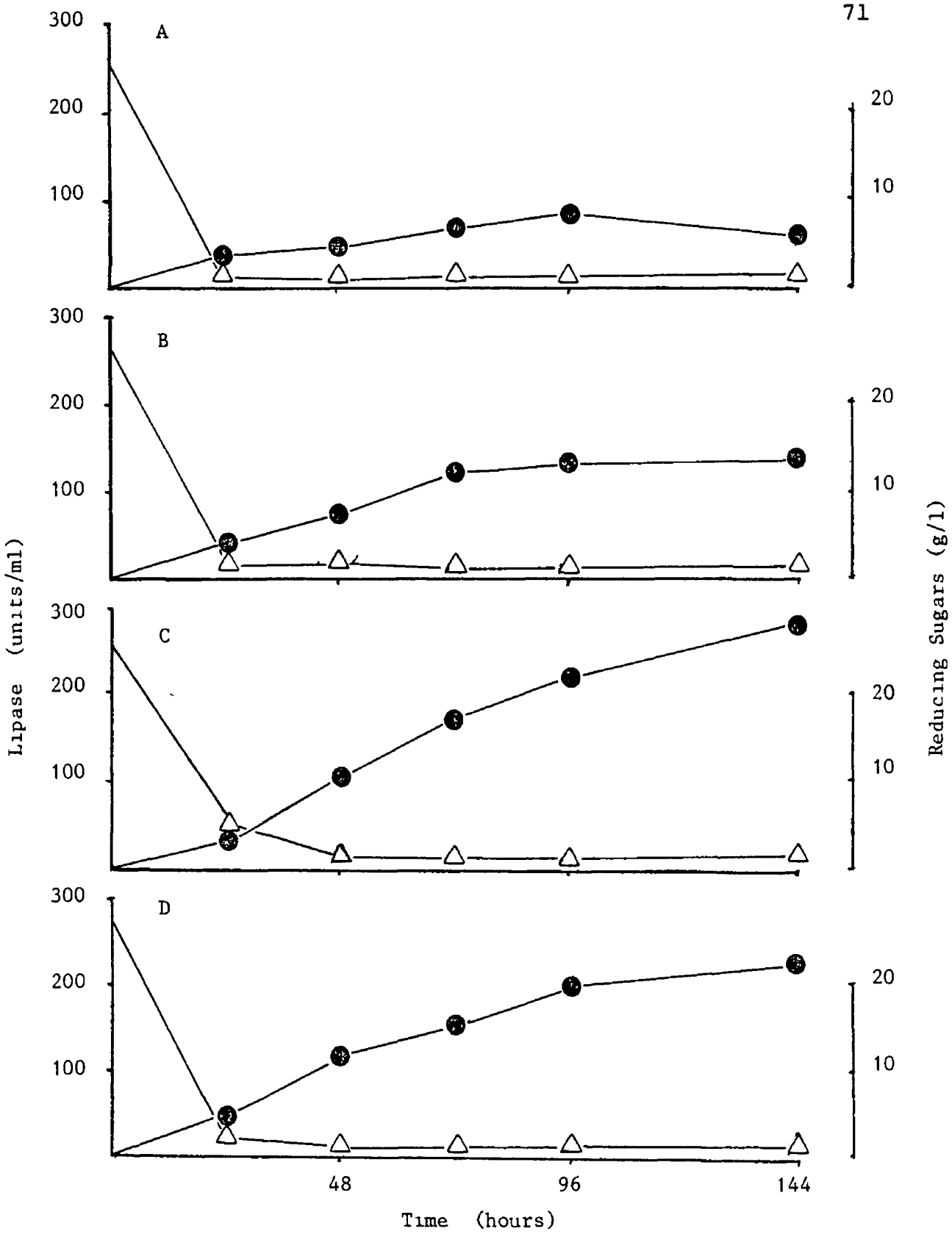

Figure 3 8 Effect of chemical grades and/or zinc on lipase production (●) and reducing sugars (Δ) in R arrhizus cultures in Medium D (1 l) in 2-l conical flasks, incubated at 30°C and 150 r p m Medium D was formulated using A. Analar chemicals, B. A.R chemicals + 2 mg/l $ZnSO_4$ $7H_2O$, C General Purpose Reagent chemicals, D G.P.R. chemicals + 2 mg/l $ZnSO_4.7H_2O$ Vegetative inocula (10% v/v) were grown as standard (Section 3.2.1)

3.2.7. Enhancement of lipase yields by addition of fatty substances.

Lipase production by Rhizopus species has been reported to be affected by addition of fatty materials to the culture medium (Volkova & Lebedeva, 1979). In particular, fatty components of soya beans have been shown to enhance lipase yields (Aisaka & Terada, 1979; Wagenknecht et al., 1961). Rhizopus species do not have an absolute requirement for fats in order to produce lipase,as has been shown for Candida paralipolytica (Desnuelle, 1972; Ota et al., 1968). Some experiments were conducted adding fatty supplements to Medium D and the effect on the lipase fermentation monitored. The results of several fermentations are shown in Table 3.10. Tween-80 was found to increase the lipase yield. This may be due to enhanced enzyme release because of surfactant effects on the fungal membranes (Reese & Maguire, 1969). Olive oil was shown to reduce yields and this is in agreement with previous reports (Akhtar et al., 1974). Addition of soya flour had little effect on lipase yields.

3.2.8. Effect of pH on lipase production.

Experience accumulated during this work indicated that a pH pattern of: 6-6.4 at t=0, dropping to ~5.80 at t=24 and rising to pH 6.5 subsequently, was optimal for enzyme production. Difficulty was experienced in

Table 3.10. Enhancement of lipase yields by addition of fatty substances

Flask no.	1.		2.		3.		4.		5.	
Medium D Fatty additive:	Control		Soya flour (full fat) 10 g/l		Olive oil 1 ml/l		Olive oil 10 ml/l		Tween 80 1 ml/l	
Time (hours)	pH	Lipase (u/ml)	pH	Lipase (u/ml)	pH	Lipase (u/ml)	pH	Lipase (u/ml)	pH	Lipase (u/ml)
0	6.41	-	6.38	-	6.42	-	6.47	-	6.39	-
72	6.28	134.0	6.28	230.5	6.26	163.0	6.11	130.0	6.27	178
145	6.44	265.0	6.45	258.0	6.42	240.0	3.37	203.0	6.45	307.0

R. arrhizus cultures were grown in 2-litre conical flasks with 1-litre Medium D (modified as shown); 10% (v/v) vegetative inoculum (Section 3.2.1) and incubated at 30°C and 150 r.p.m.

conducting definitive experiments related to pH. If pH of the medium was set to 7-7.5 at t=0, it quickly dropped to 6.0 during the first 24 hours incubation. Similarly, media set to pH 5-5.5 rose quickly in the same period to ~6.0. Such fermentations produced suboptimal lipase yields when compared to control cultures.

3.3. GROWTH FROM VEGETATIVE INOCULA AND SCALE-UP OF THE PROCESS.

For a fermentation process to achieve economic viability, it must be conducted at a scale which matches the cost of manufacture with the value of the product (Stowell & Bateson, 1983). The inoculum development process often requires several transfers of vegetative inocula to build up sufficient volume for the plant-scale production fermenter. The inoculum volume is designed to reduce lag periods in the production fermenter (Aunstrup et al., 1979). A typical inoculum development would be as follows: stock cultures are used to inoculate several flasks. These are incubated until sufficient biomass is produced, and, with the culture still actively growing, transferred to a pilot-fermenter containing the same medium. Further incubation is conducted in this fermenter, and when substantial biomass has been produced, this is used to inoculate the production fermenter (Wang et al., 1979). In fungal fermentations, the vegetative growth obtained by cultivating spores may be referred to as primary inoculum cultures, and growth obtained by subculturing these in the same medium, as secondary inocula. The purpose of inoculum media is to produce active biomass for transfer into other media, with the minimum of lag in subsequent growth. Such media may differ from production media, which are designed for optimal product yields.

The scale of operation envisaged for the lipase fermentation was dictated both by economic considerations and by plant facilities available at Biocon Ltd., Ireland. A final production volume of 2,000 litres in each of a number of 3,000-litre vessels was envisaged. In this section experiments were carried out to characterize growth of secondary inocula in flasks and in fermenters. Further experiments were then conducted to design an inoculum development procedure for the lipase process in the plant-scale fermenters.

3.3.1. Experiences with secondary inocula in flasks.

In standard primary inoculum cultures, using 100 ml Medium A in 250 ml conical flasks with standard incubation conditions, growth remained dispersed only up to 20 hours incubation (Section 3.1.2). Coalescence of growth was shown to be delayed if larger flasks or slower shaker-table speeds (100 instead of 150 r.p.m.) were used. Similarly, when higher spore levels were used ($2x10^5$ instead of $1x10^4$ spores/ml of culture), growth remained dispersed in the medium longer. When primary cultures, having reasonably dispersed growth, were used as 10% (v/v) inocula for the same medium, secondary cultures invariably exhibited coalescence or clumping. Primary inocula grown in the presence of Span-20 have been shown to be more dispersed (Section 3.1.3). However, secondary cultures with Span-20 present

coalesced almost as badly as did standard cultures. Nevertheless, secondary cultures in standard medium were shown to be capable of lipase production in shake-flasks, in spite of their morphology.

3.3.2. Experiences with secondary inocula in stirred laboratory fermenters.

Operational difficulties were encountered in attempting to transfer coalesced flask cultures through inoculation ports (2.1 cm diameter) on the 7.5-litre capacity Laboferm fermenters. Dispersed primary cultures could be transferred more easily. Many fermentations were attempted with 5 litres of Medium A in Laboferm fermenters operated at 30°C, 600 r.p.m. stirring rate and 0.5 v/v/m aeration rate. Inocula tested included: primary cultures produced from either $1x10^4$ or $2x10^5$ spores/ml culture, and direct transfer of spores to the fermenter at $4x10^3$ spores/ml culture. In all cases, growth in these fermenters attached itself to walls, baffles and impellers after 24 hours (Figure 3.9). It proved impossible to sample this biomass through sampling tubes of either 8 or 12 mm diameters. Some fermentations were attempted in which baffles and impellers were removed and aeration/agitation supplied using a ring sparger situated near the base of the Laboferm fermenter (ring sparger: 6 cm diameter with 12 perforations); (Barker & Worgan, 1981. Kiese et al., 1980). In each fermentation tried, growth remained in

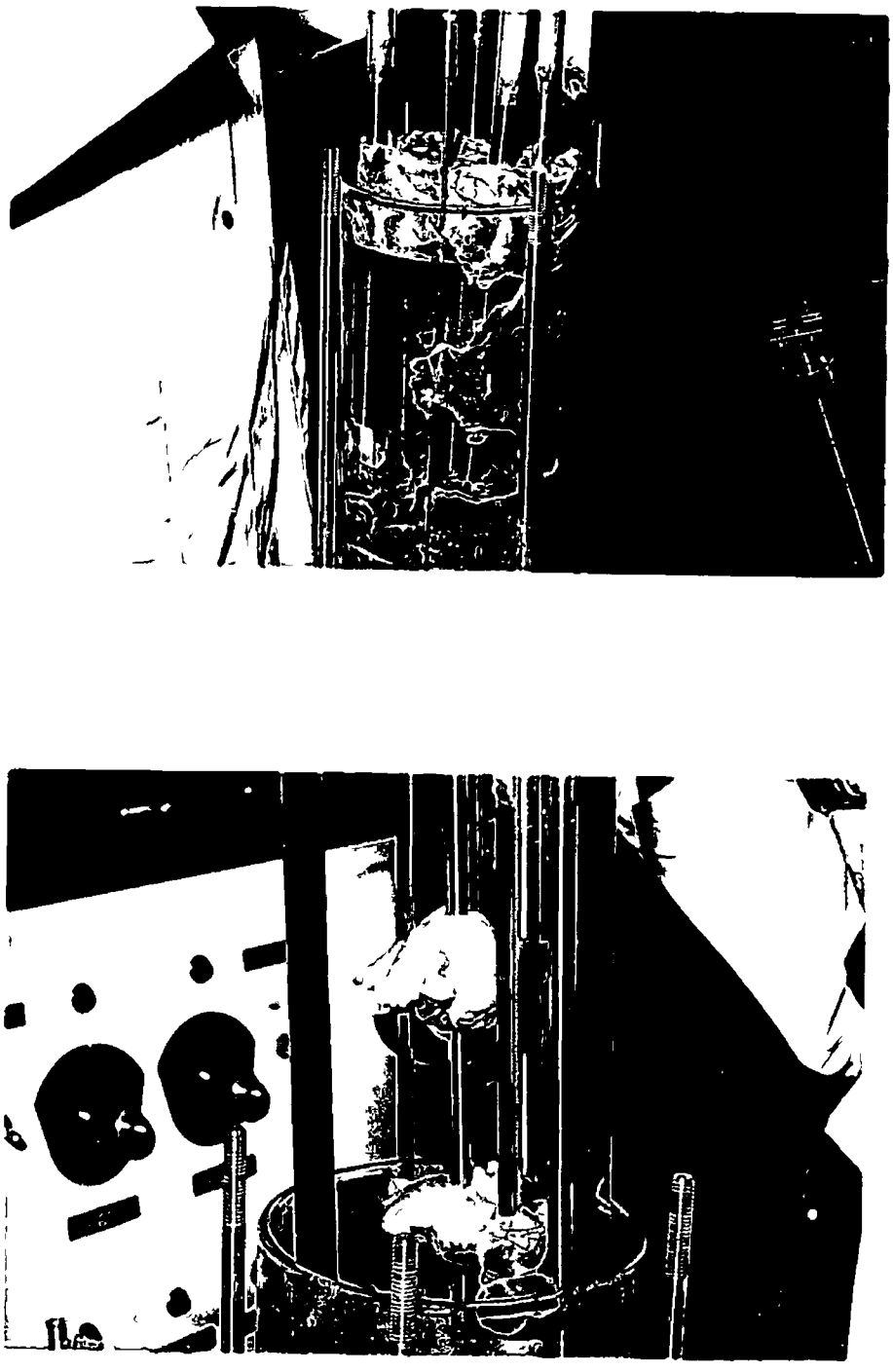

Figure 3.9. Growth of R.arrhizus attaching to walls, baffles and impellers in Laboferm fermenters.

the medium but could not be transferred or sampled using standard tubing.

3.3.3. Experiences with secondary inocula in pilot-scale fermenters.

It was decided to test if secondary inocula could be developed in a pilot-scale fermenter (250-litre) using Medium A. The fermenter dimensions are given on section 2.6. Primary inocula were developed in shake-flasks (Medium A) and used to inoculate a 250-litre fermenter containing the same medium. After further incubation, this fermenter was used to inoculate a plant-scale fermenter containing Medium D (Section 2.7.4). The fermentation stages are summarized as follows:

Culture stage	Vessel type	Vessel Capacity (l)	Medium (Section 2.7)	Medium volume-l	Agitation (r.p.m.)	Aeration (v/v/m)	Inoculum age(hours)	Inoculum type
Primary inoculum:	shake-flask	2	A	1	150	-	-	spores $1x10^4$/ml
Secondary inoculum	Fermenter	250	A	200	230	0.5	24	2% (v/v) primary culture
Production	Fermenter	3,000	D	2,000	104	0.5	26	10% (v/v) secondary culture

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When flask inocula were transferred to the pilot fermenter severe foaming occurred which necessitated intermittant cessation of agitation. Large volumes of anti-foam (type 'Kg', Biocon Ltd.) were ineffective at controlling the problem. Microscopic examination of

samples showed that torn fragments of mycelia were present. Nevertheless, the contents of this pilot fermenter were transferred to the production fermenter. However, no growth, sugar utilization or lipase production took place. It was concluded that shear forces caused by agitation in the pilot fermenter had destroyed the biomass.

These preliminary observations on growth of R. arrhizus identified a number of problems related to designing an inoculum development procedure for the lipase process. These problems included: the tendency of primary and secondary cultures in flasks to coalesce on prolonged incubation; the tendency of such growth to cling to baffles and impellers of laboratory fermenters; the difficulty experienced in transferring such growth aseptically and the susceptibilty of R. arrhizus to fragmentation by impeller shearing forces. A screening trial was initiated using primary cultivation in shake-flasks to develop a medium giving dispersed growth of R. arrhizus.

3.3.4. Studies on the effect of medium constituents on culture morphology.

Medium constituents and inoculum levels have frequently been cited in the literature as affecting growth morphology of fungi (Metz & Kossen, 1977; Whitaker &

Long, 1973). Many different media were tested as primary 24-hour cultures in shake-flasks, using two levels of spore inocula and standard conditions (Section 2.4). Higher spore inocula ($2x10^5$ instead of $1x10^4$ spores/ml of culture) have been shown to enable growth to fill the growth medium (Section 3.3.1). The results are presented in Table 3.11, and growth morphologies obtained are illustrated in Figures 3.10 and 3.11.

The results show that R. arrhizus was capable of assuming a diverse range of morphologies in different media. Levels of casein and glucose in Medium A did not affect morphology significantly. However, they did affect the level of biomass produced (Section 3.1.3). Initial pH of the medium affected morphology very little also. When casein and glucose levels were varied in Media 4 and 5, pH changed very little during growth but morphology was unaffected. Similarly, when the initial pHs were adjusted in Media 6, 7, 8 and 9, no significant effect on growth morphology was observed. Supplements added to Medium 1 were generally without effect on growth morphology. Addition of Span-20 at two levels dispersed growth somewhat, but this has been shown to coalesce on transfer to fresh media (Section 3.3.1).

Alternative nitrogen sources had the most profound effect on growth morphology. Sources such as soya peptone and corn-steep-solids gave similar results to casein hydroysate. Yeast extract gave an unusual

11.	Glucose, 45;	Casein, 13;	Soya bean meal, 10	(a)	6.30	3.78	(3)
				(b)	6.30	4.75	(4)
12.	Glucose, 45;	Casein, 13;	Solka-floc, 10	(a)	6.30	3.85	(3)
				(b)	6.30	4.40	(4)
13.	Glucose, 45;	Casein, 13;	Span-20, 0.2% (v/v)	(a)	6.06	4.10	(3)
				(b)	6.06	4.80	(4)
14.	Glucose, 45;	Casein, 13;	Span-20, 0.4% (v/v)	(a)	6.04	4.13	(3)
				(b)	6.04	4.20	(4)
15.	Glucose, 45;	Casein, 13;	Olive oil, 2.5% (v/v)	(a)	6.30	3.80	(3)
				(b)	6.30	4.23	(4)
16.	Glucose, 45;	Yeast extract, 3;	-	(a)	5.96	2.90	(2)
				(b)	5.96	3.10	(2)
17.	Glucose, 45;	Soya peptone, 5;	-	(a)	6.10	3.55	(3)
				(b)	6.10	3.32	(4)
18.	Glucose, 45;	Corn steep solids, 10;	-	(a)	6.24	3.90	(3)
				(b)	6.24	3.93	(4)
19.	Glucose, 45;	Soya flour, 10; (full fat)	-	(a)	5.93	3.60	(3)*
				(b)	5.93	3.60	(4)*

Notes: spore inoculum levels: (a)= 1×10^4/ml culture; (b)= 2×10^5/ml culture.

Growth morphology: numbers in brackets for Media 1-18 refer to Figure 3.10;
*for Medium 19, they refer to Figure 3.11.

Cultures were grown from spores, using 100 ml medium in 250 ml conical flasks, and incubated at 30°C and 150 r.p.m. for 24 hours.

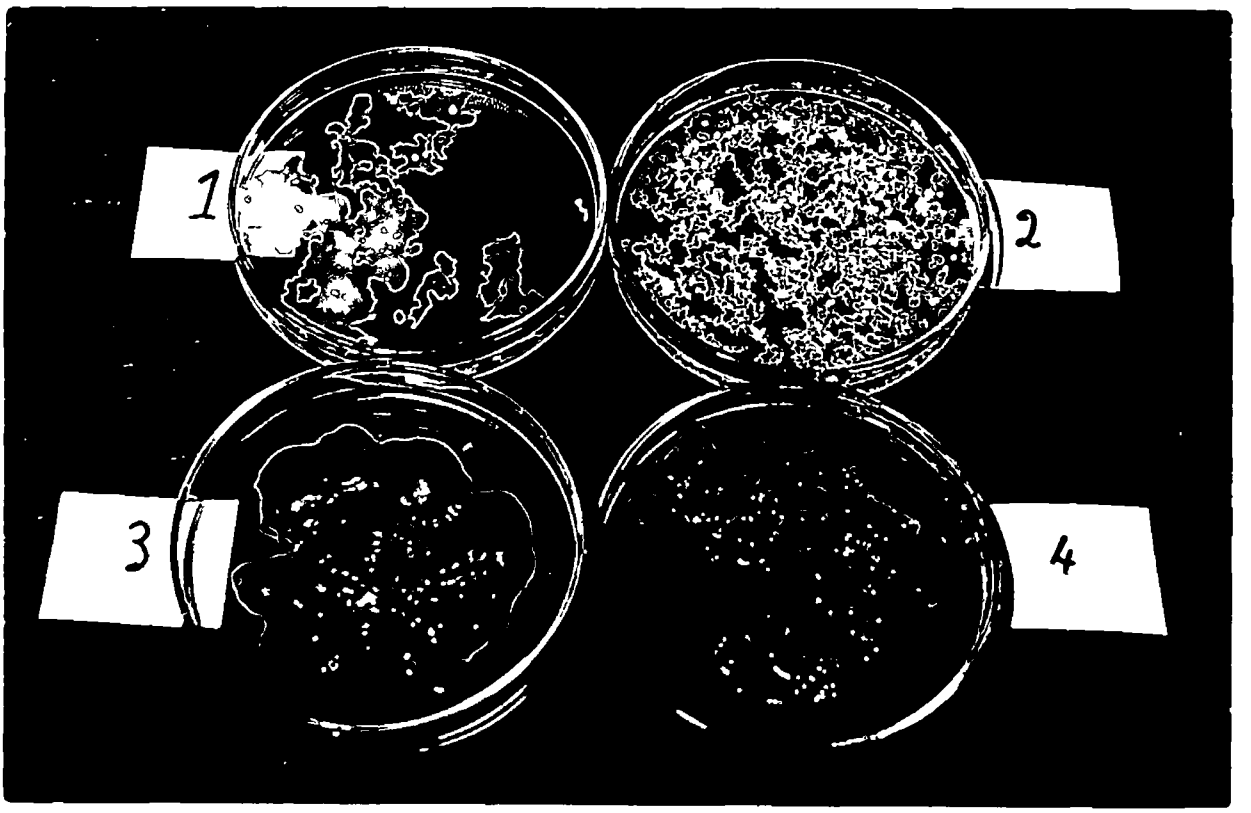

Figure 3 10 Growth morphologies of R.arrhizus in shake-flasks using a range of media

1. Pelleted growth.
2. Discrete pellets
3 Clumped, coalesced growth.
4. Clumpy growth.

Figure 3.11 Growth morphologies found in shake-flask cultures of R arrhizus using variations of Medium C.

1 /2. Discrete pellets.

3 Clumpy growth.

4. Dispersed growth.

morphology in which growth was packed into many discrete pellets or rice-like marbles, each less than 1 cm in length. The most significant result obtained related to the dispersed growth obtained in Medium 19 containing soya flour as a nitrogen source. On dilution of a 5 ml aliquot of this growth in 100 ml water, complete dispersion of the mycelial filaments was observed. This did not occur with growth from other media. Spore inoculum affected growth morphology in each medium. Generally, the higher inoculum gave heavier growtn, usually filling the growth medium. In Medium 19, however, the dispersion of growth was observed only when the higher spore inoculum level was employed. It was decided to investigate the growth in Medium 19 (soya inoculum or Medium C) further.

3.3.5. The effect of varying soya medium components on growth morphology.

Primary shake-flask cultures were then used to investigate the effect of varying soya medium component levels on culture morphology. Two levels of spore inocula ($1x10^4$ and $2x10^5$ /ml culture) were used again. Cultures were grown using standard conditions (Section 2.4). Growth morphology and pH were determined after 24 hours incubation.

The results are presented in Table 3.12 and in Figure 3.11.

Table 3.12. The effect of varying soya medium components on growth morphology

Medium No.	Medium constituents (g/l) Glucose	Soya flour (full-fat)	$CaCO_3$	Spore inoculum	pH initial	pH final	Growth morphology
1.	10	10	-	(a)	6.28	3.77	(4)
				(b)	6.28	4.05	(4)
2.	20	10	-	(a)	6.01	3.68	(4)
				(b)	6.01	3.69	(4)
3.	30	10	-	(a)	5.95	3.52	(3)
				(b)	5.95	3.66	(4)
4.	45	10	-	(a)	5.90	3.52	(3)
				(b)	5.90	3.52	(4)
5.	45	10	10	(a)	7.30	6.40	(1-2)
				(b)	7.30	5.73	(1-2)
6.	10	20	-	(a)	6.33	4.45	(3)
				(b)	6.33	4.80	(3)
7.	20	20	-	(a)	6.12	3.95	(3)
				(b)	6.12	4.25	(3)
8.	30	20	-	(a)	6.11	3.65	(3)
				(b)	6.11	3.70	(3)
9.	45	20	-	(a)	6.07	3.60	(3)
				(b)	6.07	3.73	(3)
10.	45	20	10	(a)	7.28	6.00	(1-2)
				(b)	7.28	5.50	(1-2)

Notes: Spore inoculum levels: (a)=1×10^4/ml culture
(b)=2×10^5/ml culture

Growth morphology: Numbers in brackets refer to Figure 3.11.

Cultures were grown from spores, using 100 ml media in 250 ml conical flasks, and incubating at 30°C and 150 r.p.m. for 24 hours.

Only two of the soya medium variants gave suitably dispersed growth - Media 3 and 4. In these media, growth dispersion was obtained only when $2x10^5$ spores/ml culture was used as inoculum. When lower levels of glucose were used growth became clumpy. Such growth did not disperse itself on dilution. This may be due to the higher biomass level produced. Similarly, addition of higher levels of soya flour resulted in clumpy and apparently heavier biomass. It should be noted that dry weight analysis could not be used to determine biomass in soya medium cultures. This was due to the insoluble nature of the medium and also to the fact that dispersed mycelia produced tended to block filter papers, preventing filtration. For this reason rates of growth were estimated by visual inspection of samples and by the rate of consumption of reducing-sugars. Addition of calcium carbonate cannot be used to control pH if severe pelleting of the growth is to be avoided (Media 5 and 10). Medium 4 (Table 3.12) was adopted as standard soya inoculum medium.

3.3.6. Primary and secondary growth in soya medium and its production capability.

Since it was not possible to measure biomass in soya medium using the dry weight techique, it was decided to assess growth using residual reducing-sugar levels. Flask cultures were inoculated with $2x10^5$ spores/ml of

culture and incubated under standard conditions (Section 2.4). Samples were removed after different incubaton periods for pH and reducing-sugar analysis. Replicate secondary cultures (inoculum and production media) were inoculated at these sampling times also using 10% (v/v) inocula. These cultures were then incubated under standard conditions. The results are presented in Table 3.13.

In both primary and secondary cultures, growth was demonstrated to be dispersed. In each case samples could be removed using an inverted 10 ml pipette (3 mm orifice). On dilution in water a finely dispersed suspension of mycelia was formed. Reducing-sugars were utilized between 12 and 30 hours incubation, with pH dropping to 2.5. Secondary cultures from younger primary inocula (15-19 hours) utilized more reducing-sugars than did older primary inocula (24-30 hours). There was no apparent difference in biomass levels however.

The results also show that inocula grown in soya medium were capable of good lipase production, up to 320 units/ml, under the conditions used. Secondary cultures were superior in this regard compared with the other cultures tested. Secondary casein medium cultures were clumped on transfer, whereas soya medium cultures remained dispersed (Figures 3.10 and 3.11).

Table 3.13. Reducing sugar and pH patterns in primary and secondary cultures using soya medium, and production capability

	Primary Culture			Secondary cultures at t=20 hours		
Time (hours)	pH	Residual Reducing sugars(g/l)	Lipase production u/ml at t=118 hours	pH	Residual Reducing sugars(g/l)	Lipase production u/ml at t=118 hours
15	3.69	33.20	-	3.33	13.6	-
19	3.41	12.71	216	3.34	13.9	326
24	3.34	8.35	-	3.46	15.7	-
30	3.32	2.51	-	3.52	17.4	-

Cultures were grown in soya medium (Medium C) using 250 ml conical flasks with 100 ml medium.

Primary cultures were inoculated with 2×10^5 spores/ml of medium.

Secondary cultures were inoculated with 10 ml of primary culture.

Production cultures were grown in 2-litre flasks and 1 litre Medium D from 10% inocula growth. Incubation was conducted at 30°C and 150 r.p.m. for periods indicated.

3.3.7 Growth of R. arrhizus in laboratory fermenters using soya medium.

Having established that soya medium allows dispersed growth of R. arrhizus through several transfers, attempts were made to use this medium in stirred laboratory fermenters. Spores and primary cultures were used as inocula and some fermenters were run under different conditions or modified as described previously (Section 3.3.2). These modificatons were designed to reduce the chances of growth becoming entangled onto fermenter parts. In each fermentation, growth was assessed by measuring reducing-sugars and pH, and also by visual inspection of samples. The experimental variations and the results are presented in Table 3.14.

In all these fermenters R. arrhizus was found to grow as a dispersed,homogeneous suspension (Figure 3.12). This is in contrast to inoculum fermenters tried previously using the casein inoculum medium (Figure 3.9). Primary cultures developed in the fermenters exhibited a lag period in growth compared with secondary cultures. This was due to the time required for spore germination and out-growth. Appreciable growth did not appear in these fermenters until after 24 hours incubation. Some growth appeared to attach itself to the baffles and impellers, but the biomass remained largely dispersed in the medium. Agitation at 450 r.p.m. produced a finer dispersion of growth compared with 300 r.p.m. Secondary

Table 3.14. Growth of R. arrhizus in laboratory fermenters using soya medium

Inoculum:	6x10 spores/ml medium		6x10 spores/ml medium		500 ml, 15 hour flask culture		500 ml, 15 hour flask culture		500 ml, 15 hour flask culture		500 ml, 15 hour flask culture	
Fermenter:	Standard		Standard		Standard		Standard		Baffles removed		Baffles/impellers removed aerated via ring sparger	
Agitation: (r.p.m.)	300		450		300		450		300		450	
Aeration: (v/v/m)	0.5		0.5		0.5		0.5		0.5		1.0	
Time (hours)	pH	Reducing sugars (g/l)	pH	Reducing sugars (g/l)	pH	Reducing sugars (g/l)	pH	Reducing sugars (g/l)	pH	Reducing sugars (g/l)	pH	Reducing sugars (g/l)
0	5.50	45.0	5.90	45.0	5.60	45.0	5.70	45.0	5.80	45.0	5.60	45.0
23	3.20	42.0	3.55	41.0	3.35	13.8	3.35	9.1	3.34	41.0	3.40	11.3
30	3.55	17.6	3.35	3.7	3.30	7.6	3.31	10.4	3.30	18.7	3.35	1.4
50	3.40	2.5	3.49	7.5	3.50	1.2	3.20	13.2	3.30	2.0	3.47	1.0

Inoculum cultures were grown in 1-litre conical flasks with 500 ml Medium C inoculated with $2x10^5$ spores/ml medium, and incubated at 30^{o}C and 150 r.p.m. for 15 hours. Labofern (7.5-litre) fermenters were used with 5 litres of Medium C incubated at 30^{o}C.

Figure 3.12 Samples of dispersed growth of R.arrhizus in Medium C in Laboferm fermenters.

cultures in fermenters grew out well to give a dispersed suspension of mycelia. In the fermenter with 450 r.p.m. agitation a residual amount of sugars was detectable but no observable difference in growth density was noted. Removing the baffles from the fermenter caused a lag period in sugar utilization, and growth was found to be less dense compared with fermentations with baffles present. This may have been due to the poorer mixing and aeraton patterns in this fermenter. In fermentations in which aeration and agitation were supplied using only a ring sparger, good sugar utilization and growth were found. In all stirred fermenters only a small amount of wall growth was observed above the medium line due to deposition of solid medium particles. This did not represent a serious problem, however. Samples were easily removed via 8 mm diameter tubing (Figure 3.12). When examined microscopically growth was seen to be present as loose hyphae not forming clumps or pellets.

3.3.8. Agitation and its effect on growth in laboratory fermenters.

Previous attempts at growing R. arrhizus in a pilot fermenter were unsucessful due to the susceptibility of the mycelia to fragmentation by impeller shear (Section 3.3.3). Impeller tip-speed gives a measure of shear forces generated and may be calculated from rotation speed and impeller diameter (Wang et al., 1979). Tip-speed in the pilot fermenter was calculated as 241

$cm.s^{-1}$ (Section 2.6). In this experiment secondary cultures were grown in laboratory fermenters with Medium C at two levels of agitation. Growth density and sugar utilization were compared. The experimental conditions and results obtained are presented in Table 3.15.

The culture developed with 600 r.p.m. agitation was noticeably less dense or heavy with biomass, compared with the culture with 450 r.p.m. agitation. Residual sugar levels also showed a considerable lag period in cultures given higher agitation. These results indicate that R. arrhizus is susceptible to impeller speed above 118 $cm.s^{-1}$.

3.3.9. Effect of inoculum, pH and aeration levels on growth in laboratory fermenters.

In operating an industrial fermentation process it is often convenient if the initial flask inoculum can be developed in as small a volume as possible (Section 3.3). For example, if a 200-litre pilot fermenter is to be given a 10% (v/v)vegetative inoculum, twenty 2-litre conical flasks with 1 litre inoculum media would need to be grown. Reducing the level of inoculum to 2% (v/v) would entail cultivating only four such flasks, reducing the contamination risk on transfer. In viscous mould cultures aeration can become limiting very quickly (Wang & Fewkes, 1977). In the following experiment lower (2% v/v) inoculum was compared with the standard level (10%

Table 3.15. Effect of agitation on growth of R. arrhizus in laboratory fermenters

Fermenter agitation:

Agitation rate (r.p.m.)	450		600	
Impeller tip speed ($cm.s^{-1}$)	118		158	
Time (hours)	pH	Reducing sugars (g/l)	pH	Reducing sugars (g/l)
0	6.05	45.0	6.01	45.0
24	3.28	18.8	3.28	34.6
36	3.29	12.3	3.00	25.5

Inoculum cultures were grown in 1-litre flasks with 500 ml Medium C, inoculated with 2×10^5 spores/ml medium, and incubated at 30°C and 150 r.p.m. for 15 hours.

Laboferm (7.5-litre) fermenters were used with 5 litres of Medium C, aerated at 0.5 v/v/m, and incubated at 30°C.

v/v) for growth in two fermenters. A third fermenter was given standard inoculum and aerated at a level of 1.0 v/v/m. The experimental conditions and results are presented in Table 3.16. Use of a 2% (v/v) inoculum did not adversely affect the apparent density of growth obtained, compared with a culture given a 10% (v/v) inoculum. Some degree of lag was observed in sugar utilization, however. The lower level of vegetive inoculum was adopted in further experiments.

Aeration at 1.0 v/v/m did not enhance the level of biomass obtained as assessed visually. However, a larger residual quantity of reducing-sugars was detectable.

When a 10% (v/v) vegetative inoculum at pH ~3.0 was transferred to fermenters containing soya medium, the pH dropped from 6.0 to 4.5. When a 2% (v/v) inoculum was used the pH dropped only to ~5.10. An experiment was conducted in which fermenters were inoculated with 2% (v/v) vegetative inocula and the post-inoculation pH adjusted aseptically. It was shown that heavier growth and better sugar utilization was obtained when the pH was readjusted to ~6.5. It was not possible to measure biomass using dry weights, so the benefit of the apparently increased biomass obtained must be weighed against the operational difficulties of adjusting pH aseptically. This consideration applies especially to larger scale fermenters where the risk of contamination is amplified. One solution might be to adjust the pH of

Table 3.16. Effect of low inoculum, and aeration level, on growth of R. arrhizus in laboratory

Inoculum:	500 ml, 24-hour culture		100 ml, 24-hour culture		500 ml, 24-hour culture	
Fermenter Conditions:						
Aeration v/v/m:	0.5		0.5		1.0	
Time (hours)	pH	Reducing sugars (g/l)	pH	Reducing sugars (g/l)	pH	Reducing sugars (g/l)
0	5.62	45.0	5.58	45.0	5.62	45.0
24	2.62	13.6	2.54	23.7	2.59	22.2
42	2.67	16.8	2.60	18.0	2.65	22.7

Inoculum cultures were developed in 1-litre flasks with 500 ml Medium C inoculated with $2x10^5$ spores/ml, and incubated at 30°C and 150 r.p.m. for 24 hours. A 100 ml aliquot of a replicate culture was used as a 2% inoculum.

Laboferm (7.5 litre) fermenters with 5 litres of Medium C were used, agitated at 450 r.p.m. and incubated at 30° C.

the medium prior to sterilization.

3.3.10. Effect of age of inoculum on growth in laboratory fermenters.

Characteristics of the inoculum can affect subsequent growth capabilities (Section 3.1). In this experiment flask inocula were developed using a range of incubation periods and then transferred into laboratory fermenters. Growth was evaluated by pH, sugar analysis and also by visual inspection of samples. The experimental conditions and results are presented in Table 3.17.

The results indicate that a 36 hour inoculum resulted in poorer utilization of sugars, with a higher level remaining after 42 hours fermentation. Growth in this fermenter was much less dense compared with the other fermenters. Little difference was observed in growth density in the other fermenters. This implies that a range of incubation periods may produce adequate inocula, provided this period is not prolonged beyond 30 hours. This is a useful feature for an industrial fermentation where precise timing of transfers is not always possible due, for example, to equipment malfunctioning or to delays in processing materials.

Table 3.17. Effect of age of inoculum on growth in laboratory fermenters

Flask inocula. Age (hours)	18		24		30		36	
Reducing sugars (g/l)	36.4		7.4		1.7		1.0	
Fermentation: Time (hours)	pH	Reducing sugars (g/l)	pH	Reducing sugars (g/l)	pH	Reducing sugars (g/l)	pH	Reducing sugars (g/l)
0	5.42	45.0	5.45	45.0	5.40	45.0	5.46	45.0
24	2.74	25.9	2.50	31.3	2.53	30.8	2.63	27.7
42	2.48	18.2	2.50	23.6	2.43	21.9	2.73	26.1

Inoculum cultures were developed in Medium C, 500 ml in 1-litre conical flasks, inoculated with 2×10^5 spores /ml medium, and incubated at 30°C and 150 r.p.m. for periods shown. 100 ml aliquots were transferred as inocula from replicate flasks(2%, v/v).

Laboferm (7.5-litre) fermenters were used with 5 litres of Medium C, agitated at 450 r.p.m., aerated at 0.5 v/v/m. and incubated 30°C.

3.3.11. Soya flour levels in soya medium and their effect on growth in fermenters.

Secondary growth in soya medium, whether in flasks or in fermenters, has been observed to be much less dense compared with growth obtained in primary cultures. Higher soya flour levels, when used for primary culture development, have been shown to cause growth to become clumpy (Section 3.3.5). This may have been partly due to an increase in biomass produced. An experiment was conducted in which the level of soya flour in secondary fermenters was varied to investigate if an increase in biomass could be obtained. The experimental conditions and results are presented in Table 3.18. The results indicate that increasing the soya flour level in the fermenters allowed greater utilization of the reducing sugars. The growth obtained in the fermenter with 20 g/l soya flour was by far the best obtained, being heavy, filling the medium completely, yet not attaching itself to baffles or impellers.

3.3.12. Experiences with secondary cultures, using soya medium in pilot-scale fermenters.

An attempt was then made to grow *R. arrhizus* in a pilot-scale fermenter (250-litres) using soya medium. The dimensions of this fermenter have been described previously (Section 2.6). Flask inocula and fermenter conditions were as follows:

Table 3.18. Soya flour levels in Medium C, and their effect on growth in fermenters

Fermenter medium:

Soya flour (g/l)	10		15		20	
Glucose (g/l)	45		45		45	
Time (hours)	pH	Reducing sugars (g/l)	pH	Reducing sugars (g/l)	pH	Reducing sugars (g/l)
0	5.53	45.0	5.62	45.0	5.71	45.0
24	2.70	31.3	2.75	28.9	2.78	18.0
41	2.92	24.8	2.85	20.4	2.96	9.0

Inoculum cultures were developed in 1-litre conical flasks with 500 ml Medium C, inoculated with 2×10^5 spores/ml medium, and incubated at 30°C and 150 r.p.m. for 24 hours. 100 ml aliquots were used to inoculate each fermenter (2% v/v).

Laboferm (7.5-litre) fermenters were used, with 5 litres of media (as above), as agitated at 450 r.p.m., aerated at 0.5 v/v/m and incubated at 30°C.

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Culture stage	Vessel: type	Volume (l)	Medium	Volume (l)	Agitation (r.p.m.)	Aeration (v/v/m)	Inoculum age (hours)	Inoculum type
Primary inoculum:	shake-flask	2	C	1	150	-	-	spores: 2×10^5/ml
Secondary culture:	pilot-fermenter	250	C (enriched)	200	230	0.5	24	2% v/v primary culture

(Note: Medium C, enriched contained 20 g/l soya flour)

Excessive foaming occurred and the mycelia were found to have been fragmented by impeller shear in the pilot-fermenter as had occurred in previous experiments (Section 3.3.3).

The agitation equipment on this fermenter was not designed to allow for variable-speed operation so an alternative mode of operation was sought. A survey of the literature revealed that much work had been done with air-lift and tower fermenters, in which agitation and aeration were supplied solely by forced aeration (Greenshields & Smith, 1971; Smith & Greenshields, 1974). These cultivation methods have been used to cultivate filamentous fungi in large volumes (Barker & Worgan, 1981; Malfait et al., 1981). Although the equipment used was of a specialized design, it was decided to try using the existing pilot-scale fermenter in this way.

A fermention was set up exactly as outlined above except that no agitation was used in the fermenter. Instead, the aeration rate was increased from 0.5 v/v/m to 1.0 v/v/m. The results of one such fermentation are presented in Table 3.19. The results show that R. arrhizus could be grown to high biomass levels under these conditions. Utilization of sugars occurred almost as rapidly as in laboratory stirred fermenters. Good mixing was observed with no settling of medium components. The capacity of the biomass to produce lipase in subsequent production fermenters was investigated at a later stage of the project (Section 3.4.).

Table 3.19. Growth of R. arrhizus in a pilot-scale fermenter

Time (hours)	pH	Reducing-sugars (g/l)	Growth observations
0	6.30	48.4	-
6	5.81	42.8	Mycelia visible
12	5.12	39.5	Microscopically:
15	4.41	38.5	Light growth visible
18	3.37	35.9	
24	2.60	26.8	Dense, dispersed growth
30	2.41	23.9	

For experimental conditions see text.

3.3.13.

Section 3.3 describes how major problems in the process development were identified and solved. These problems related to the tendency of R. arrhizus primary cultures to clump in flasks causing difficulties in inoculating fermenters aseptically. Growth in secondary cultures also clumped giving rise to baffle, impeller and wall growth in fermenters. It was found to be impossible to aseptically transfer this growth from the fermenters. Finally, the organism was shown to be extremely susceptible to shear in stirred fermenters. These problems were overcome by screening a range of media for one which would allow dispersed growth. Soya medium was then identified and growth in flasks and fermenters optimized in a further series of experiments. Using these findings a pilot-scale culture was successfully developed by replacing mechanical agitation with aeration to mix the fermenter medium.

3.4. LIPASE PRODUCTION IN STIRRED FERMENTERS, AND SCALE-UP OF THE PROCESS.

Initial attempts at growing *R. arrhizus* in production medium (Medium D, Section 2.7.4) in Laboferm fermenters were not successful (Section 2.6). The experimental conditions tried were as follows:

Culture stage	Vessel type	Volume (l)	Medium type	Volume (l)	Agitation (r.p.m.)	Aeration (v/v/m)	Inoculum: type	Inoculum: age (hours)
Primary	Shake-flask	1.0	C	0.5	150	-	2×10^5 spores/ml	-
Secondary	Laboferm fermenter	7.5	D	5.0	600	0.5	10% (v/v) primary culture	19

Either no growth was obtained, perhaps due to the intensity of agitation, or any growth which did occur clung to baffles, impellers and walls, as described previously with casein medium (Section 3.3.2). The production medium (Medium D) contains large amounts of heavy, insoluble materials such as ground maize and calcium carbonate (Section 2.7.4). Much of this material remained attached to the base of the Laboferm fermenters after autoclaving, despite the application of intense agitation before cooling. Other problems encountered included persistent bacterial contamination and fouling of sample-lines and air-spargers with growth and medium components.

In this section, experiments were conducted to solve these problems and to develop a plant-scale lipase production process.

3.4.1. Lipase production in Microferm laboratory fermenters.

Microferm (16-litre) fermenters have an advantage over Laboferm (7.5-litre) fermenters in that their dimensions are obviously greater, allowing better clearance for fungal growth between walls, baffles and impellers (Section 2.6). Microferm fermenters are sterilizable in-situ with constant agitation. This feature prevented attachment of maize components to the fermenter base. These units were also found to facilitate aseptic operation. In the first experiment, a 10% (v/v) inoculum was developed using Medium C in shake-flasks under standard conditions (Sections 2.4 and 2.7.3). Lipase production, pH and growth morphology were then monitored in production medium (Medium D) in Microferm fermenters (Sections 2.6, 2.7.4 and 2.8.2). The experimental conditions and results are presented in Figure 3.13.

The results show that R. arrhizus is capable of producing high yields of lipase in stirred fermenters, up to 370 u/ml in 90 hours. Growth occurred as a thick porridgy suspension with mycelia dispersed throughout (Figure 3.14). None of the growth was attached to walls, baffles or impellers. Reducing sugars were depleted to

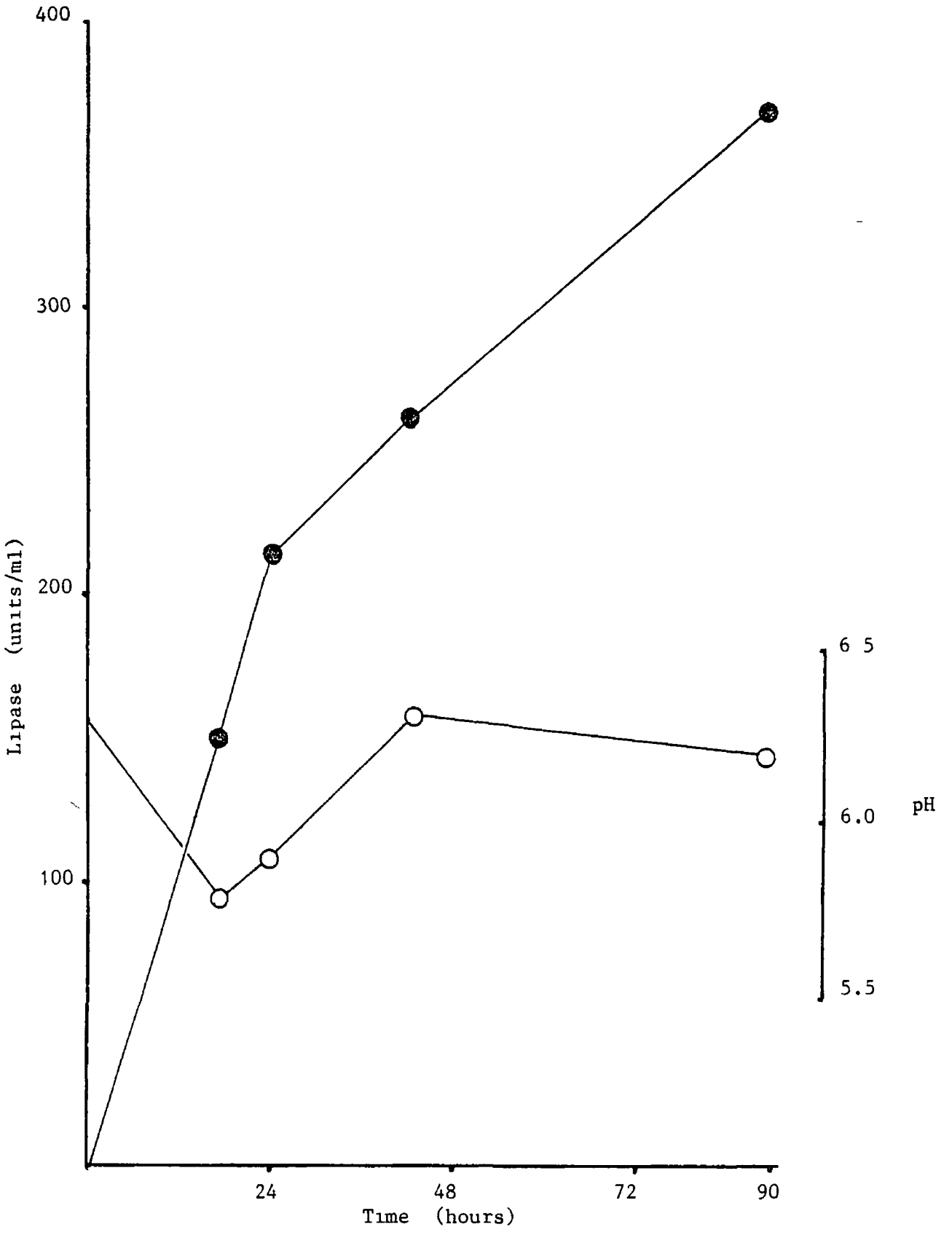

Figure 3 13. Effect of incubation time on lipase production (●) and pH (○) of R.arrhizus cultures in Microferm fermenters containing Medium D (10 l) incubated at 30°C with 200 r p m agitation and 0 5 v/v/m aeration Vegetative inoculum (10% v/v) was grown with 1 l Medium C in a 2-l conical flask, inoculated with 2 x 10^5 spores/ml, and incubated at 30°C and 150 r.p.m for 24 hours

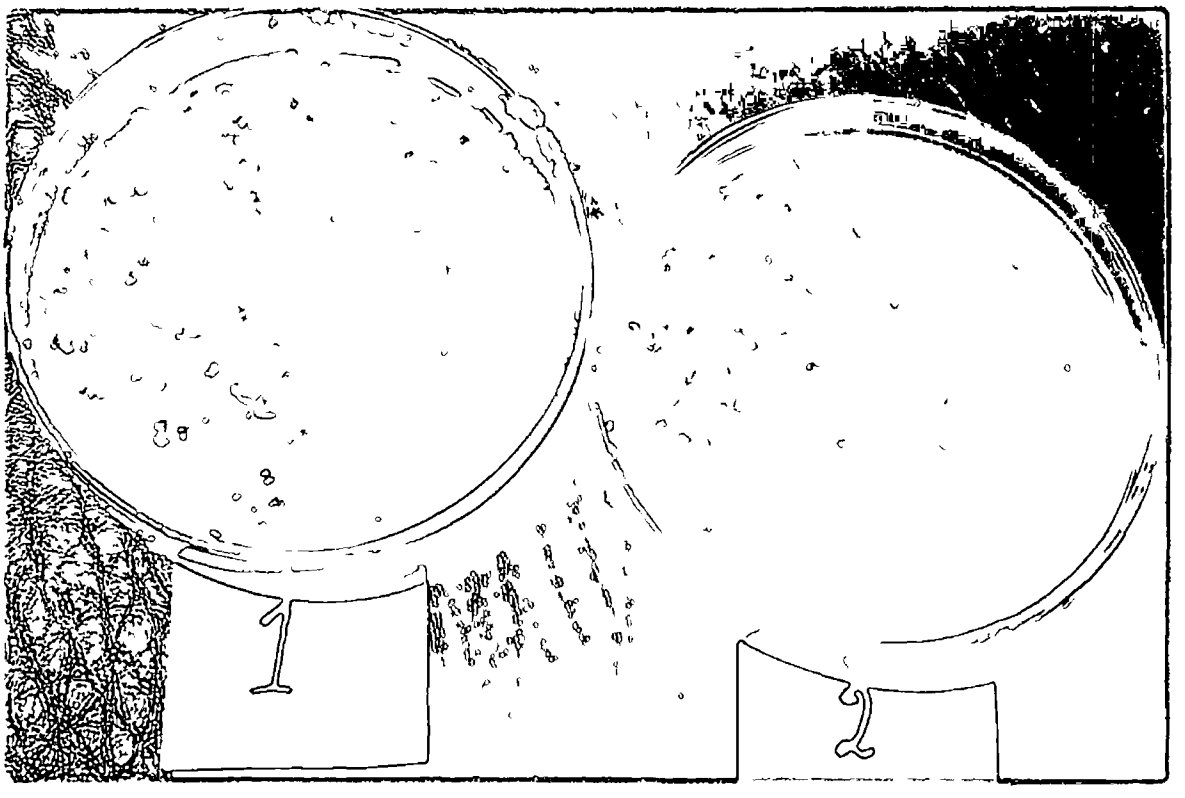

Figure 3.14 Samples of porridgy growth of R.arrhizus in Medium D in Microferm fermenters

below 5 g/l after 17 hours fermentation. Lipase production was much more rapid in fermenters than had previously been observed in flasks (Section 3.2.1). Presumably, this was due to the better aeration and mixing patterns in fermenters.

3.4.1.1. Effect of aeration on lipase production in laboratory fermenters.

Fungi are obligate aerobes and all fungal processes require an optimum level of aeration. In stirred fermenters this aeration is supplied by a combination of sparged aeration and impeller mixing. The presence of mould mycelia and solid particles in high concentrations have been shown to impair aeration efficiency in fermenters (Blakebrough & Hamer, 1963; Brierley & Steel, 1959; Wang & Fewkes, 1977). Low oxygen levels have been shown to adversely affect lipase production in R. delemar (Giuseppin, 1984). In this experiment the effect of aeration rates ranging from 0.5 v/v/m to 1.5 v/v/m on lipase production was examined. The other experimental conditions were as described in Section 3.4.1. The results are presented in Figure 3.15.

These show that raising the aeration rate from 0.5 v/v/m to 1.5 v/v/m increases the lipase yield. However, for some reason, the yields obtained in fermenter A were not as high as those previously obtained (Section 3.4.1). Lipase activity in each fermenter appeared to be less

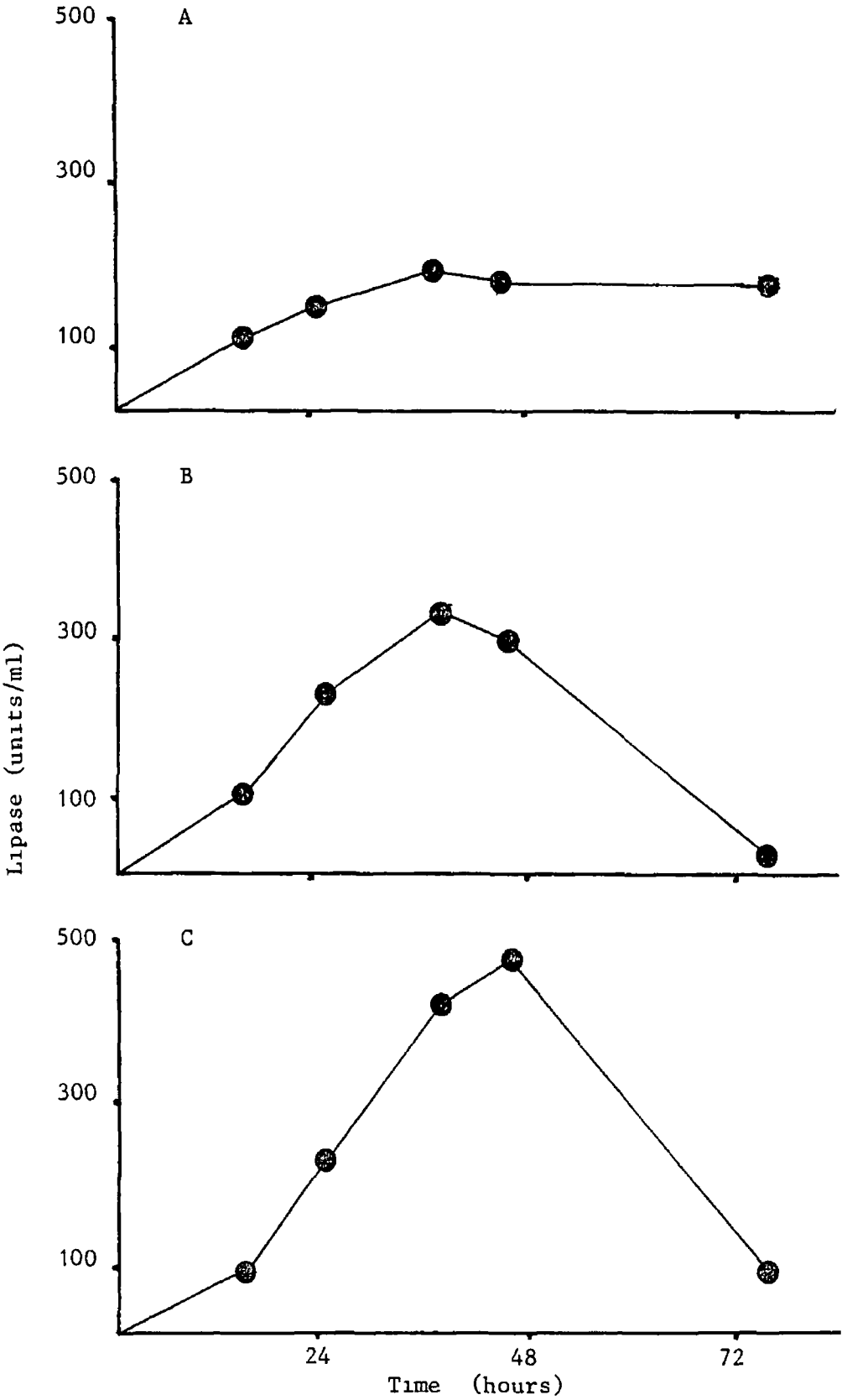

Figure 3 15 Effect of aeration rate on lipase production (●) by cultures of R arrhizus in Microferm fermenters containing Medium D (10 l), incubated at 30°C with 200 r p m agitation Aeration levels used were· A 0.5 v/v/m, B. 1.0 v/v/m, C 1.5 v/v/m Vegetative inocula (10% v/v) were developed as standard (Section 3 4.1)

stable also. No bacterial contamination was detectable. Growth occurred as thick, porridgy suspensions in each case, with growth in fermenters B and C appearing heavier then in A. pH patterns were similar in each fermenter, starting at 6.2 and dropping to 5.8 after 15 hours, before rising to 6.45 at the end of the fermentation. Each fermentation had an initial reducing-sugar level of 22 g/l. Sugar utilization patterns differed in that levels in fermenters B and C remained above 20 g/l until after 15 hours incubation, whereas in fermenter A levels were reduced to 8 g/l before this time. Lipase instability may have been caused by autolysis of biomass, which may have released proteolytic enzymes. Biomass in each fermenter became noticeably thinner towards the end of the fermentation.

3.4.1.2. Effect of agitation on lipase production in laboratory fermenters.

It has been shown previously that *R. arrhizus* is sensitive to shear from impellers (Sections 3.3.3 and 3.3.8). However, agitation has an important function in stirring and aeration of fermenter contents. In this experiment lipase production was examined in Microferm fermenters using different levels of agitation. Three agitation speeds were tried: 200, 300 and 400 r.p.m., with tip-speeds of 73.3, 100 and 146.6 $cm.s^{-1}$, respectively. The other experimental conditions were as described in Section 3.4.1. An aeration rate of 1.5

v/v/m was used. The results are presented in Figure 3.16.

These show that increasing the agitation rate from 200 r.p.m. to 300 r.p.m. resulted in a lag period in enzyme production occurring. Higher final yields were obtained at the higher agitation rate. Growth in this fermenter was not as heavy as that occurring in the fermenter with lower agitation. No growth, reducing sugar utilization and little lipase production were observed in fermenter C, which was given 400 r.p.m. agitation. No bacterial contamination was detectable. It was concluded that the mycelia had been sheared by the impellers, as had occurred previously in laboratory fermenters (Section 3.3.8). This was confirmed by the appearance of mycelial fragments in fermenter samples.

Yields of enzyme were somewhat lower in fermenter A than had been found previously under the same conditions (Section 3.4.1.1). Two factors, pH and initial reducing-sugar levels, may have affected yields. Due to the complexity of the production medium (Medium D) these two parameters were difficult to control in fermenters (Section 2.7.4). In this experiment pH was at 6.5 initially and did not drop below 6.0 during the first 24 hours incubation, and rose gradually to 6.80 at the end of the fermentation. In contrast, the previous fermentation showed a pH pattern beginning at 6.3, dropping to 5.8 after 15 hours incubation, before

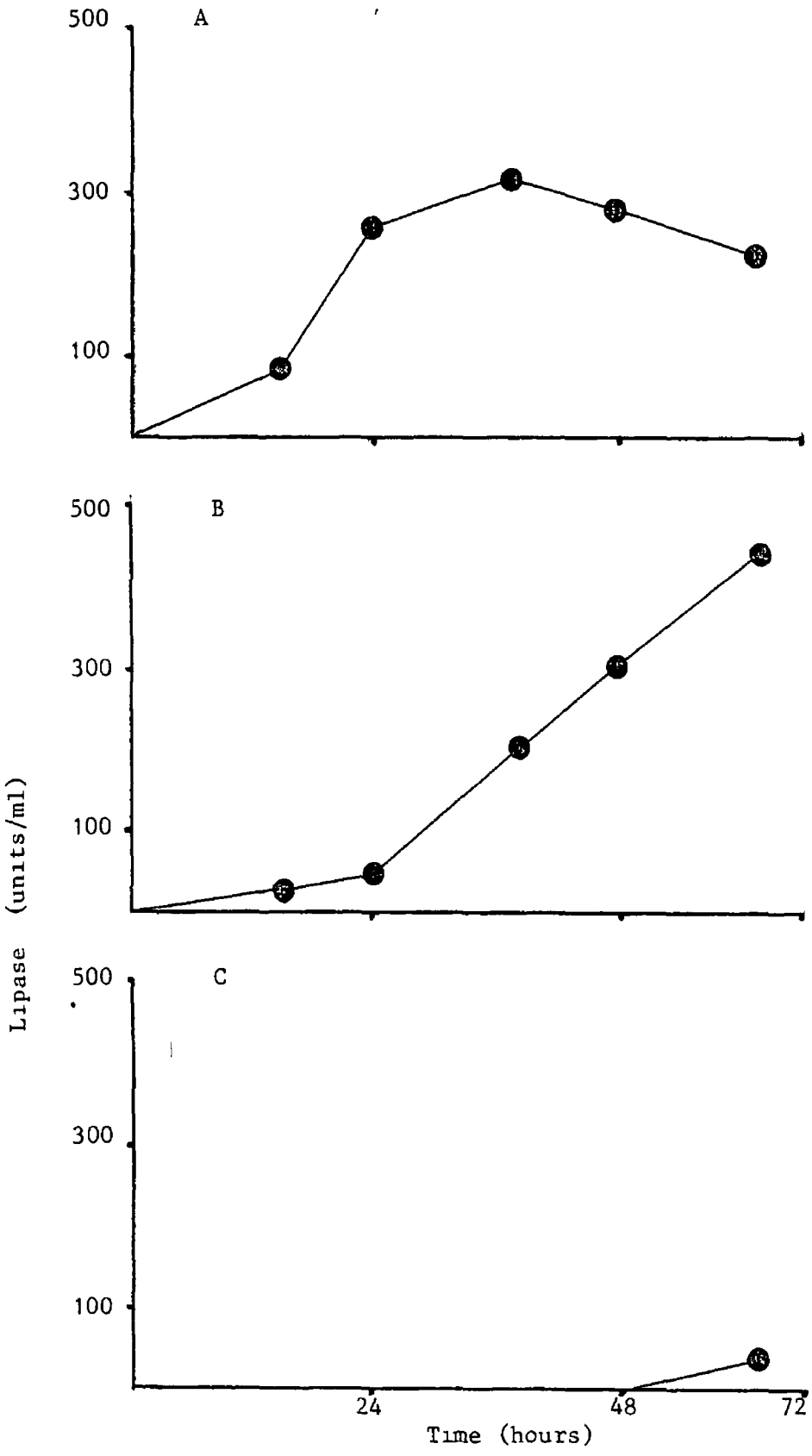

Figure 3 16. Effect of agitation rate/impeller tip speed on lipase production (●) by R arrhizus cultures in Microferm fermenters containing Medium D (10 l), incubated at 30°C with 1 5 v/v/m aeration Agitation levels used were A 200 r p.m (73.3 cm.s^{-1}), B. 300 r.p.m. (100 cm s^{-1}), C. 400 r p m (146 6 cm s^{-1}) Vegetative inocula (10% v/v) were developed as standard (Section 3 4 1).

rising gradually to 6.45 (Section 3.4.1.1). A lower initial reducing-sugar level was observed also in this fermentation (22 g/l compared with 30 g/l). Lipase production in flasks has previously been shown to be sensitive to both reducing-sugar levels and to pH (Section 3.2.4 and 3.2.8).

3.4.2. Use of secondary fermenter inocula for lipase production in fermenters.

Sections 3.3.7 to 3.3.11 give an account of how secondary inocula were grown using soya medium (Medium C) in Laboferm fermenters. In this experiment, secondary growth was developed in a Microferm fermenter and used to inoculate a further Microferm unit containing production medium (Medium D). The experimental conditions were as follows:

Culture stage	Vessel type	Volume (l)	Medium type	Volume (l)	Inoculum: type	Inoculum: age (hours)	Agitation (r.p.m.)	Aeration (v/v/m)
Primary inoculum:	Shake-flask	2	C	1	spores 2×10^5/ml	-	150	-
Secondary inoculum:	Microferm	16	C	10	10% (v/v) primary culture	24	300	0.5
Production	Microferm fermenter	16	D	10	10% (v/v) secondary culture	24	200	0.5

- - - - - - -

Production of lipase was monitored in the production fermenter and reached 360 u/ml after 85 hours

incubation. This experiment demonstrates that R. arrhizus is capable of lipase production even after transfer through a secondary fermenter cultivation stage.

An experiment was then carried out to investigate the effect of varying the secondary fermenter cultivation stage on lipase production. In one secondary fermenter Medium C was enriched to yield higher biomass levels as previously described (Section 3.3.11). The experimental conditions were as follows:

Culture stage	Vessel type	Volume (l)	Medium: type	Volume (l)	Inoculum: type	Inoculum: age (hours)	Agitation (r.p.m.)	Aeration (v/v/m)
Primary inoculum.	Shake-flask	1.0	C	0.5	spores $2x10^5$/ml	-	150	-
Secondary inoculum	Labofemm fermenter	7.5	C (or enriched)	10	2% (v/v) primary culture	24	450	0.5
Production	Microfemm fermenter	16.0	D	10	10% (v/v) secondary culture	24 or 30	200	1.5

Note: Enriched soya medium: 20 g/l, soya flour (instead of 10 g/l); glucose 45 g/l, pH 6.0.

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Lipase production was then monitored and the results are presented in Figure 3.17.

The results show that R. arrhizus is capable of producing high levels of lipase under the conditions

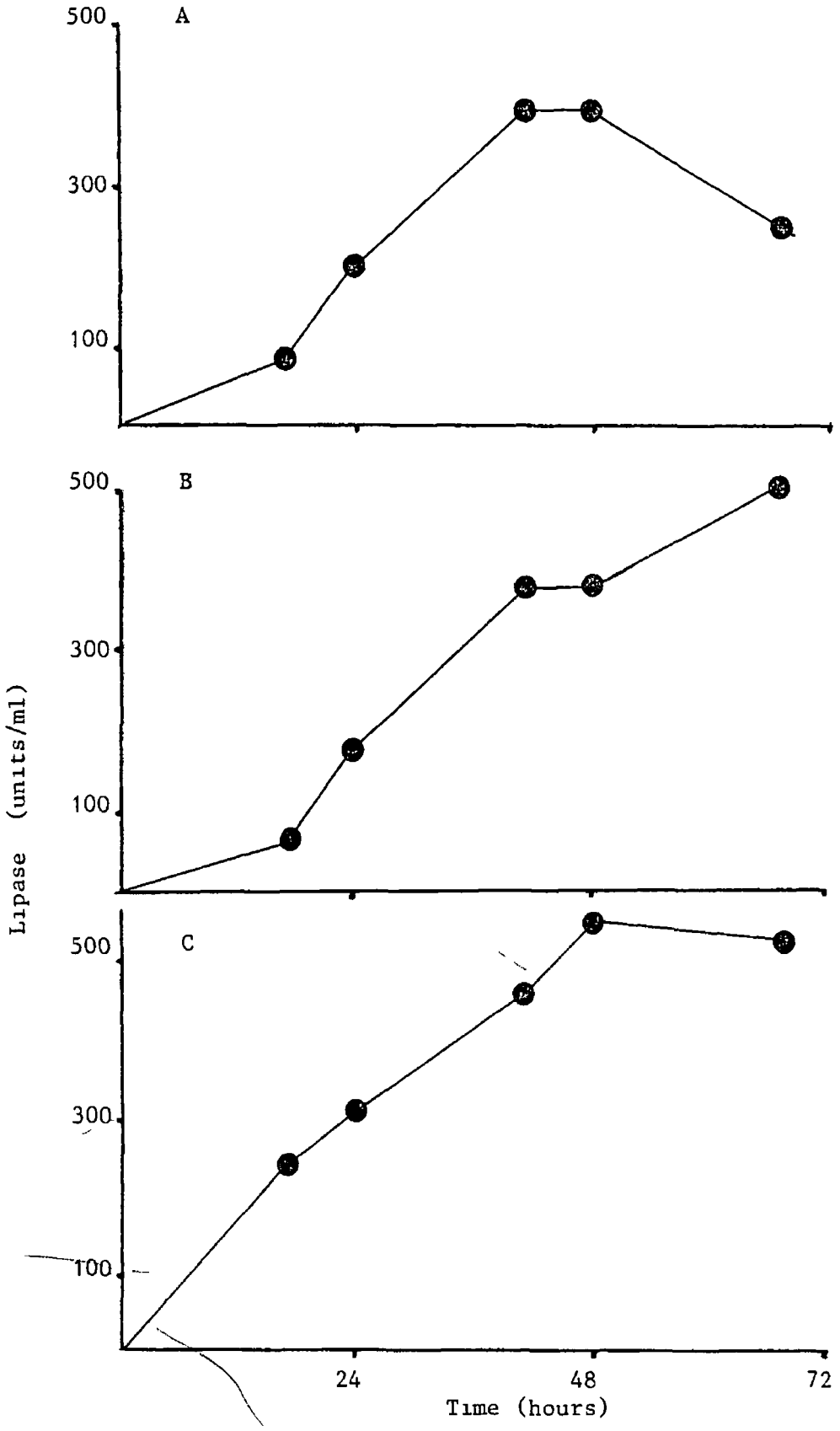


Figure 3 17 Effect of different secondary fermenter inocula on lipase production (●) by R arrhizus cultures in Microferm fermenters containing Medium D (10-1)

A 24 hour fermenter inoculum, B 30 hour fermenter inoculum,

C 24 hour fermenter inoculum (Medium C, enriched)

See text for experimental conditions

used. Starting the secondary inoculum fermenters with a 2% (v/v) vegetative inoculum allowed better subsequent production, compared with using a 10% (v/v) inoculum. In each fermentation the target yield of 400 units/ml was approached, with fermenter C producing over 550 units/ml. In general, the production phase appears to peak at 48 hours, after which time the lipase yields tended to decline. After this period growth appeared to become thinner also. The age of the standard inoculum (Medium C) did not affect the fermentation pattern very much. Use of higher soya flour levels (20 g/l instead of 10 g/l) appeared to be beneficial to enzyme yields. Sugar utilization by this culture was very rapid with sugars being depleted to below 5 g/l in 17 hours, at which time the levels in fermenters A and B were still ~22 g/l.

3.4.3. Experiences with lipase production in plant-scale fermenters.

Section 3.3.12. describes how a 200-litre pilot-scale secondary inoculum was developed. This was used to inoculate plant-scale fermenters of 3,000-litres gross capacity containing 2,000 litres of production medium (Medium D). Previous experiments have shown that *R. arrhizus* is sensitive to impeller tip-speeds above 110 $cm.s^{-1}$ (Sections 3.3.8 and 3.4.1.2). When the standard impeller speed was used in the plant-scale fermenter containing production medium, no growth or

lipase production occurred (Section 2.6). In another experiment, aeration at 1.5 v/v/m was tried, without agitation, to test if this would be sufficient to aerate and mix the fermenter contents. However, poor mixing of solid maize particles resulted in poor growth and enzyme yields (less than 20 u/ml in 24 hours).

In other experiments the impeller speed was reduced to 72 r.p.m. (170 cm.s^{-1} tip-speed) with an aeration rate of 1.5 v/v/m. These conditions allowed good growth of R. arrhizus and a typical lipase production pattern is presented in Figure 3.18. Almost 320 units/ml were obtained after only 36 hours incubation, even though pH remained below 6.0. Reducing-sugars were depleted from 21 g/l to less than 5 g/l after 18 hours incubation. In further plant-scale fermentations yields of up to 550 units/ml were routinely obtained after 48 hours incubation. The economic enzyme target-yield had been achieved.

3.4.4.

Section 3.4 describes how problems related to growth of R. arrhizus in production fermenters were solved. These problems were caused by medium components and fungal mycelia attaching to fermenter parts, reducing both growth and enzyme yield potential. Dispersed growth and good lipase production were obtained in Microferm fermenters. Further experiments demonstrated that lipase

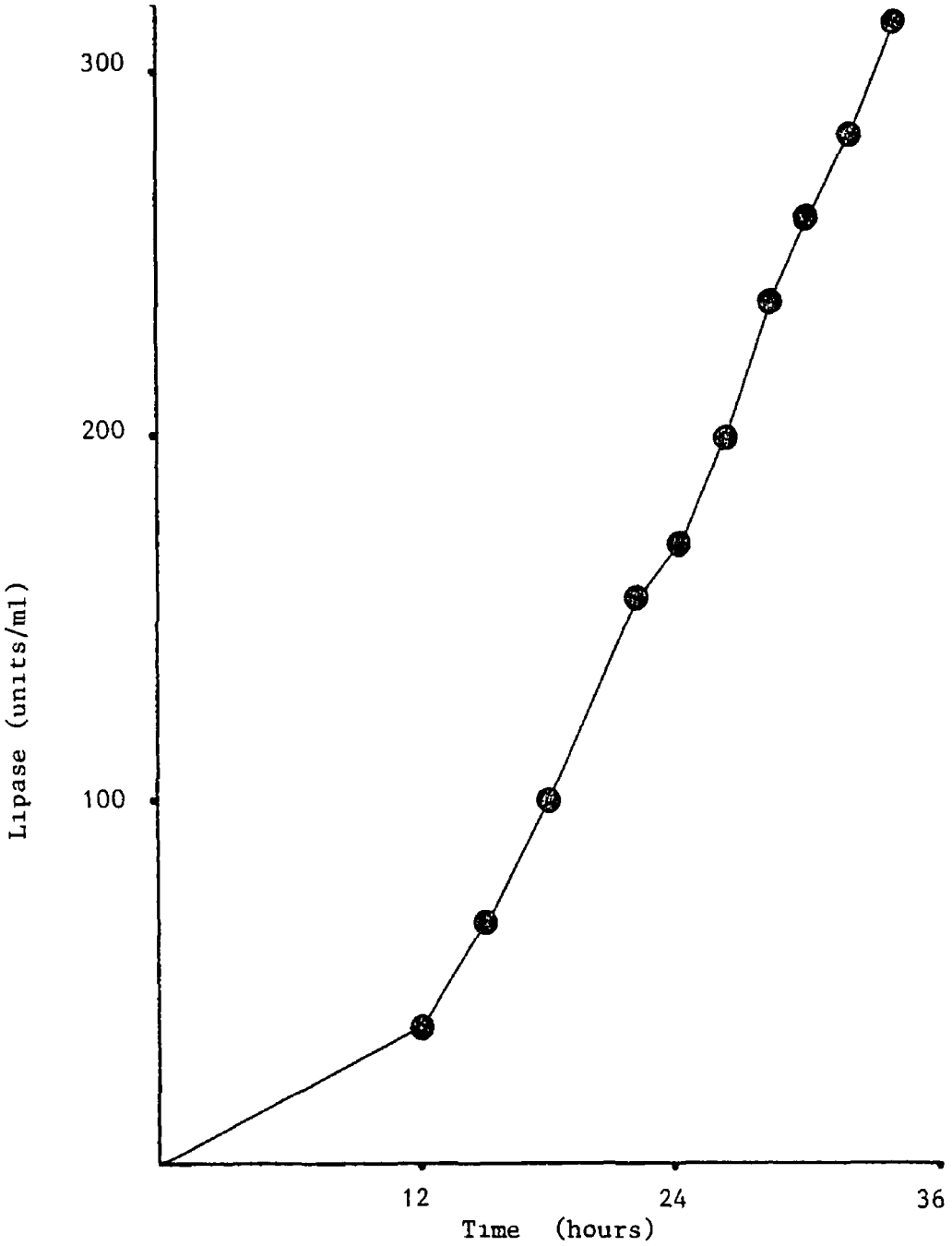


Figure 3.18 Effect of incubation time on lipase production (●) by R.arrhizus in a plant-scale fermenter (3,000-l) containing Medium D (2,000 l) incubated at 30°C with 72 r p m agitation and 1.5 v/v/m aeration Vegetative inoculum (10% v/v) was developed in a pilot-scale fermenter (250-l) containing modified Medium C inoculated with standard flask inoculum See text for details (Section 3 4.3).

production was affected by levels of aeration and agitation. An experimental inoculum development process was conducted on a laboratory-scale and good enzyme production was demonstrated. Finally, lipase production was achieved on the plant-scale with yields exceeding the economic level of 400 units/ml.

The final process consists of development of a vegetative primary flask inoculum from spores in soya medium, transfer at 2% (v/v) to a prefermenter, containing enriched soya medium, using incubation conditions with a low shear-rate. A 10% (v/v) prefermenter inoculum is then used for the production fermenter which is operated at a reduced agitation rate. Incubation periods should optimally be around 24 hours for flask inoculum and for prefermenter growth, and 48 hours for lipase production.

3.5. DEVELOPMENT OF A RAPID LIPASE ASSAY.

During the preliminary work for this project a lipase assay was used with olive oil as substrate (Section 2.8.2.1; Naher, 1974). The lipase enzyme reacts with the olive oil emulsion releasing fatty - acids from triglycrides. The assay was conducted at 37°C and pH 7.0 with constant stirring. These free fatty - acids lowered the pH of the reaction mixture and this was titrated back to pH 7.0 by addition of sodium hydroxide from a burette. To estimate the additional amounts of fatty-acids released, but not ionized at pH 7.0 (i.e. not measured by the pH meter), the reaction mixture was titrated to pH 9.0 at the end of the 10 minute reaction period. The number of moles of sodium hydroxide added is a direct measure of moles of fatty-acids released. One unit of activity requires one mole of fatty-acids to be released per minute, per ml of enzyme added (Náher, 1972). Sodium taurocholate must be added to act as a fatty-acid acceptor to prevent enzyme inhibition, while calcium ions are added to aid the ionization of long-chain fatty-acids. Gum arabic is added to stabilize the olive oil emulsion. As may be seen in Section 2.8.2.1., the assay involved preparation of many solutions, some requiring over-night storage before use. It was found that the assay as a whole was too cumbersome to be used as a rapid routine test for many lipase samples.

The literature on the subject was examined and several authors had noted the advantages of using tributyrin as a lipase assay substrate (Desnuelle, 1972; Sémériva et al., 1971). These include the ability of tributyrin to form an emulsion when agitated vigorously on a magnetic stirrer (Section 2.8.2.2). The emulsion is unstable when agitation is stopped, but reforms readily when stirring is restarted. This property is apparently due to the short fatty acids (C-5) occurring in tributyrin, in contrast to those occurring in olive oil (Merck Index, 1976). Another advantage, related to lipase assays at pH 7.0, is that the fatty-acids released (butyric acid) have a pK_a of 4.5 . This means that at pH 7.0 all free fatty-acids are ionized and therefore titratable (Laboureur & Labrousse, 1966). These acids are freely soluble in water and so do not require a fatty acid acceptor molecule.

A lipase assay was then developed using the titrimetric principle of the olive oil assay, i.e. release of fatty acids was titrated potentiometrically using sodium hydroxide. The reaction vessels were the same (100 ml beakers), but only a 30 ml reaction volume was used. An aqueous emulsion of tributyrin was prepared by stirring vigo rously on a magnetic stirrer and then quickly dispensing this into reaction vessels. These were equilibrated to 37^{o}C and pH 7.0 and 1 ml of enzyme dilution added at t=0, again stirring vigorously to maintain the emulsion. The titrations were then

conducted for different periods.

A substrate level of 5% (v/v) was adopted when it was shown that increasing this to 10 or 15% did not effect enzyme activity. Initially, the diluent used for the substrate contained 0.1m NaCl to aid emulsification. Later, 0.1m $CaCl_2$ was also added, as high levels of calcium are present in lipase production media (Sections 2.7.4 and 2.7.5). Calcium ions were not found to be stimulatory in this assay, but they have previously been implicated in lipase stability (Laboureur & Labrousse, 1966). Lipase activity on tributyrin was found to be lower than on olive oil, under the conditions used. The sensitivity of the assay was increased by using 5 mM NaOH in a 10 $\pm$ 0.02 ml burette, instead of 10mM in a 50 $\pm$ 0.1 ml burette. To further speed up the procedure, assays were conducted for 5 minutes only, although the reaction rate was shown to be linear up to 15 minutes. When different dilutions of a standard lipase preparation were assayed, titers were obtained in proportion to the dilution used. Background activity, i.e. when no enzyme was added to the reaction mixture, was shown to be minimal. Finally, using lipase samples from several cultures of R. arrhizus, activity units obtained using tributyrin and olive oil assays were compared (Sections 2.8.2.1 and 2.8.2.2). The results are shown in Table 3.20. These show that good correlation between the assays was obtained. Other experiments, using commercial lipase preparations, showed good

correlations also.

The tributyrin assay was found to be more efficient because fewer solutions had to be prepared. Enzyme diluent (10 g/l NaCl) and substrate diluent (0.1m $NaCl/CaCl_2$) were prepared in bulk. The substrate emulsion was formed in 100 ml quantities and dispensed. Sodium hydroxide was prepared by diluting 0.5M NaOH with cooled, boiled water. Finally the assay was conducted for 5 minutes only. This assay was then used to analyse for lipase in fermentation development work (Sections 3.2, 3.3 and 3.4), and also to determine the properties of different lipase preparations (Section 3.6).

Table 3.20. Comparison of tributyrin and olive oil assays for estimating lipase activity

Sample	Olive oil (Units/ml)	Tributyrin (Units/ml)
A	22.0	21.0
B	23.2	18.5
C	129.0	125.0
D	146.0	135.0
E	89.0	92.5
F	81.0	83.8

3.6. PROPERTIES OF SOME LIPASE ENZYMES.

Enzymes isolated from different sources differ in characteristics such as optimum pH and temperature for activity or stability (Godfrey & Reichelt, 1983). These properties may vary according to assay procedures used or purity of the enzyme preparation. In this section, the properties of lipases from three sources were compared. These sources were: lipase produced by R. arrhizus in our laboratory, a Sigma London R. arrhizus lipase preparation (No. L4384), and a Sigma London porcine pancreatic lipase preparation (No. L3126).

3.6.1. Comparison of temperature-activity patterns.

Using the tributyrin assay, lipase activity was determined at different incubation temperatures for each enzyme preparation (Section 2.8.2.1). The results are presented in Figure 3.19.

The results indicate that the temperature-activity curves for the two R. arrhizus lipase preparations were similar. Their optimum temperature was around 30°C. The optimum temperature quoted in the literature is 37°C (Laboureur & Labrousse, 1964, 1966; Mauvernay et al., 1970). These optima were obtained using an olive oil assay, however. It was shown, using the olive oil assay, that higher activity was obtained at 37°C compared with

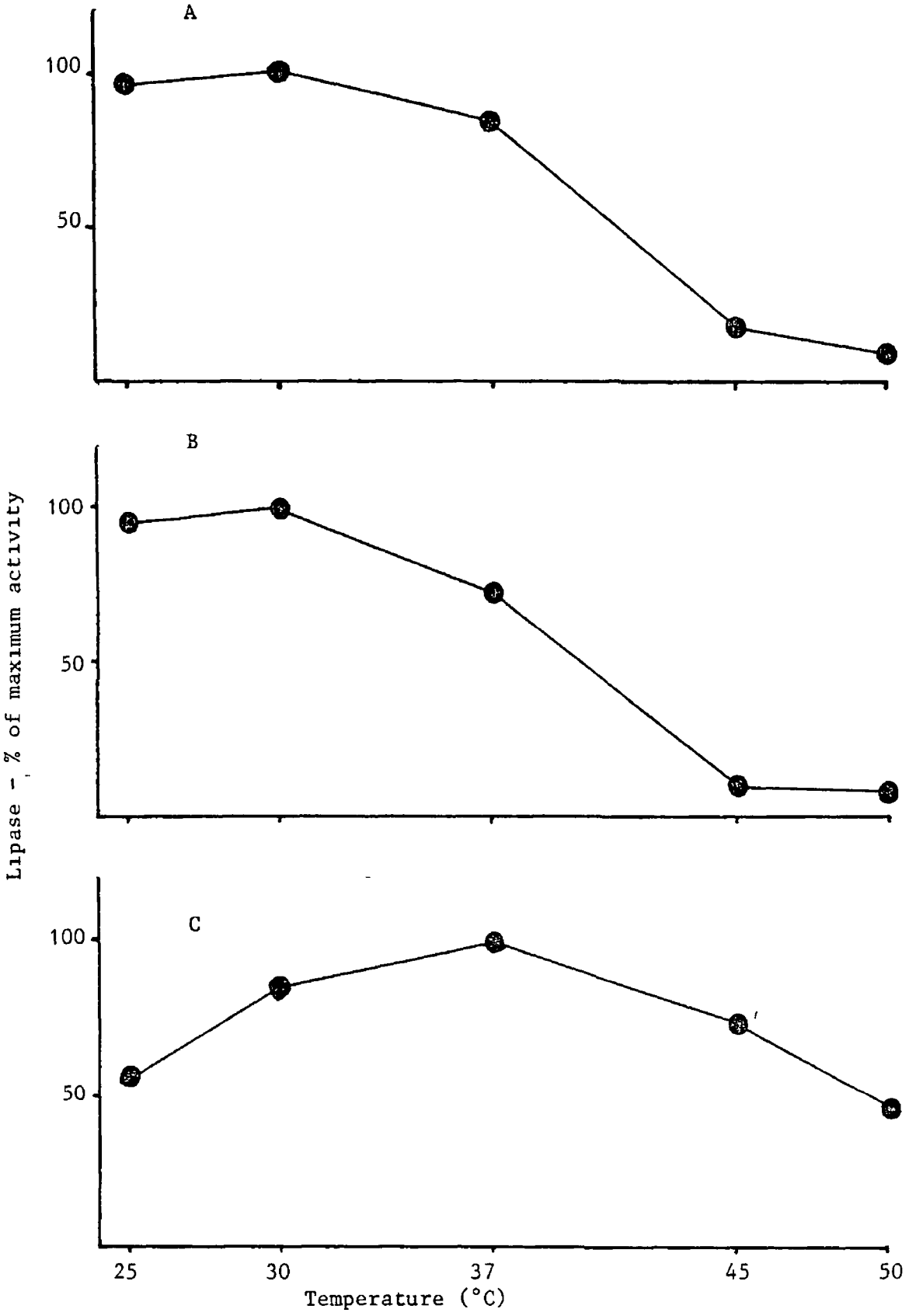


Figure 3.19 Temperature activity curves for different lipase preparations A R arrhizus laboratory culture supernate, B. R.arrhizus lipase, Sigma, London (L 4384), C Porcine pancreatic lipase, Sigma, London (L 3126)

30 °C for both R. arrhizus lipase preparations (Section 2.8.2.2). This confirms that the conditions of assay can affect optimum reaction conditions. The temperature-activity curve for pancreatic lipase differed from that for R. arrhizus lipase. The optimum temperature was shown to be 37 °C using the tributyrin assay. Again, previous results have shown the optimum temperature to be 50 °C (Godfrey & Reichelt, 1983). However, no confirmatory tests were carried out using the olive oil assay.

3.6.2. Comparison of pH-activity patterns.

The pH of the tributyrin assay was varied from 5.0 to 9.0 and the effect on the activity of the lipase preparations determined. Activity at pH 3.5 was determined using the olive oil assay. The tributyrin assay proved unreliable below pH 5.0, when the titration had to be brought to pH 7.0 after incubation, to allow all the butyric acid released to be determined potentiometrically (Section 3.5). The results are presented in Figure 3.20.

The R. arrhizus lipase preparations showed maximum activity at pH 7.0. The curves differed in that the Sigma lipase (B)was more sensitive to higher pH, when activity was significantly reduced. The literature has reported that R. arrhizus lipase is unusual in that it exhibits secondary peak of activity at pH 3.5 (Laboureur

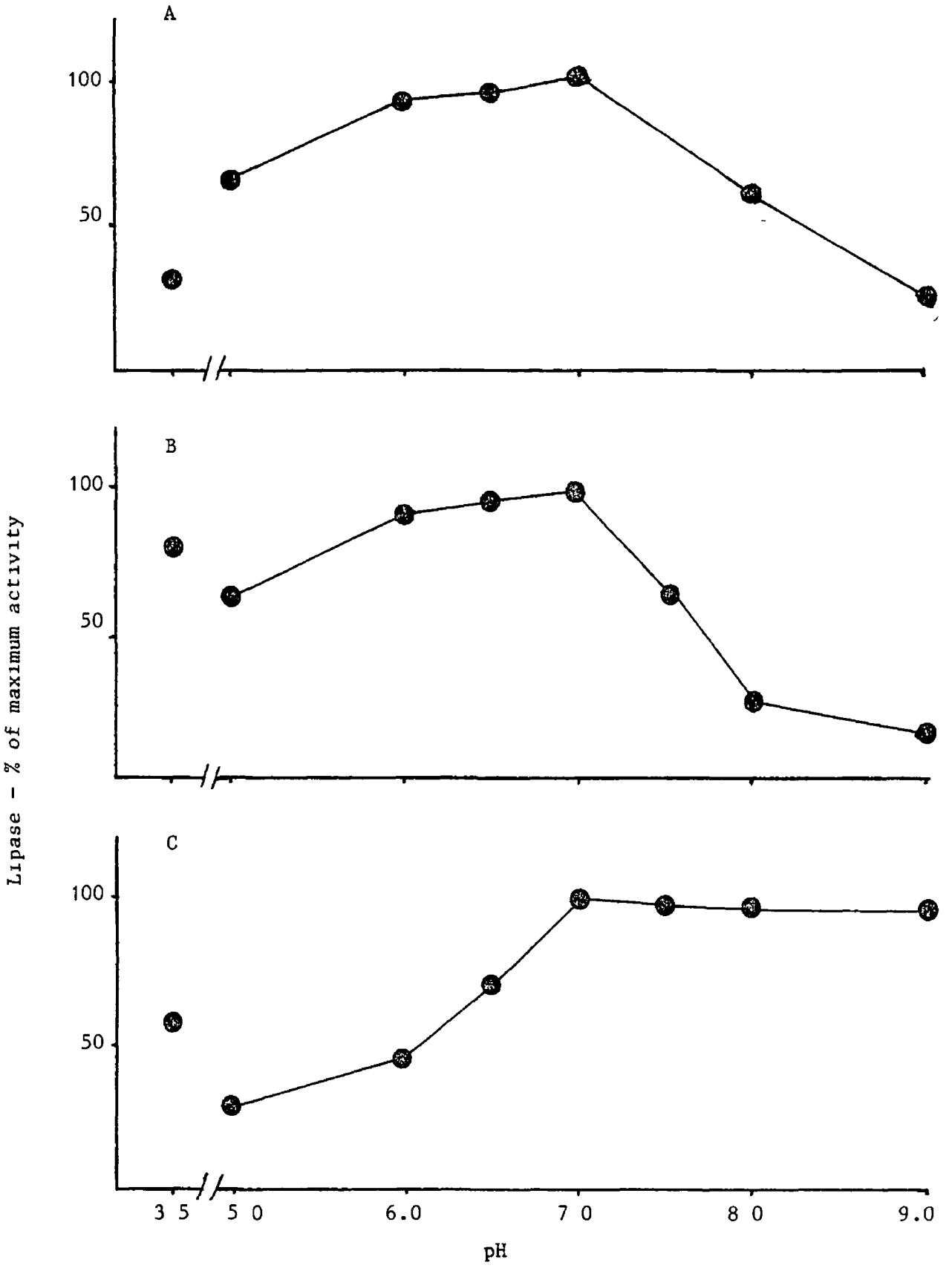


Figure 3 20 pH - activity curves for different lipase preparations.

A R arrhizus - laboratory culture supernate, B R.arrhizus lipase, Sigma, London (L 4384), C Porcine pancreatic lipase, Sigma, London (L 3126)

& Labrousse, 1964, 1966; Mauvernay et al., 1970). However, this was not found for the R. arrhizus culture lipase. It is difficult to find an explanation for this, unless this strain of R. arrhizus differs from that used to prepare the Sigma lipase. Porcine pancreatic lipase showed a marked preference for pH 7.0 and above. A broad maximal activity peak was observed with maximum activity occurring at pH 7.0. Some residual activity was noted at pH 3.5, also.

3.6.3. Effect of temperature on lipase stability.

To examine the effects of temperature on lipase stability, enzyme preparations were incubated at pH 7.0 and the residual lipase activity was determined. The enzyme preparations were diluted in 0.1 M Tris-HCl buffer pH 7.0 to approximately 80 units/ml (Appendix II). These mixtures were sampled at zero time, and after four hours incubation at different temperatures, for lipase analysis using the standard tributyrin assay. The percentage residual lipase activity was calculated for each incubation temperature. The results are presented in Figure 3.21.

The lipase activity of the R. arrhizus laboratory culture was stable up to 45^{o}C , when over 80% of activity remained. This was in contrast to the Sigma fungal lipase which was sensitive to storage at temperatures exceeding 30^{o}C. This latter finding is in agreement with

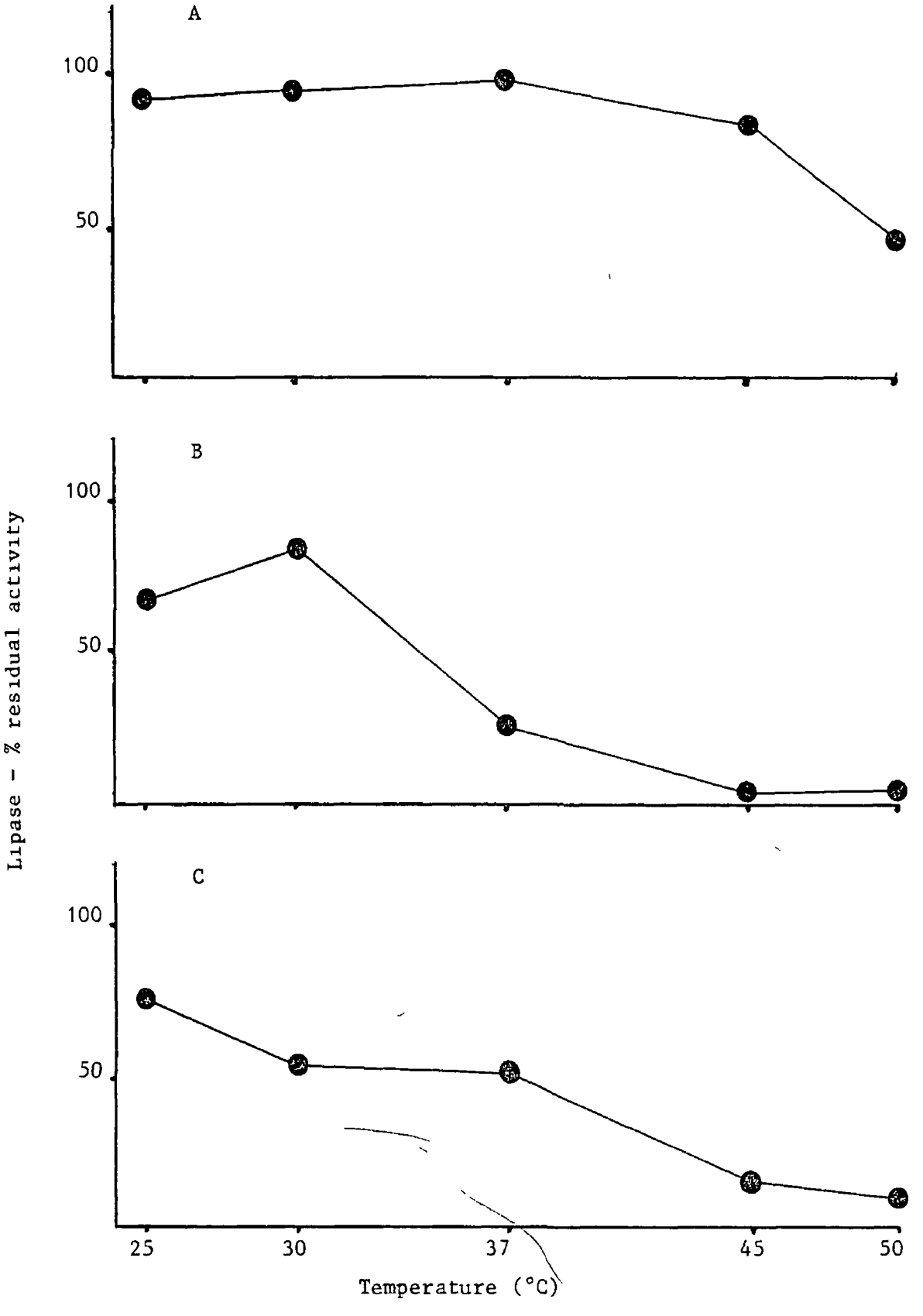


Figure 3 21. Effect of temperature on lipase stability after 4 hours incubation at pH 7.0 for A R arrhizus laboratory culture supernate, B R arrhizus lipase, Sigma, London (L 4384), C Porcine pancreatic lipase, Sigma, London (L 3126)

previous reports. The difference may be accounted for by the fact that the R. arrhizus laboratory culture lipase was unpurified and contained a high level of calcium ions. Calcium ions were not added to the enzyme mixtures during these trials. Alternatively, these different properties may indicate again that different lipase proteins were being produced by different strains of R. arrhizus. Porcine pancreatic lipase retained only half of its initial activity up to 37°C, above which the loss of activity was more marked.

3.6.4. Effect of pH on lipase stability.

To examine the effects of pH on lipase stability, enzyme preparations were incubated at 37°C over a pH range 3-8, and the residual lipase activity determined. The enzyme preparations were diluted to 80 units/ml in appropriate buffers and incubated for 4 hours. Lipase activity was determined at zero and after 4 hours, and the percentage residual activity calculated. The buffers used were 0.1 M Tris-HCl for pH 7 and 8, and 0.1 M citrate for pH 3-6 (Appendix II). The results are presented in Figure 3.22.

The results show that the lipase of the R. arrhizus culture was stable at pH 4 and above. The Sigma fungal lipase retain 56% activity at pH 4 and 99% activity at pH 5.0. At higher pH values the residual activity dropped markedly. The porcine pancreatic lipase was more

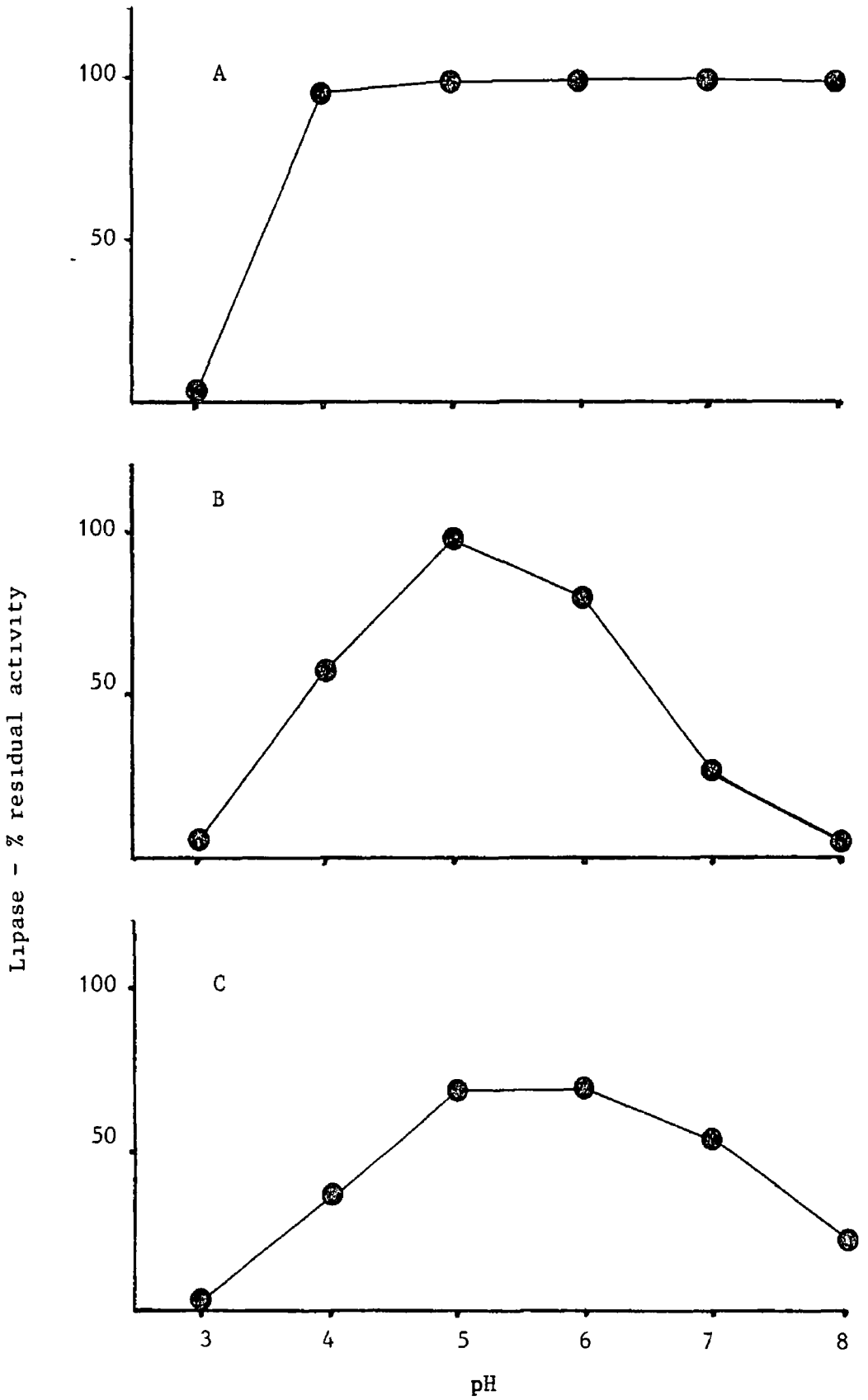


Figure 3.22. Effect of pH on lipase stability after 4 hours incubation at 37°C for A R arrhizus - laboratory culture supernate, B R arrhizus lipase, Sigma, London (L 4384), C Porcine pancreatic lipase, Sigma, London (L 3126)

stable at pH 5-6 than at higher or lower values. A minimum of 30% activity was lost under all conditions of incubation tested, which may indicate that the preparation was contaminated with a protease enzyme.

3.7. FACTORS AFFECTING MORPHOLOGY OF R. ARRHIZUS IN SUBMERGED CULTURE.

The conditions most often implicated in affecting fungal growth morphology include level and type of inoculum, and growth medium and conditions (Metz & Kossen, 1977; Whitaker & Long, 1973). These findings have been confirmed in the case of R. arrhizus in different sections of this thesis (Sections 3.1, 3.3.4 and 3.3.5).

The aim of this section of work was to further investigate the effect of medium constituents on morphology. It was decided to determine if specific components added to complex or defined media would induce pelleted or filamentous growth. These components were selected from the literature on fungal growth and morphology. The ultimate aim of the work was to gain a greater understanding of the factors causing and preventing pellet formation by R. arrhizus.

Many authors have published results on fungal growth morphology and have described types of growth obtained qualitatively and quantitatively (Burkholder & Sinnott, 1945; Clark, 1962; Steel et al., 1954; Testi-Campasano, 1959). The types of growth morphologies found in the course of this work on R. arrhizus did not conform to previous classifications. A key was drawn up to facilitate description of growth morphologies. Macro- and microscopic observations were made on each culture

(Section 2.9). A description of each morphology type is presented in Table 3.21 and some representative photographs of growth are shown in Figure 3.23.

Table 3.21. Key to degree of clumping or pelleting in *R. arrhizus* (refer also to Figure 3.23)

Growth Type:

1. Discrete pellets.

Growth centres - round or variable in shape;
size variable but generally about 1 mm diameter;
filling medium with up to 3×10^4/l.
Microscopically: centres smooth and dense, although some lighter, more filamentous pellets were also encountered having a clumpy gross morphology.

2. Clumped, coalesced growth.

Growth coalesced or clumped into one fungal mass, not generally filling medium.
Microscopically: dense, smooth growth.

3. Clumped, pelleted growth.

Centres of growth discernible on dilution with sizes ranging from 1 cm to several cms, with a range appearing in each culture.

continued over,

continuation

Microscopically: dense growth centres.

4. Dispersed, pelleted growth.

Centres of growth discernible on dilution with sizes ranging from 1mm to several mms in any given culture.
Microscopically: growth less dense and more filamentous.

5. Dispersed, filamentous growth.

Growth dispersed and filling medium, visible on dilution as a turbid suspension.
Microscopically: many single hyphae, some light filamentous conglomerates.

3.7.1. Growth and morphology of R. arrhizus in complex media.

3.7.1.1.(a). Comparison of growth in different media.

Three complex media were compared, using a range of component concentrations, for growth morphology under standard conditions (Section 2.4). A spore inoculum of $2x10^5$ spores/ml was used to inoculate 250 ml conical flasks containing 100 ml media. Growth morphology was examined after 24 hours incubation. The results are presented in Table 3.22.

Figure 3 23 Growth morphologies of R.arrhizus in shake-flasks.

1./2. Discrete pellets.

3. Clumpy growth.

4. Dispersed growth

Table 3.22. Growth morphology of R. arrhizus in complex media

Medium	Nitrogen Source (g/l) Casein	Peptone	Yeast Extract	Glucose (g/l)	pH t=0	pH t=24	Growth Morphology Type
1.	20.0			45.0	5.47	3.54	Clumped,
2.	10.0			45.0	5.61	2.82	coalesced growth (2)
3.	5.0			45.0	5.60	2.82	
4.	2.5			45.0	5.68	2.83	Discrete pellets (1)
5.	1.0			45.0	5.45	2.69	
6.	20.0			10.0	5.93	5.09	
7.	20.0			5.0	6.03	5.94	Clumped,
8.		20.0		45.0	5.63	3.58	coalesced growth (2)
9.		10.0		45.0	5.81	2.93	

10.	5.0		45.0	5.77	2.85	
11.	2.5		45.0	5.66	2.81	Clumpy growth (1) (see text)
12.	1.0		45.0	5.40	2.98	
13.	20.0		10.0	6.43	6.63	
14.	20.0		5.0	6.60	7.16	
15.		20.0	45.0	5.80	4.85	
16.		10.0	45.0	5.85	3.69	
17.		5.0	45.0	5.48	2.75	Clumped, coalesced growth (2)
18.		2.5	45.0	5.69	2.52	
19.		1.0	45.0	5.42	2.56	
20.		20.0	10.0	6.03	6.91	
21.		20.0	5.0	6.19	7.22	

Note For morphology, refer to key (Section 3.7). Cultures were grown in 250 ml conical flasks with 100 ml medium and inoculated with $2x10^5$ spores/ml. Incubation was conducted at 30^oC, 150 r.p.m. for 24 hours.

Results show that R .arrhizus grows predominantly in a clumped, coalesced form under the conditions used. At lower levels of casein (1-5 g/l), discrete, dense pellets were formed. At similar levels of peptone, filamentous growth centres (1 mm) were discernible on dilution only, the gross morphology occurring as clumped growth. This is in contrast to the other media used. This feature of peptone medium growth morphology was later shown to occur erratically, some cultures not being dispersed by dilution.

3.7.1.1.(b). Effect of varying peptone medium components on growth.

The effect of varying peptone media components on biomass yield and growth morphology was then examined in 24-hour cultures (Section 2.4; Table 3.23). The results indicate that increasing the peptone concentration increased the level of biomass obtained. In all cultures, clumped growth was obtained. However, at lower peptone levels (1-5 g/l), growth centres were discernible on dilution as before (Section 3.7.1.1.a). Within this range, there appeared to be an inverse relationship between the level of peptone and numbers of growth centres ocurring. Glucose was only minimally utilized at the lowest peptone level (1 g/l) and only a low yield of biomass was produced. This suggests that the cultures were limited by the nitrogen source. It is possible that these results indicate that coalescence

Table 3.23. Effect of varying peptone medium components on growth of *R. arrhizus*

A. Effect of varying peptone levels.

Medium	Peptone (g/l)	Glucose (g/l)	pH t=0	pH t=24	Dry weight (mg/100ml)	Residual sugars (g/l)	Pellets (No./ml)	Growth morphology
1.	20.0	45	5.78	3.72	495.2	1.9	-	Clumped coalesced growth (2)
2.	10.0	45	5.78	2.98	344.0	10.2	-	
3.	5.0	45	5.74	2.75	194.6	38.8	80	Clumpy growth (1) (see text)
4.	2.5	45	5.62	2.78	104.1	40.0	138	
5.	1.0	45	5.33	2.98	44.5	45.0	>300	

B. Effect of varying glucose levels.

1.	5.0	45	5.74	2.75	194.6	38.8	80	Clumpy growth (1) (see text)
2.	5.0	30	5.95	2.85	170.7	20.9	160	
3.	5.0	20	6.15	2.80	177.4	10.9	200	
4.	5.0	10	6.48	2.84	173.7	1.3	>300	
5.	5.0	5	6.60	3.28	175.2	<0.1	>300	

Note: For morphology key, refer to Section 3.7.

Cultures were grown in 250 ml conical flasks with 100 ml medium, inoculated with 2×10^5 spores/ml, and incubated at 30°C, 150 r.p.m. for 24 hours.

and pelleting occurs in a sequence, ranging from large numbers of growth centres, to the formation of a single fungal mass. The effect of glucose concentration was examined using a single peptone concentration (5 g/l). Neither biomass yield nor growth morphology were affected by glucose levels ranging from 5-30 g/l. However, lower levels of glucose resulted in higher numbers of growth centres. Again, it is possible that at glucose levels of 5 g/l and 10 g/l, some limitation was being placed on growth although biomass levels were not affected. Biomass levels obtained from 45 g/l glucose were slightly higher with fewer growth centres occurring.

Peptone medium containing: soya peptone, 5 g/l; glucose, 20 g/l; pH 6.0 (Medium B.2), was selected for use in further experiments on growth morphology.

3.7.1.2. Effect of culture medium supplements on growth of R. arrhizus.

Many medium supplements have been cited in the literature as affecting fungal growth morphology (Metz & Kossen, 1977). These include oils and lipids, detergents and polymers (Baig et al., 1972; Kobayashi & Suzuki, 1972 a, b; Reese & Maguire, 1969; Takahashi et al., 1965). A range of these additives were added to peptone medium (Medium B.2) and the effect on growth morphology of R. arrhizus was examined under standard conditions

(Section 2.4). The results are presented in Table 3.24. The results show that Triton X-100 and sodium deoxycholate caused the formation of discrete pellets. Oleic acid increased the biomass yield but did not alter the growth morphology. A similar result was observed with polyethylene glycol.

3.7.1.3. Effect of culture medium viscosity on growth of R. arrhizus.

Viscosity has been shown to affect fungal growth morphology by several workers (Metz & Kossen, 1977; Takahashi et al., 1960 a, b). In this experiment, a range of viscous agents were added at different concentrations to peptone medium (Medium B.2). Cultures were inoculated with $2x10^5$ spores/ml and incubated under standard conditions (Section 2.4). The initial viscosity of each medium was determined (Section 2.10). After 24 hours incubation, the dry weight and morphology of growth was determined. The results are presented in Table 3.25.

The results indicate that, in general, growth morphology was more dispersed in media with increased viscosity. The anionic polymers bear a negative charge, so increasing their concentration affects the number of acidic groups present, as well as viscosity. Non-ionic polymers do not bear a net charge and so their effects on morphology may be more directly attributed to

Table 3.24. Effect of medium supplements on growth morphology of R. arrhizus

Medium Supplement	% (v/v)	pH t=0	pH t=24	Dry weight	Growth: Morphology
Control		5.92	3.20	180.0	Clumpy growth (1)(Sect 3.7.1)
Triton X-100	0.1	5.98	4.20	158.0	Discrete pellets (1)
Span-20	0.1	5.92	3.50	157.0	Clumped, coalesced growth (2)
Span-20	0.4	5.90	3.60	179.0	Clumped, pelleted growth (3)
Sodium deoxycholate	0.1 (g/l)	5.97	3.20	190.7	Clumped, coalesced growth (2)
Sodium deoxycholate	0.6 (g/l)	5.80	3.80	156.5	Discrete pellets (1)
Oleic acid	0.1	5.85	3.32	215.8	Clumped, coalesced growth (2)
Soya bean oil	2.0	5.86	3.18	n/m	
Peanut oil	2.0	5.90	3.18	n/m	
Polyethylene glycol	3.0 (g/l)	5.85	3.31	213.8	
Polyvinylalcohol	3.0 (g/l)	5.90	3.27	178.3	

Notes: n/m = not measured, due to interference with measurement.

Growth morphology: refer to key in Section 3.7.

Cultures were grown in peptone medium (Medium B.2), 100 ml in 250 ml conical flasks, inoculated with 2×10^5 spores/ml, and incubated at 30°C and 150 r.p.m. for 24 hours.

B. Non-ionic polymers:

12. M-cellulose	10.0	4.53	6.23	2.60	196.0	Clumped, coalesced growth (2)
13. M-cellulose	20.0	14.69	6.30	2.67	n/m	Clumped, pelleted growth (3)
14. M-cellulose	30.0	38.75	6.30	2.60	n/m	Dispersed, pelleted growth (4)
15. Dextran	20.0	3.20	5.78	3.00	187.1	Clumped, coalesced growth (2)
16. Dextran	30.0	5.02	6.13	3.00	186.3	
17. Dextran	40.0	7.59	6.15	3.10	182.8	Clumped, pelleted growth (3)
18. Polyethylene oxide	5.0	1.78	6.20	3.22	201.4	Clumped, coalesced growth (2)
19. Polyethylene oxide	10.0	4.30	6.40	3.26	196.1	Clumped pelleted growth (3)
20. Polyethylene oxide	15.0	9.44	6.60	3.30	207.6	
21. Polyethylene oxide	20.0	18.41	6.50	3.20	191.4	Dispersed, pelleted growth (4)

Notes: n/m = not measured due to interference with measurement.

Growth morphology: refer to key in Section 3.7.

Cultures were grown in peptone medium (Medium B.2), 100 ml in 250 ml conical flasks, inoculated with 2×10^5 spores/ml, and incubated at 30°C and 150 r.p.m. for 24 hours.

viscosity. Sodium alginate was the most effective polymer at dispersing growth, giving filamentous growth at 20 g/l (9.05 cP). Other polymers (such as carboxymethyl cellulose and Carbopol-934) required higher concentrations and/or viscosities to achieve similar levels of dispersion. Anionic polymers had a significant pH-buffering effect. However, other results have indicated that pH does not affect pelleting in R. arrhizus (Sections 3.3.4 and 3.7.1.4). Biomass yields tended to be higher when growth was more dispersed. In some cases, some of the apparently higher biomass levels may have been due to polymers attaching to the mycelia and not being removed by the washing procedures used (Section 2.5).

3.7.1.4. Studies on the effect of metal ions on growth morphology.

Metals such as manganese, zinc and iron have been reported to affect fungal growth morphology (Choudhary & Pirt, 1965; Clark 1962; Clark & Lentz, 1963). Divalent cations, such as magnesium and calcium, have been implicated in promoting yeast flocculation, a phenomenon similar to fungal pelleting (Atkinson & Daoud, 1976; Mill, 1964 b).

In a series of experiments, magnesium ions (1.25 mM $MgSO_4$) were added to peptone cultures (Medium B.2) and incubated under standard conditions (Section 2.4).

Inoculum used was $2x10^5$ spores/ml. The metal ions were added at different times during incubation. A series of cultures were also examined in which the initial pH was adjusted aseptically prior to inoculation. The results are presented in Table 3.26.

The results show that growth morphology was affected by addition of magnesium ions. Growth was changed from an amorphous, clumped morphology to one with many discrete pellets. This process ocurred even with ion additions after 12 hours incubation, when the spores had germinated and hyphae had formed (Section 3.7.1.1). With magnesium added at zero time, this pelleting was unaffected by the initial pH of the medium. A similar effect was observed when 1.25 mM $CaCl_2$ was added to the medium. When $CaCO_3$ was added at this level, the effect on morphology was similar, with the carbonate apparently being taken up by the pellets giving them a chalky appearance.

3.7.1.5. Effect of polymers and magnesium ions on growth morphology.

An experiment was then conducted to test if addition of polymers would reverse the pelleting effect of magnesium ions in peptone medium. Polymers at different concentrations were tested in peptone medium (Medium B.2) containing 1.25 mM $MgSO_4$. Cultures were inoculated with $2x10^5$ spores/ml and incubated under standard

Table 3.26. Effect of adding 1.25 mM $MgSO_4$ on growth morphology of R. arrhizus in peptone media

Time (hours) of $MgSO_4$ addition	pH t=o	pH t=24	Growth morphology
Control (No Mg^{++})	6.0	2.75	Clumpy growth (1)(Section 3.7.1)
0	5.95	2.70	
4	6.10	2.80	
8	6.00	2.78	
12	6.05	2.75	
0	7.00	2.54	Discrete pellets (1)
0	5.85	2.50	
0	4.96	2.50	
0	3.90	2.45	
0	2.73	2.35	

Note: For growth morphology see key in Section 3.7.

Cultures were grown in 250 ml conical flasks with 100 ml (Medium B.2) inoculated with $2x10^5$ spores/ml, and incubated at 30^0C and 150 r.p.m. for 24 hours. Sterile additions of $MgSO_4$ were made at times indicated, and cultures with different initial pHs were adjusted aseptically before inoculation using sterile 0.1M HCl or NaOH.

conditions (Section 2.4). The results are presented in Table 3.27.

Anionic polymers dispersed growth of R. arrhizus in the presence of magnesium ions, as if no ions had been added (Section 3.7.1.3). Methyl cellulose, a non-ionic polymer, did not disperse growth in the presence of magnesium ions at 5-20 g/l. These results illustrate the dual role anionic polymers appear to play in affecting fungal morphology: viscosity and cation absorption. It would appear that the presence of divalent cations has a greater influence on morphology than viscosity. The properties of some polymers are considered in Section 3.7.3.

3.7.1.6. Effect of maize solids on growth morphology.

When the ingredients of lipase production medium (Medium D) are examined, it will be seen that large quantities of $MgSO_4$, $CaCl_2$ and $CaCO_3$ are added (Section 2.7.4). Yet experiments using peptone medium (Medium B.2) have indicated that even small amounts of magnesium can severely pellet growth of R. arrhizus (Section 3.7.1.4). Despite this, growth in production fermenters remained dispersed and porridgy (Sections 3.2.1 and 3.4.1). This morphology is not readily classified using the morphology key which was drawn up for soluble peptone media (Section 3.7). The main difference between peptone medium (Medium B.2) and lipase production medium (Medium

Table 3.27. Effect of polymers and metal ions on growth morphology

Medium supplement (g/l)	Viscosity (cP)	$MgSO_4$ (1.25 mM)	Growth Morphology
Control 1 (no Magnesium)	1.06	-	Clumpy growth (1) (Section 3.7.1)
Control 2 ($MgSO_4$, 1.25 mM)	1.06	+	Discrete pellets (1)
A. An ionic polymers:			
Na-alginate 20		+	Dispersed,filamentous growth (5)
Carbopol-934 3	>2000	+	Dispersed,pelleted growth (4)
CM-cellulose 5	1.81	+	Clumpy growth (1) (Section 3.7.1)
CM-cellulose 10	3.54	+	
CM-cellulose 20	9.58	+	
CM-cellulose 30	27.40	+	Dispersed,pelleted growth (4)
B. Nonionic polymers:			
M-cellulose 5	2.31	+	Discrete pellets (1)
M-cellulose 10	4.57	+	
M-cellulose 20	15.91	+	
M-cellulose 30	48.0	+	Clumped,pelleted growth (3)

Note: For morphology key see Section 3.7.

Cultures were grown in 250 ml conical flasks with 100 ml peptone medium (Medium B.2, supplemented as indicated), inoculated with 2×10^5 spores/ml, and incubated at $30^{\circ}C$ and 150 r.p.m. for 24 hours.

D) is that the latter contains 45 g/l maize solids. These have been reported to enable Aspergillus species to grow dispersed (Smiley et al., 1967). When added to peptone medium (Medium B.2) R. arrhizus was shown to grow in a dispersed manner similar to production medium cultures. This dispersion occurred even in the prescence of $CaCl_2$ and $CaCO_3$. Maize particles may chelate ions from solution preventing pelleting. Microscopic observations on samples have shown that the mycelia appear to bind to the maize particles and use these as growth centres. Perhaps this prevents too many mycelia binding together to form pellets or clumps.

3.7.1.7. Comparison of growth in peptone and soya media.

Soya medium (Medium C) has been shown to yield dispersed growth of R. arrhizus (Section 3.3.5). This growth may be classified as Type 5 or dispersed, filamentous growth (Section 3.7). Growth in this medium has also been shown to be affected by addition of 100 mM $CaCO_3$ which caused the formation of pellets (Section 3.3.5). Addition of 100 mM $MgSO_4$ has been shown not to affect growth in soya medium, whereas addition of the same level of $CaCO_3$ caused pelleting. This is in contrast to findings with peptone media where both $MgSO_4$ and $CaCO_3$ caused pelleting, even at very low levels (Section 3.7.1.4). The solid, insoluble components in soya medium may bind mycelia or divalent cations preventing pellet formation,

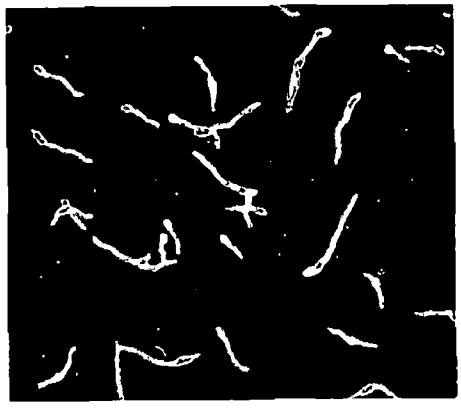
as was postulated for media containing maize solids (Section 3.7.1.6). The structures of hyphae formed in these media are compared in Section 3.7.1.8.(b).

3.7.1.8. Microscopic observations on growth of R. arrhizus.

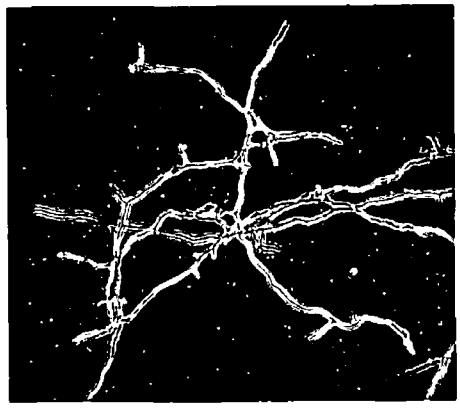
3.7.1.8.(a). The sequence of events leading to pellet formation.

In order to establish the sequence of events leading to pellet formation, an experiment was conducted in which samples were removed from cultures periodically during incubation and examined microscopically (Section 2.9). Samples removed were wet-mounted and photographed under a phase-contrast microscope. Some representative photomicrographs of fungal mycelia at different incubation times in casein medium (Medium A), incubated under standard conditions (Section 2.4), are presented in Figure 3.24.

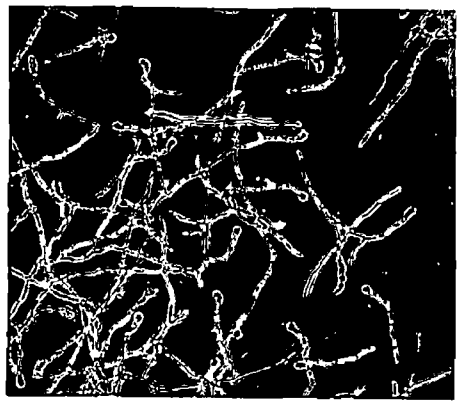
Germination of spores occurred in most media after 6 hours incubation. Hyphae elongated and branched, forming mycelial conglomerates from 9 hours on. Within any given sample, a range of clump sizes were encountered. Coalescence continued in different media to various degrees and led to typical final morphologies. In many cases, spore aggregation was also observed. Addition of magnesium ions to cultures induced pelleting whether



1.



2.



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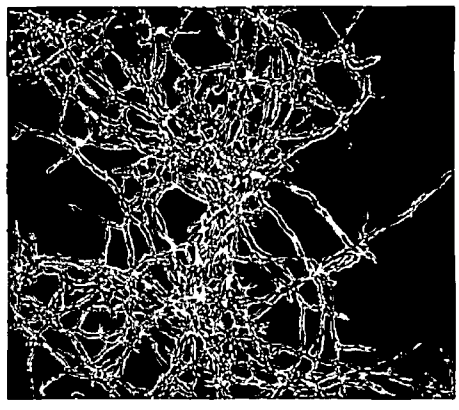

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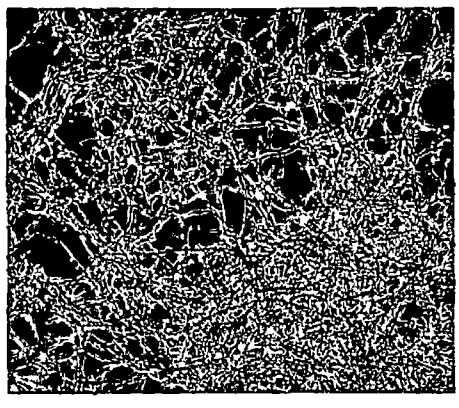

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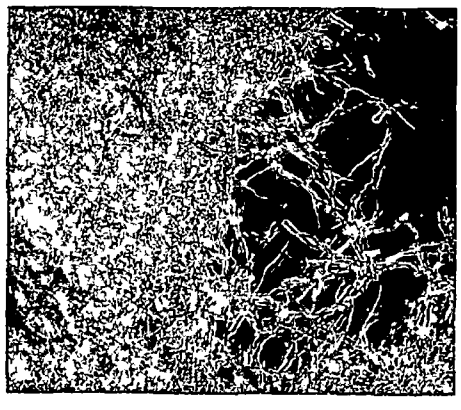

6.

Figure 3.24 Photomicrographs (magnification x 25) showing growth of R.arrhizus in Medium A (standard conditions) from spores.

1 and 2. t= 6 hours, 3 - 6. t = 9 hours and later.

added at zero time or up to 12 hours incubation (Section 3.7.1.4). These results suggest that pelleting may occur by either spore or mycelial aggregation, or both.

3.7.1.8.(b). Hyphal structures formed in different media.

When samples were removed from cultures in different media and examined by microscopy, a wide variation was observed in hyphal dimensions. This was especially marked in media containing polymers where hyphae formed were particulary long and broad. To quantify these observations, an experiment was conducted using culture media yielding different final morphologies. Samples were removed after 12 and 24 hours incubation under standard conditions (Section 2.4). Using wet-mounted samples, a micrometer attached to a phase-contrast microscope (x100) was used to measure single hyphae for total length, branching pattern and branch lengths (Section 2.10). At least 20 representative hyphae were measured for each sample and the average lengths calculated. The results are presented in Table 3.28.

The results show that R. arrhizus hyphae grew much longer in media supplemented with polymers. It would appear that long, more frequently branching hyphae are associated with dispersed growth. Shorter, less branched hyphae were observed mainly in media leading to pelleted growth. Hyphae in polymer-supplemented media appeared

different media.

Hyphae t=12			Hyphae t=24			
Secondary branches		Total	Primary branches		Secondary branches	
No.	Length (μm)	length (μm)	No.	Length (μm)	No.	Length (μm)
		173.3	6 (few free hyphae)	55.0	2	25.0
-	-		(no free hyphae)			
2	50.0	387.5	6	71.0	2	26.6
6	113.6	725.0	9	201.7	2	50.0
8	25.0	800.0	7	350.0	4	230.0
1	25.0	837.5	14	54.0	7	12.5

supplemented as indicated) or in soya media (Medium C), inoculated with 2×10^5 spores/ml, and incubated at 30°C.

broader under the microscope with cytoplasma filling the whole length. Hyphae in media giving pelleted growth appeared to be thinner, but the different dimensions were below the sensitivity of the micrometer used (1 μm). Hyphae formed in soya medium were shorter at t=12 compared with media supplemented with polymers. At t=24 however, longer hyphae were observed with a higher frequency of branching.

3.7.2. Growth and morphology of R. arrhizus in defined media.

Use of defined or mineral-salts media allows the examination of microbial growth in known chemical environments. Complex media contain many undefined ingredients which can have interacting effects on microbial growth. With defined media, all the components added are known, and may be varied independently. In this section the effect of polymers, metal ions and chelating agents on the growth of R. arrhizus in a mineral-salts medium is considered.

3.7.2.1. Effect of polymers on growth in defined medium.

In these experiments defined medium (Medium F) was supplemented with sodium alginate or carboxymethyl cellulose. The viscosities of the resulting media were determined (Section 2.10). Enzyme activity against the

polymers was screened for in culture supernates using the Bioglucanase assay method (substrate levels: CM-cellulose: 10 g/l; Na-alginate: 5 g/l ; see Appendix I).

A range of glucose levels were used, and the culture biomass and morphology were determined after 32 hours incubation, under standard conditions (Section 2.4). The experimental results are presented in Table 3.29. No growth was observed in media with sodium alginate or carboymethyl cellulose as sole carbon source.

None of the culture supernates showed polymer-hydrolysing activity, even when reaction mixtures were incubated for 24 hours. These results indicate that R. arrhizus is not capable of degrading these polymers. Growth in defined medium containing 10 and 20 g/l glucose was poor, with no growth occurring with 5 g/l glucose. When polymers were added to these media, growth was very heavy and dispersed, even with 5 g/l glucose. Growth was heavier in media containing sodium alginate compared with carboxymethyl cellulose. Growth in these media increased with increasing glucose concentration.

In the presence of polymers, growth was of a dispersed, filamentous morphology, compared with the discrete pellets formed in their absence. Some cultures were so finely dispersed that filter papers used for dry weight

Table 3.29. Effect of polymers on growth of R. arrhizus in defined media

Medium: pH 6.20				Culture:		
Glucose (g/l)	Supplement	(g/l)	Viscosity (cP)	pH	Growth morphology	Dry weight (mg/100 ml)
0	Na-alginate	5	2.35	6.73	NG	-
0	CM-cellulose	10	4.00	6.80	NG	-
5	-		0.99	6.50	NG	-
5	Na-alginate	5	1.69	3.64	Dispersed, filamentous growth (5)	324.7
5	CM-cellulose	10	2.76	4.45		183.7
20	-		1.06	3.00	Discrete pellets (1)	90.0
20	Na-alginate	5	1.80	3.23	Dispersed, filamentous growth (5)	Not filterable
20	CM-cellulose	10	3.00	3.76		306.9
45	-		1.10	3.00	Discrete pellets (1)	105.0
45	Na-alginate	5	1.64	3.25	Dispersed, filamentous growth (5)	645.7
45	CM-cellulose	10	3.25	3.65		388.2

Notes: NG = No growth occurred; for growth morphology see key in Section 3.7.

Cultures were grown in defined medium (Medium F, supplemented as indicated), 100 ml in 250 ml conical flasks, inoculated with 2×10^5 spores/ml, and incubated at 30^{o}C, 150 r.p.m. for 32 hours.

determination were blocked. The drop in culture pH was reduced slightly in the presence of polymers. Initial viscosity was also increased, but not to high values. It seems unlikely that these two factors could have affected the culture morphology so significantly.

In another experiment, defined media containing 10 g/l glucose, were supplemented with a range of polymers and the effect on growth determined under standard conditions. These results are presented in Table 3.30. In cultures containing polymers, growth yields were increased several-fold and the morphology was dispersed. This was observed in the case of the non-ionic polymer, methyl cellulose, also.

3.7.2.2. Effect of metal ions and chelating agents on growth in defined media

It has previously been reported that fungal growth morphology can be affected by metal ions and chelating agents (Choudhary & Pirt, 1965). An experiment was conducted to determine the effect of different metal ion levels on growth of R. arrhizus. Defined medium (Medium F, glucose 20 g/l) was used and incubated under standard conditions after inoculation with 2×10^5 spores/ml (Section 2.4). The results are presented in Table 3.31.

Reducing levels of magnesium in the medium resulted in poorer growth yields and in a clumped growth morphology.

Table 3.30. Effect of polymers on growth of R. arrhizus in defined media

Medium			Culture.		
Supplement	(g/l)	Viscosity (cP)	pH	Dry weight (mg/100 ml)	Growth Morphology
-		1.00	3.00	59.6	Discrete pellets (1)
Na-Alginate	5.0	1.90	3.64	328.8	Dispersed,
CM-Cellulose	10.0	1.76	4.38	204.1	filamentous
Carbopol-934	0.8	1.74	3.10	194.6	growth
M-Cellulose	10.0	4.85	2.48	197.2	(5)

Cultures were grown in defined media (Medium F, 10 g/l glucose, supplemented as indicated), 100 ml in 250 ml conical flasks inoculated with 2×10^5 spores/ml. Incubation was conducted at 30 °C and 150 r.p.m. for 48 hours.

Note: For morphology key see Section 3.7.

Table 3.31. Effect of metal ion levels on growth of *R. arrhizus* in defined medium

Medium: pH 6.20			Culture:		
No.	MgSO (mM)	MnSO (mM)	pH	Dry weight (mg/100ml)	Morphology
1.	1.25	0.20	2.20	272.0	Discrete pellets (1)
2.	0.60	0.20	2.27	198.9	Clumped, coalesced growth (2)
3.	0.30	0.20	2.40	168.6	
4.	1.25	0.10	2.20	284.9	
5.	1.25	0.05	2.20	274.6	
6.	0.60	0.10	2.20	246.6	

Cultures were grown in defined media (Medium F, modified as above) 100 ml 250 ml culture flasks, inoculated with 2×10^5 spores/ml, and incubated at 30°C and 150 r.p.m. for 48 hours.

Lower levels of manganese ions also resulted in a clumped growth morphology, but growth yields were not significantly affected. This observation was also made when both magnesium and manganese levels were reduced (medium no. 6).

In another experiment, defined media were supplemented with different levels of E.D.T.A., a metal chelating agent. Cultures were inoculated with 2×10^5 spores/ml and incubated under standard conditions (Section 2.4). The results are presented in Table 3.32.

Addition of 1.0 and 0.5 mM E.D.T.A. did not affect growth yield or morphology. When media were supplemented with 0.25 mM E.D.T.A. growth was filamentous and dry weight was significantly higher. Other observations made, using E.D.T.A. in peptone medium (Medium B.2), may help to explain these results. At 0.05 mM E.D.T.A., growth in peptone medium occurred as discrete pellets. This implies that E.D.T.A. may act in a manner similar to magnesium ions in inducing pelleting (Section 3.7.1.4.). The results in defined media may be explained by the balance of the pelleting versus the chelating effect of E.D.T.A. (which removes divalent cations from solution). The enhanced growth yield may reflect the improved metabolic efficiency associated with dispersed growth.

Table 3.32. Effect of E.D.T.A. on growth on defined medium

Medium: (pH 6.20) No.	E.D.T.A. (mM)	pH	Culture: Dry weight (mg/100ml)	Morphology
1.	-	2.20	272.0	Discrete pellets (1)
2.	1.00	2.21	271.5	
3.	0.50	2.27	276.3	
4.	0.25	2.24	316.5	Discrete, filamentous growth (5)

Cultures were grown in defined media (Medium F, supplemented as above) 100 ml 250 ml flasks, inoculated with 2×10^5 spores/ml, and incubated at $30^{\circ}C$ and 150 r.p.m. for 48 hours.

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3.7.3. Investigation of the properties of some polymers.

In earlier sections it has been noted that polymers may affect fungal morphology by a combination of cation binding and viscosity (Section 3.7.1.5). It has also been observed that anionic polymers have significant pH-buffering effects on cultures, in contrast with non-ionic polymers (Section 3.7.1.3).

3.7.3.1. The pH-buffering effect of polymers.

In this experiment, peptone media (Medium B.2) supplemented with different polymers, were titrated with HCl (0.1 M) to pH 5.5 and 4.5. The number of moles of acid required was calculated for each medium. The results are presented in Table 3.33. The buffering capacity of methyl cellulose (a non-ionic polymer) was negligible. Carbopol-934 had the highest buffering capacity per unit weight.

In the course of this work, it was noted that pH affected the viscosity of Carbopol-934 solutions. In an experiment, a solution of Carbopol-934 (3 g/l) in peptone medium (Medium B.2) was adjusted to different pHs and the resulting viscosity measured. These results are presented in Table 3.34. At low pHs (below 4.0) viscosity was low, but increased rapidly to >2000 cP at pH 6.0. When readjusted to pH 4.0 viscosity was lowered. These results imply that *R. arrhizus* cultures grown in this medium have a low final viscosity (Section

Table 3.33. The pH-buffering effect of polymers

Peptone medium supplement	(g/l)	m moles/l of HCl added to reduce pH from 6.0 to: pH 5.5	pH 4.5
-		1.12	2.66
Na-alginate	20	1.90	11.34
CM-cellulose	30	3.26	31.14
Carbopol-934	3	13.00	37.60
M-cellulose	30	1.52	4.09

Peptone media (Medium B.2, supplemented as indicated) were titrated to pH 5.5/4.5 with 0.1M HCl.

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Table 3.34. Effect of pH on the viscosity of Carbopol-934 in peptone medium

	pH	Viscosity (cP)
A.	3.78	4.68
B.	4.00	8.33
C.	5.00	720.00
D.	6.00	>2,000
E. Readjusted to	4.00	4.04

Carbopol-934 (3 g/l) in peptone medium (Medium B.2) was measured for viscosity at different pHs using a Brookfield Digital Viscometer. pH was adjusted using NaOH or HCl solutions.

this medium have a low final viscosity (Section 3.7.1.3). Further experiments have shown that R. arrhizus will grow dispersed (Type 4) in this medium at an initial pH of 4.0 (viscosity 4.0 cP). This result provides further evidence that viscosity is not essential for dispersal of growth by polymers. It seems unlikely that pH-buffering alone could affect morphology, particulary when other results are considered (Sections 3.3.4, 3.7.1.3 and 3.7.1.4).

3.7.3.2. The ability of polymers to absorb metal ions.

It has been demonstrated that polymers reverse the pelleting effect of magnesium ions on fungal growth morphology (Section 3.7.1.5.). It was suggested that this effect could be due to the absorption of the metal ions. Solutions of polymers in distilled water were used in dialysis bags to determine if they were capable of absorbing magnesium ions from solution and of retaining ions added to them. The external solutions, in which dialysis bags were suspended, were stirred continuously during a 17-hour incubation period at room temperature. Levels of magnesium were estimated using a Solochrome-Black titration method (Section 2.8.3). The results are presented in Table 3.35.

The results show that the polymer solutions, with the exception of methyl cellulose, are capable of absorbing magnesium ions. For each anionic polymer tested, the

Table 3.35. Ability of polymers to absorb magnesium ions

Distribution of Mg^{++} ions during dialysis.

Polymer	(g/l)	Internal polymer solution (20ml) Total Mg^{++} (μ moles)	External solution (40ml) Total Mg^{++} (μ moles)
A. Initial distribution:		50	0
Equilibrium distributions:			
Control (d H_2O)	-	18.80	31.20
Na-alginate	20	41.18	8.68
CM-cellulose	30	48.65	1.25
Carbopol-934	3	47.74	2.28
M-cellulose	30	24.36	25.74
B. Initial distribution:		0	100
Equilibrium distributions:			
Control (d H_2O)	-	37.60	62.40
Na-alginate	20	94.50	5.61
CM-cellulose	30	94.00	5.95
Carbopol-934	3	91.20	8.80
M-cellulose	30	41.79	58.11

Polymer solutions were dialysed against water with constant agitation for 17 hours at room temperature. $MgSO_4$ was added either to the polymer solution, or to the external solution. Mg^{++} ions were determined using a Solochrome-Black titration method (Section 2.8.3).

distribution of ions occurred significantly away from the external solution. While full equilibrium had not been achieved in the incubation times employed, distribution values for controls and methyl cellulose solutions were similar. This indicated that the non-ionic polymer was not capable of absorbing divalent cations.

3.7.4.

In Section 3.7 a number of medium supplements were shown to affect pelleting of R. arrhizus. High nitrogen levels were shown to increase biomass and coalescence of growth. Addition of Triton X-100, sodium deoxycholate and metal ions to complex media caused the formation of discrete pellets. Viscosity-conferring polymers were shown to disperse growth, although the degree of dispersion varied with the type of polymer used. Some of these polymers were shown to reverse the pelleting effect of magnesium ions. Maize solids were shown to disperse the growth of R. arrhizus cultures, even in the presence of high levels of metal ions. Growth was compared in peptone and soya media and hyphae formed were shown to be significantly longer in the latter medium, yielding dispersed growth. R. arrhizus cultures in defined media were affected by the presence of polymers. These were shown to disperse growth and to significantly increase biomass, while not being degraded themselves. E.D.T.A. also dispersed growth in defined

media, but only at one specific level. In general, R. arrhizus may form pelleted growth either by spore or hyphal agglomeration. Long, well-branched hyphae gave dispersed filamentous growth, whereas shorter hyphae led to a clumped growth morphology. Some chemical properties of a selection of polymers were investigated to determine if some explanation might be found for their effect on growth of R. arrhizus. The presence of divalent cations appeared to be important in determining the morphology of growth. These ions were absorbed by anionic polymers which may, in part, account for their effect on the growth of R. arrhizus in complex and defined media.

4. DISCUSSION.

In common with findings with other species of Rhizopus, lipase production by R. arrhizus was shown to be repressed in the presence of critical levels of reducing-sugars (Section 3.2.4; Aisaka & Terada, 1979; Akhtar et al., 1974; Laboureur & Labrousse, 1966; Macrae, 1983). Lipase is produced mainly after growth has ceased and nutrients have been utilized. This would suggest that a form of catabolite repression controls lipase production (Demain, 1972; Paigen & Williams, 1970). Use of partially hydrolysed maize was found to give optimum yields compared with using fed-batch fermentations or slowly metabolizable carbohydrates (Section 3.2.4). Casein hydrolysate was found to be a suitable nitrogen source for the fermentation, although other sources have been cited in literature (Aisaka & Terada, 1979; Mauvernay et al., 1970). Supplementary nitrogen sources were found in some cases to repress enzyme production (Section 3.2.5.2). This may be a form of catabolite repression where exessive nitrogenous nutrients repress enzyme synthesis (Demain, 1970).

When highly refined medium ingredients were used it was shown that trace metals limited the fermentation (Section 3.2.6). This emphasizes the importance of such elements for optimum fungal growth (Foster & Waksman, 1938; Wegener & Romano, 1963). In an industrial fermentation plant it is unlikely that such limitation

would occur as the trace metals would be supplied not only in cruder grade raw materials, but also in the water used to batch media. pH 6.0 was shown to be optimal for lipase production by R. arrhizus (Section 3.2.8). This is in agreement with previous findngs for other Rhizopus species (Aisaka & Terada, 1979; Fukumoto et al., 1966; Laboureur & Labrousse, 1966).

In these previous studies, no reference was made to problems associated with fungal growth morphology. During work for this thesis, it was found necessary to grow R. arrhizus in a dispersed form to allow growth in fermenters to occur (Section 3.3). Nevertheless, pelleted growth was shown to be capable of producing lipase when it dispersed itself in production media (Section 3.3.6).

In fermenters, lipase production by R. arrhizus was enhanced when aeration levels were increased, as had been shown previously for R. delemar (Section 3.4.1.1; Giuseppin, 1984). R. arrhizus was shown to be especially sensitive to shear from fermenter impellers above tip speeds of 100 $cm.s^{-1}$ (Sections 3.3.8 and 3.4.1.2). Previous reports concerning R. nigricans showed this organism to be susceptible to shear from impellers above tip-speeds of 310 $cm.s^{-1}$ (Hanisch et al., 1980). P. chrysogenum has been reported to be sheared by impellers also, but this did not effect the viability of the mycelia (Dion et al., 1954; Suijdam & Metz, 1981).

P. chrysogenum has been shown to break apart at the septae or cross-walls leaving individual cell compartments intact (Savage & Vander Brook, 1946). However, since Rhizopus species are coencytic or aseptate, shear forces may disrupt cellular integrity if the cell walls are damaged (Alexopoulos & Mims, 1979). This reasoning also implies that the same number of growth units are present in inoculum flasks as are present in the final production and secondary growth cultures. This number may be reduced by cellular fragmentation or pelleting of hyphae. Inoculum age and volume was shown to affect growth in secondary fermenters (Sections 3.3.9 and 3.3.10). Production of lipase was also affected by inoculum characteristics (Section 3.4.2; Calam, 1976; Meyrath & Suchanek, 1972). This emphasizes the need to standardize such procedures in order to obtain reproducible fermentation results.

Scale-up of the lipase process was achieved by a combination of trial and error, and a knowledge of factors affecting fungal growth (Sections 3.3.12 and 3.4.3). A systematic approach would require more equipment and time then was available during this project. Such an approach could possibly examine oxygen transfer rates, power input and mixing times at each scale of operation, bearing in mind the characteristics of non- newtonian fungal fermentation broths (Einsele, 1978; Wang & Fewkes, 1977; Wang et al., 1979). However, recommendations for changing plant equipment would need

to be justified economically by considerably improved yields.

Further improvements in the lipase yields from this process could be achieved by further medium development and by selection and improvement of strains (Calam, 1972; Johnston, 1975; Sultanova & Zabirov, 1979). Further medium development could be undertaken in laboratory fermenters, as conditions and production time-courses approximate more closely to those occurring in plant-scale equipment (Sections 3.2.1, 3.4.1 and 3.4.3). In parallel studies, the purification and extraction methods could be optimized for each medium type.

In comparing enzyme preparations from different sources, observed properties may be influenced by assay methods, purity of the preparation and methods used to produce the enzyme (Godfrey, 1983). Key characteristics of enzymes such as temperature/pH - activity and stability may be used to finger-print enzymes (Fulbrook, 1983). Such properties are important also when considering particular applications for enzyme preparations (Godfrey & Reichelt, 1983). It should be noted that enzymes are more stable in the presence of their substrates, which may enhance their suitability for any given application (Godfrey, 1983). Stability optima tend to be broader than activity optima, due to the reversible effects of any given storage pH or temperature. Conditions for

optimal activity affect the conformation of the active site and the availability of substrate. pH profiles are considered to be more specific then temperature profiles (Fulbrook, 1983).

Temperature activity curves showed the two R. arrhizus lipase preparations to be similar, whereas pH-activity profiles showed showed significant differances (Sections 3.6.1 and 3.6.2). In particular, the characteristic secondary pH 3.5 optimum was not found in the R. arrhizus culture preparation (Benzonana, 1973; Laboureur & Labrousse, 1964, 1966). These differences may be attributed to different strains and production methods used and also to the relative impurity of the culture preparation. Purified (Sigma London) R. arrhizus lipase was much less tolerant to storage at higher pHs and temperatures than the culture lipase (Sections 3.6.3 and 3.6.4). Again, this may be attributed to the purity of the preparations used. The presence of other proteins, ions and sugars in the culture preparation may have stabalized the lipase proteins. The purified lipase proteins might have been more stable in the presence of other proteins or of a substrate triglyceride. Porcine pancreatic lipase, in common with other pancreatic lipases, was shown to be most active at 37^{o}C and at pHs above 7.0 (Sections 3.6.1 and 3.6.2; Godfrey, 1983; Naher, 1974). Stability curves for pancreatic lipase may be somewhat suspect as the preparation used had some contaminating protease

activity (Sections 3.6.3 and 3.6.4.). Therefore, the observed effects may be due partly to the activity of this protease at different pHs and temperatures.

Pelleting of R. arrhizus was shown to be affected by inoculum, medium components and various supplements. Levels of spore inocula were shown to affect growth morphology in different media. In casein media spore inoculumof $1x10^2$/ml was shown to give poor pelleted growth, whereas higher inocula allowed more dispersed growth (Section 3.1.2). Similarly, growth in other complex media was shown to be more dispersed when inocula of $2x10^5$ spores/ml were used, (Section 3.7.1). Growth of R. arrhizus was dispersed and filamentous in soya medium when $2x10^5$ spores/ml were used as inoculum, but became pelleted or clumped when $1x10^4$ spores/ml were used (Section 3.3.5). These findings are in agreement with previous results which showed that higher spore inocula gave less pelleted growth (Camici & Sermonti, 1952; Steel et al., 1954; Testi-Campasano, 1959).

It has been shown that addition of cations to peptone media results in pelleted growth of R. arrhizus (Section 3.7.1.4). It may be that the spore inoculum at higher levels is less likely to pellet in standard media because insufficient cations are present. At lower spore levels, pelleting may proceed either by spore or mycelial interactions (Section 3.7.1). In soya medium, the ions may be chelated by the solid materials present.

In defined media, levels of mineral ions and chelating agents affected morphology of R. arrhizus (Section 3.7.2.2). E.D.T.A. at one specific level (0.25 mM) was shown to disperse growth, whereas higher levels gave pelleted growth. This result is difficult to explain, but it could be that at a specific level of free ions to E.T.D.A. ratio, pelleting is prevented (Choudhary & Pirt, 1965). The presence of cations has been shown to affect yeast flocculation, which is a phenomenon similar to fungal pelleting in that cell-cell interactions are involved (Atkinson & Daoud, 1976; Galbraith & Smith, 1969; Lyons & Hough, 1971; Rainbow, 1966; Stewart, 1975). In yeast flocculation particular cellular components have been implicated in cross-linking cells (Amri et al., 1982; Jayatissa & Rose, 1976; Miki et al., 1982 a, b; Mill, 1964 a, b). No cellular components has so far been implicated in fungal pelleting, although colonial mutants of Neurospora crassa have been shown to have chemically altered cell walls (Galbraith & Smith, 1969; Terra & Tatum, 1963).

Fungal spores bear a net negative charge at physiological pHs (Douglas et al., 1959; Galbraith & Smith, 1969). R. arrhizus vegetative biomass has been shown to absorb a wide variety of positive ions and the functional groups enabling such uptake have been studied (Tobin et al., 1984). Divalent cations must, therefore, overcome the repulsive forces of negatively-charged spore or mycelial surfaces to facilitate pelleting.

Pelleting of R. arrhizus was shown, moreover, to be independant of the medium pH, in contrast with previous results with A. niger and P. chrysogenum (Section 3.3.4; Galbraith & Smith, 1969; Pirt & Callow, 1959).

Bacterial aggegation is frequently caused by the production of polysaccharides (Atkinson & Daoud, 1976; Cadmus et al., 1976; Govan et al., 1981; Jansson et al., 1983; Stanley & Rose, 1967; Unz & Farrah, 1976). However, in general, fungi grow more dispersed in the presence of polymers (Elmayergi et al., 1973; Elmayergi, 1975; Suijdam et al., 1980; Takahashi et al., 1960 a, b). This effect may be due to the masking of surface charges on cells as some polymers have been shown to coat cellular surfaces (Elmayergi & Moo-young, 1973). R. arrhizus has been shown to grow more dispersed in the presence of polymers (Sections 3.7.1.3., 3.7.1.5 and 3.7.2.1). No absolute correlation was demonstrated between viscosity and the degree of dispersion. Non-ionic polymers, such as polyethylene polymers and methyl cellulose, were less effective at dispersing growth compared with anionic polymers such as sodium alginate, carboxymethyl cellulose and Carbopol-934 (Section 3.7.1.3; Elmayergi et al., 1973; Trinci, 1983). Nevertheless, physical separation of spores and mycelia may contribute to dispersion of growth. Anionic polymers were shown to be capable of absorbing divalent cations from solution (Section 3.7.4.3). This property may have contributed to preventing pelleting of R. arrhizus in

the presence of magnesium ions, where non-ionic polymers, which cannot bind ions, were less effective (Section 3.7.1.5). Carbopol-934 at pH 6.0 was shown to have a high viscosity, but to have low viscosity at pH 4.0 (Section 3.7.4.4). R. arrhizus was shown to grow more dispersed in its presence at either pH, demonstrating that viscosity is not of paramount importance in dispersing growth with this polymer (3.7.3.1). Previous reports have suggested that this polymer disperses spores by charge repulsion (Elmagergi, 1975).

In defined media biomass production by R. arrhizus was increased several-fold in the presence of polymers at low levels (Section 3.7.2.1). The efficiency of conversion of nutrients to biomass appeared to be enhanced by the polymers. Carbopol-934 has been reported to increase mass transfer into fungal mycelia, and to increase respiration and glucose consumption (Elmayergi & Moo-Young, 1973; Moo-Young et al., 1969). Growth in a filamentous form is more efficient than pelleted growth in terms of oxygen and nutrient transfer (Huang & Bungay, 1973; Phillips, 1966).

The presence of maize solids has been shown to disperse growth of R. arrhizus even in the presence of calcium and magnesium ions (Section 3.7.1.6). Similar observations have been made previously with A. niger (Smiley et al., 1967). From microscopic observations on

samples, these cultures remained dispersed because mycelia apparently used the solid particles as alternative loci for forming colonies. It is possible that the solid particles may chelate free ions from solution also. Addition of oils to cultures of R. arrhizus did not affect morphology, in contrast to findings with other fungi (Section 3.7.1.2; Baig et al., 1972; Kobayashi & Suzuki, 1972 b). Some surfactants, such as Triton X-100 and sodium deoxycholate caused pelleting of R. arrhizus, whereas others, such as Span-20, had only a slight dispersing effect (Section 3.7.1.2; Takahashi et al., 1965). These supplements appear to vary in their effect on different fungal species.

The sequence of events leading to fungal pelleting appears to involve spore or mycelial agglomeration, or both (Section 3.7.1; Cocker & Greenshields, 1977; Galbraith & Smith, 1969; Metz & Kossen, 1977). Experiments with R. arrhizus have shown that pelleting may be induced by addition of magnesium ions, even when added to cultures after up to 12 hours incubation (Section 3.7.1.4). Morphology of individual hyphae has been shown to be longer and more branched in media giving dispersed growth (Section 3.7.3; Pirt & Callow, 1959). In contrast with previous observations, mechanical stirring of R. arrhizus cultures did not affect its morphology, but excessive shear was shown to destroy the biomass (Sections 3.3.2, 3.3.3, 3.3.8,

3.3.12, 3.4.1.2 and 3.4.3; Compasano et al., 1959; Dıon & Kaushal, 1959; Suıjdam & Metz, 1981).

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7. APPENDIX I.

DETERMINATION OF COMMERCIAL ENZYME ACTIVITIES.

1. Protease determination for Fungal Protease (Biocon Ltd, Ireland)

Principle:

This procedure is for the determination of proteolytic activity expressed as Haemoglobin Units on a Tyrosine basis (H.U.T.), since tyrosine is used for preparation of a standard curve. The test is based on a 30-minute hydrolysis of a haemoglobin substrate at pH 4.7 and 40 ^{o}C. Unhydrolysed substrate is precipitated with trichloroacetic acid and removed by filtration. Solubilized haemoglobin is determined spectrophotometrically.

Reagents and solutions:

1. Haemoglobin substrate:

Haemoglobin, suitable as protease substrate, is used in the assay (Sigma London Ltd). To 10 ml water, 5g of haemoglobin is added and stirred to dissolve. The pH is reduced to 1.7 using 0.3 M HCl solution. After 10 minutes the pH is readjusted to 4.7 by addition of 0.5 M sodium acetate and the solution is diluted to 250 ml

with water. This solution is stable for 5 days when refridgerated.

2. Enzyme dilution buffer:
Mix 25 ml of 2 M sodium acetate solution with 50 ml 1 M acetic acid and dilute to 1 litre.

3. Trichloroacetic acid solution:
Dissolve 140 g of trichloroacetic acid in 75 ml water and dilute to 100 ml.

4. Stock tyrosine solution:
Dissolve 100 mg of L-tyrosine (Sigma, London Ltd) in 60 ml 0.1 M HCl. Dilute to 1 litre with water. Solution contains 100µg/ml of L-tyrosine.

Procedure:

Pipette 10 ml of substrate into a series of test tubes; one for each enzyme test, one for each enzyme blank and one as a substrate blank. The sample is prepared by diluting in buffer to give between 9 and 22 H.U.T. units per ml. Substrate and sample tubes are equilibrated to 40°C. To each enzyme test solution, 2 ml of sample is added, and to the substrate blank solution, 2 ml of buffer is added. The tubes are stoppered and shaken gently to mix the contents. Incubation of each tube is conducted for exactly 30 minutes, when 10 ml of trichloroacetic acid is added. The tubes are shaken

vigorously and allowed to cool to room temperature for one hour, during which time the tubes are shaken at 10 minute intervals. Enzyme blanks are prepared by incubating substrate (10 ml) and enzyme dilutions separately at 40°C for 30 minutes. Trichloroacetic acid solution (10 ml) is added to the substrate solution and shaken well. Enzyme dilution (2 ml) is then added, shaken and allowed to cool, with intermitent shaking as before, for 1 hour. All the tube contents are then filtered through Whatman No.42 filters, refiltering the first half of filtrate through the same paper. The absorbance of each filtrate is determined at 275 nm on a Pye-Unicam SP6-550 spectrophotometer. The enzyme blank reading is subtracted from each enzyme test, with the spectrophotometer set at zero using the substrate blank. A standard curve of L-tyrosine absorbance is obtained by diluting the stock tyrosine solution to give 75, 50 and 25 g/ ml tyrosine. This should give a straight line and the value for 70 μg/ ml, divided by 63.5, should give 0.0084 (equivalent to 1.10 μg/ml).

Calculation:

One H.U.T. unit is defined as that amount of enzyme which will, under standard conditions, give an hydrolysate, in one minute, whose absorbance at 275 nm is equivalent to that of 1.1 μg/ml tyrosine in 6 mM HCl.

$$\text{H.U.T.} = \frac{A_{\text{test}}}{A_{11\mu g/ml\ \text{tyrosine}}} \times \frac{\text{Reaction volume(ml)}}{\text{Time (mins)}} \times \frac{\text{Enzyme dilution}}{2}$$

(units/ml)

$$= A \times \text{Enzyme dilution} \times 43.7$$

2. α-amylase determination for Canalpha (Biocon Ltd., Ireland).

Principle:

The method is based on starch degradation by enzyme hydrolysis at pH 5 and 40°C. Starch forms a blue colour with iodine which weakens as the starch is broken down. The colour change is followed spectrophotometrically and related to enzyme activity.

Reagents and solutions:

1. Acetate buffer:
8.2 g of sodium acetate is dissolved in 50 ml water, the pH adjusted to 5.0, and the solution is diluted to 100 ml

2. Substate solution:
1 g Lintner starch (B.D.H. England) is stirred into 20 ml water, and added to 20 ml boiling water to dissolve. This is then cooled, 15 ml acetate buffer is added, and the mixture is diluted to 144 m) with water (make daily).

3. Stock iodine solution:

5.5g Iodine and 11 g potassium iodide are dissolved in 100 ml water and diluted to 250 ml (make daily).

4. Dilute iodine solution:

1 ml stock solution and 10 g potassium iodide are dissolved and diluted to 250 ml. Make daily.

Procedure:

To a series of test tubes, 20 ml of substrate solution is added, one for each enzyme test. The enzyme sample is diluted in water and all tubes are equilibrated to 40 C. To another series of test tubes, 5 ml of dilute iodine solution is added, several for each enzyme test. At zero time 10 ml of the enzyme dilution is added to a substrate tube and mixed by inversion. At definite time intervals, pipette 1 ml of the reaction mixture into dilute iodine solution. This stops the reaction and develops the colour complex. As this colour changes from blue-black to a lighter blue, sample at 0.5 minute intervals. Samples are measured for absorbance at 617 nm on Pye-Unicam SP 6-550 spectrophotometer. Optical density is plotted against time and the time required to obtain O.D. 0.5 is estimated. This time (t) should fall between 10 and 20 minutes, otherwise an appropriate dilution is selected and the test repeated.

Calculation:

$$\text{Units/ml} = \frac{1430}{\text{Volume of enzyme (ml)} \times t} = \frac{143}{t}$$

3. Amyloglucosidase determination for Amylo (Biocon Ltd., Ireland).

Principle:

Unit enzyme activity is measured as the amount of enzyme required to release 1mg of glucose from starch in 1 minute. Buffered starch at pH 5.0 and 40°C is used as substrate. Glucose released is estimated using a dinitrosalycylic acid method (D.N.S.), (Miller, 1959).

Reagents and solutions:

1. Acetate buffer:
8.2 g of sodium acetate is dissolved in 50 ml of water, the pH adjusted to 5.0, and the solution is diluted to 100 ml.

2. Substrate solution:
Soluble starch (B.D.H., England) 1g, is slurried in 20 ml water and added to 60 ml boiling water to dissolve it. The mixture is cooled, 5.6 ml acetate buffer is added and diluted to 100 ml with water (make daily).

3. D.N.S. solution:

3,5-dinitrosalycylic acid (10 g), potassium sodium tartarate (300 g), and sodium hydroxide 16 g, are all weighed and dissolved in 600 ml water by heating (without boiling). The solution is cooled and diluted to 1 litre.

4. Glucose stock solution:

A solution is made of 5 g/l glucose,with 0.2 g/l sodium azide as preservative.

5. Glucose standards:

The stock solution is diluted to give 0.1-1.5 g/l

Procedure:

The enzyme solution is diluted in water. Substrate solution (1 ml) is added to a series of test tubes, one for each enzyme test. A substrate blank is prepared by adding 1 ml substrate to 1 ml water. To each enzyme test, 1 ml of sample dilution is added and incubated at 40^{o}C for 10 minutes. The reaction is stopped by adding 2 ml of D.N.S. solution. The tubes are placed in a boiling water bath for 5 minutes, cooled and 10 ml water added to each tube. The tubes are mixed and the absorbance at 540 nm measured on a Pye-Unicam SP6-550 spectrophotometer. An enzyme blank is prepared by adding 2 ml D.N.S. solution to 1 ml substrate solution, adding

1 ml enzyme dilution and treating as for enzyme test above. A standard curve of glucose concentration against absorbance at 540 nm is prepared by adding 2 ml of glucose standards to 2 ml D.N.S. solution and treating as for enzyme reaction tubes above. The quantity of glucose released from starch by the enzyme dilution is then determined.

Calculation:

$$\text{Units/ml} = \frac{\text{mg glucose released}}{\text{Time in minutes (10)}} \times \text{Enzyme dilution}$$

4. C.M-cellulase determination for Bioglucanase (Biocon Ltd., Ireland).

Principle:

A buffered suspension of carboxymethyl cellulose is reacted with the cellulase preparation, and glucose units liberated are determined. One unit is defined as that amount of enzyme which liberates 1mg of glucose per minute under the conditions specified. D.N.S. reagent is used to determine the glucose liberated.

Reagents and solutions:

1. Buffered substrate solution:
8.2 g of sodium acetate is dissolved in 50 ml water and the pH adjusted to 5.0. 1g carboxymethyl cellulose (low viscosity; Sigma London Ltd) is dissolved by heating. The solution is then cooled and diluted to 100 ml.

2. D.N.S. solution:
This is made as described for amyloglucosidase assay, 7.3., above.

3. Glucose standards:
These are made as described for the amyglucosidase assay, also.

Procedure:

1 ml aliquots of buffered substrate are dispensed into a series of test tubes and equilibrated to 40°C. Enzyme samples are diluted in water so that glucose released by the reaction does not exceed 1.0 mg (<10u/ml). 1 ml of enzyme dilution is then added to the buffered substrate and the reaction is incubated for 10 minutes at 40°C. It is terminated by addition of 2 ml D.N.S. solution. The tubes are then placed in a boiling water bath for 5 minutes, cooled, and 10 ml of water added to each tube. The tubes are mixed and absorbance at 540 nm measured on a Pye-Unicam SP6-550 spectrophotometer. An enzyme blank

is prepared by adding 2 ml D.N.S. solution to 1 ml of substrate, adding 1 ml of the enzyme dilution and then treating as for enzyme test above. A glucose standard curve is prepared by adding 2 ml standard solutions to 2 ml D.N.S. and reacting as above. The amount of glucose released due to enzyme activity is determined from this standard curve.

Calculation:

$$\text{Units/ml} = \frac{\text{mg glucose released} \times \text{Enzyme dilution}}{\text{Time in minutes (10)}}$$

Note: To determine sodium alginase activity the above procedure is used, using 5 g/l sodium alginate as substrate.

7. APPENDIX II.

LIPASE DILUTION BUFFERS.

In experiments in which lipase preparations were maintained at constant pH for incubation, the following buffers were used (Section 3.6).

1. Tris (hydroxymethyl) aminomethane-HCl (Tris-HCl) buffer - pH 7 and 8 (0.1M).

A 0.2M solution of Tris reagent is prepared by dissolving 24.2 g in water, and diluting to 1 litre. 25 ml is dispensed into a suitable beaker and the pH adjusted to 7.0 or 8.0 by addition of an appropriate volume of 0.2M HCl. The solution is then diluted to 100 ml.

2. Sodium citrate - citric acid buffer, pH 3 - 6 (0.1M).

A 0.2M solution of citric acid is prepared by dissolving 38.42 g in water and diluting to 1 litre. Sodium citrate, 58.82 g, is dissolved and diluted to 1 litre to make a 0.2M solution.

These solutions are mixed in the following proportions and diluted to 100 ml:

Buffer pH	Citric acid solution (ml)	Sodium citrate solution (ml)
3.0	46.5	3.5
4.0	33.0	17.0
5.0	20.5	29.5
6.0	9.5	40.5

7. APPENDIX III.

Research Communications.

Meetings.

"Growth characteristics of *Rhizopus arrhizus* in submerged culture".
Paper delivered to the Society for General Microbiology (Irish Branch) meeting, December 1982, at University College, Dublin.

"Studies on growth of *Rhizopus arrhizus* in submerged culture in filamentous and pelleted forms".
Poster presented at the Society for General Microbiology meeting, April 1984, at Warwick University, England.

"Effect of polymers on growth of *Rhizopus arrhizus* in complex and defined media".
Poster presented at the Society for General Microbiology meeting, April 1985, at Warwick University, England.

In addition, active participation was taken in seminar sessions at the N.I.H.E., in which different aspects of the project work were presented and discussed.