THE PRODUCTION OF SINGLE CELL PROTEIN FROM PIGGERY SLURRY

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being a thesis submitted for the degree of Doctor of Philosophy in The University of Glasgow, November 1980

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ACKNOWLEDGEMENTS

Acknowledgement is made to the Principal and Governors of The West of Scotland Agricultural College for supporting this investigation.

I am greatly indebted to Dr J Bruce for his supervision, guidance and constant enthusiasm throughout this study.

I am most grateful to Dr S Baines for providing laboratory facilities in the Microbiology Department, and for his advice during the compilation of this thesis.

My thanks are also extended to:-
Colleagues in the Microbiology Department for their technical assistance, in particular Mr P Simpson;
Dr J Dixon of the Chemistry Department for the analysis of heavy metals;
Dr W Manson of The Hannah Research Institute for performing the amino acid analysis;
Mrs S Clark for the high standard of typing.

Finally, a special debt is due to my husband for his moral and editorial support.

SUMMARY

The aim of this project is to produce from an agricultural waste, namely pig slurry, a source of protein which is suitable for feeding to animals. It is considered important that the end-product is non-toxic, free from pathogens, of a high nutritional quality and can be produced in such a way that its composition does not vary.

Analysis showed that the major carbohydrate components of pig slurry are cellulose and hemicellulose, which constitute approximately 17.5% and 22.5% of the dry weight of the slurry respectively. In this form the carbohydrate is not readily degraded by micro-organisms, and in order to overcome this problem it was decided to hydrolyse the cellulosic material with dilute sulphuric acid (H_2SO_4). Temperatures of 80°C, 90°C and 100°C and acid concentrations of between 1% and 10% w/v were investigated.

Thin-layer and paper chromatography of the liquid produced by mild H_2SO_4 hydrolysis of pig slurry indicated that xylose, arabinose, glucose, galactose and the uronic acids of glucose and galactose are released in detectable amounts. By using quantitative High Pressure Liquid Chromatographic (HPLC) analysis of the monosaccharides released by acid treatment, combined with a quantitative assessment of the residual cellulosic fractions, it was possible to optimise the hydrolysis conditions for maximum monomer release. Mild acid hydrolysis causes degradation of the hemicellulosic fraction of slurry, and produces liquid hydrolysates which contain mainly pentose sugar and large amounts of ammonium nitrogen. The α-cellulose fraction remains almost intact in the solid residue. Three hydrolysis systems were adopted for further growth work with yeast species. These were 1% H_2SO_4 at 90°C for 56 h, 1% acid at 100°C for 24 h and 5% acid at 90°C for 3 h.

A number of yeast species were selected for their ability to grow on vitamin-free media, with ammonia as a nitrogen source, and xylose and arabinose as carbon sources. Initial experiments indicated that the hydrolysate had an inhibitory effect on yeast growth. Extensive investigation into the source and nature of this inhibition showed that an aromatic phenolic breakdown product of carbohydrates was responsible for this phenomenon. It was further shown that the inhibitory effect could be overcome by dilution of the hydrolysate or treatment with activated charcoal. Of all the

yeasts tested, the growth of a species, later identified as *Candida tropicalis*, was least affected by the presence of this inhibitory compound. This yeast species was therefore used in all subsequent growth trials with diluted or charcoal-treated hydrolysate. The highest yield of yeast biomass was obtained on a diluted hydrolysate produced with 5% acid at 90°C for 3 h. The yield was such that 9 g of dry biomass were produced from each litre of raw slurry hydrolysed. The slurry used throughout this work had a solids concentration of 110 $g.l^{-1}$.

The liquid remaining after yeast growth contains a large amount of residual ammonia. It is therefore possible to add this liquid back to the solid cellulosic residue of hydrolysis as a source of nitrogen for the growth of the cellulolytic, white-rot fungus, *Sporotrichum pulverulentum*. The cellulosic constituents of the solid residues produced by the hydrolysis systems employing 1% acid were demonstrated to be only sparingly available to *Sporotrichum pulverulentum*. Treatment of pig slurry with 5% acid at 90°C for 3 h, however, disrupts the structure of the α-cellulose sufficiently to allow extensive fungal attack. Growth trials indicated that for every litre of raw slurry hydrolysed 4.6 g of fungus were produced. Finally, a process was tested in which the yeast growth stage was omitted and the fungus grown on the unseparated liquid and solid residues of hydrolysis. In fermenter growth trials yields of fungal biomass of almost 20 $g.l^{-1}$ of raw slurry were possible using this system.

Amino acid analyses of the products of microbial growth on the liquid and solid residues from acid hydrolysis of pig slurry indicated that they are potentially useful as feed supplements for pigs. It was also found that the ultimate liquid residue from microbial growth still contained significant quantities of ammonia. It is therefore considered that the liquid residue may be usefully sprayed on agricultural land.

GENERAL INTRODUCTION

Throughout history animal wastes have been recycled through the soil environment with a minimum of direct release to the water environment. The change to intensive livestock production has weakened this complimentary relationship between crop and animal husbandry. It has thus become necessary to develop methods of treating the high organic matter and nutrient loads of animal excreta, in order to prevent widespread pollution of streams, lakes and other aspects of the environment.

The method adopted for animal waste management varies depending upon the type of animal, location of production, management ability and financial resources. The proceedings of several recent symposia have extensively covered the approaches of agriculturalists to waste disposal (Proceedings of the Cornell Agricultural Waste Management Conference, 1972; Proceedings of the Third International Symposium on Livestock Wastes, 1975; Proceedings of the Eighth International Conference of the International Association on Water Pollution Research, 1977). Basically it appears that the methods suitable for the disposal and stabilisation of animal wastes, other than direct application to land surfaces, are governed by the volume of the waste to be handled, and the cost required to do so.

Recently more attention has been focused on the utilisation of animal excreta (Smith, 1973). In effect, the waste is no longer a worthless pollution hazard, but a raw material; a potential resource which can be used to advantage. Numerous studies have emphasised the possibilities of producing useful fuels from organic wastes such as animal manures. The three major methods used to convert wastes to fuel are hydrogenation, pyrolysis and bio-conversion by the growth of anaerobic microorganisms. (Hobson & Robertson, 1977; Jewell & Loehr, 1977; Maugh, 1972).

Studies by animal nutritionists have been concerned with the nutritive value of animal wastes as animal feed (Smith, 1973). The nitrogen in livestock excreta can originate from undigested food, metabolic by-products or bacterial sources. Smith (1973) reports that 80% of animal waste nitrogen is present as non-protein nitrogen (NPN). Non-ruminant animals are reported to be unable to utilise NPN efficiently (Lewis, 1972), on the other hand rumen microflora are capable of transforming NPN to amino nitrogen, which can then undergo gastric digestion.

(Bradfield, 1974; Chalupa, 1972). Rumen microflora are also capable of using cellulose and hemicellulose as carbon sources, thus providing the host animal with a source of energy. It has therefore been concluded that in general animal excreta could provide more nutrition for ruminants, than for non-ruminant animals (Pidgen & Heaney, 1969).

Numerous experiments have been conducted to evaluate various livestock excreta in the nutrition of different animals (Anthony, 1970 and 1971; El Sabban *et al*, 1970; Thomas *et al*, 1972). Both pig and poultry wastes have been added to ruminant and non-ruminant rations. It has been reported that aerobically sustained pig excreta which contains 27.7% protein can replace up to half the protein of casein or soya bean meal in the diets of rats and pigs, and support similar weight gains (Harmon *et al*, 1972). In the growth trials using pig waste with a 22% protein content however, Eggum & Christensen (1974) have observed negative effects on weight gain when 25% or more of the diet is pig excrement. Pig faeces fed to cattle and sheep has been found to have an apparent dry matter digestibility of only 30% (Hilliard, 1977). These results suggest that pig excreta is not suitable for direct refeeding. In fact, based on nutritive value and freedom from medicants, poultry waste is considered the most realistic prospect for this purpose (Smith, 1973).

The potential hazards of feeding raw untreated animal wastes are numerous. Copper toxicity in sheep fed broiler litter containing high levels of copper (Fontenot & Webb, 1974) and in sheep fed pig faeces which also has a high copper content (Stanogias & Hendrosoerkaryo, 1977) are documented cases of the refeeding of excreta being deleterious to animal health. The presence of pathogenic microorganisms, protozoan parasites, harmful drug residues, pesticides and heavy metals (Fontenot & Webb, 1975), and the accumulation of undigestible minerals, peptidoglycan and ligno-cellulose, in closed recycling systems is also a source of concern (Smith, 1973). Significant amounts of urinary steroids have been reported in the excreta of poultry (Mather & Common, 1969), and this could limit the recycling of poultry waste to reproductive livestock.

Although the simple method of direct recycling of animal wastes has received much attention, effort has also concentrated on fermentation techniques which can convert the nitrogen of excreta into products of higher nutritive value for livestock feeding (Smith, 1973). Several

species of fungi and *Streptomyces* have been successfully cultivated on the nitrogenous and carbonaceous components of the filtrates of cattle feedlot waste. The addition of a carbon source, such as whey, significantly increases the biomass yield (Weiner & Rhodes, 1974). In a process in which the filtrate of cattle feedlot waste is combined with coarsely cracked corn, the yeasts and lactic acid bacteria of the natural flora are encouraged to grow in a silage-type fermentation (Hrubant, 1975; Weiner, 1977). Singh & Anthony (1968) have investigated the growth of the yeast *Saccharomyces cerevesiae* on cattle waste filtrate in which the available carbon is increased by extracting the fibrous portion of the excreta with a 3% solution of steam heated acid.

In a patented procedure (International Farm Systems, 1974) pig excreta has been converted to a feed supplement by passing a gas mixture containing oxygen through a slurry of pig waste. The conversion process is initiated by the addition of a supplementary carbon source and the innoculation of the yeast *Candida utilis*. Even the growth of an alga *Chlorella pyrenoidosa* has been investigated as a method of reducing the polluting potential of pig waste, while producing a valuable by-product. However, the fact that the slurry must be exposed to sunlight in thin layers, combined with the reliance of this system on outdoor temperatures for incubation are major drawbacks in our temperate climate (Wilson & Houghton, 1974).

This concept of converting wastes into products of higher nutritive value has been applied to many other industrial and agricultural wastes. Attention has been focused on the need for maximum utilisation of all available resources by the many recent symposia, books, articles and reports on the world food situation. Many proposals have been made for increasing food supplies. These include improvement of land utilisation, irrigation of arid areas, use of fertilisers, control of population, distribution of surpluses, economic aid to developing countries and elimination of waste (Coppock, 1970; Kharbas & Salunkhe, 1972; Woodham, 1971).

Increase in yields by conventional methods of food production have been most noticeable in calorie rich crops. This has meant that man has constantly pushed the carbon:nitrogen ratio in his diet towards relatively higher values for carbon (Borgstrom, 1964). The quality of protein sources has only recently received serious consideration, and microbiology is seen as a means of redressing the protein calorie

balance. The use of algae, bacteria, fungi and yeasts as sources of food or feed for animals has been recognised for at least sixty years, but the potential of microorganisms as sources of nutrition is still to be fully realised.

One great advantage of microorganisms as sources of protein, compared with animals and plants, arises from their ability to produce nutrients quickly and efficiently from relatively simple media. Bacteria and yeasts, under the most favourable growth conditions, have generation times of between 0.5-2 and 1-3 h, respectively, while algae and filamentous fungi can double their masses in 2-6 and 4-13 h, respectively (Kihlberg, 1972).

The importance of microorganisms in nutrition stems from the discovery that microbial cell matter contains significant quantities of carbohydrates and lipids, and is especially rich in most B-vitamins and protein. Compared to other protein sources such as cereals and livestock, the relative simplicity of genetic modification and the short generation times of microorganisms enables strains of high nutritional quality to be developed quickly for specific cultural conditions. The independence of most microbial cultivation from climatic conditions, and the fact that the process requires relatively small areas of land and small quantities of water, confers advantages on this type of nutrient production (Kihlberg, 1972).

Since an early review by Thatcher (1954), several comprehensive reviews on Single Cell Protein (SCP) have appeared in the literature. In particular, the Proceedings of the International Symposium on Single Cell Protein (Davis, 1974) and the Symposium on Food from Waste (Birch *et al*, 1976). Many authorative articles on the subject have also been published (Dabbah, 1970; Enebo, 1970; Gaden, 1974; Ghose, 1969a; Humphrey, 1969; Kihlberg, 1972; Loftas, 1969; Marth, 1971; Rogers, 1978; Snyder, 1970; Worgan, 1973, 1974 and 1977). In assessing the types of microorganisms suitable for SCP production, many factors must be considered, including the composition of the organism, the cultural conditions, the behaviour of the organism during culturing and later processing, and its general acceptibility as a food or feed (Enebo, 1970).

Algae have a distinct advantage over non-photosynthetic heterotrophs in that they can fix atmospheric carbon dioxide. In fact algae cannot be produced economically on an autotrophic basis using conventional SCP production techniques. One of the major problems is the provision of

uniformly high light intensities in all portions of the culture medium during growth to obtain maximum yields (Priestley, 1976). In Japan the major producer of algae as a source of vitamins and growth factors for lactobacilli and green-colouring matter for fermented drinks, uses heterotrophic cultivation on glucose containing media rather than photosynthetic growth (Enebo, 1969).

Technologically feasible algal culture techniques have been developed whereby algae such as *Chlorella* species or *Scenedesmus* species can produce clear re-usable water from municipal sewage or waste water (McGarry, 1971; Oswald, 1969; Pipes & Gotaas, 1960). This type of system constitutes a significant contribution to the problem of uncontrolled waste discharges as it renders the water potable, while providing a nutritious by-product.

Nutritionally algae have a favourable composition however, the cells must be fractionated in order to render the proteins directly available for non-ruminant or human consumption. Mitsuda *et al* (1969) have concluded that fresh, dried or solvent extracted (to remove lipids and pigments) *Chlorella* species is poorly digested by humans, whereas the isolated algal protein is readily utilised. Even *Spirulina maxima*, which has been the staple food of the people in some parts of Mexico for thousands of years, has been shown to be nutritionally poor (Clement *et al*, 1967).

In recent years bacteria which can grow on methane, including *Methanococcus* and *Pseudomonas* species, have been subject to considerable investigation. Clearly the reason for this interest is to be found in the availability of vast amounts of natural gas and the low cost of this material (Bewersdorff and Dostalek, 1971; Coty, 1969; Sheehan & Johnston, 1971; Vary & Johnson, 1967; Whitenbury, 1969; Wolnak *et al*, 1967). Studies on methane-oxidising bacteria have indicated that yields of as much as 75 grams (g) dry weight for 100 g of metabolised substrate can be achieved (Senez, 1972).

Technologically mass cultivation of bacteria raises a variety of problems. In view of their small size, the bacterial cells can only be harvested by centrifugation at very high speeds. The pH of cultivation, which is normally near neutrality, means that bacterial cultures are readily contaminated by other microorganisms and bacteriophage. In addition to these general difficulties, methane-oxidising bacteria present specific problems with regard to high aeration

requirements, and the fact that a combustible mixture is fed into the fermenter.

In order to circumvent the difficulties involved in the direct conversion of methane to SCP, it has been proposed that methane should be chemically oxidised to methanol prior to bacterial cultivation (Senez, 1972). This latter compound has the two-fold advantage of being easier to handle because of its solubility in water, and of being a growth substrate for a wide variety of bacteria (Cooney & Levine, 1972; Foo, 1978, Gow *et al*, 1975; MacLennan *et al*, 1973) and some yeasts (Cooney & Levine, 1975; Levine & Cooney, 1973; Volfova *et al*, 1974). The isolation of a thermotolerant methanol-utilising yeast has been reported. This organism has the advantages of less expensive fermenter cooling, and the low pH and high temperature of cultivation render it less subject to contamination (Levine & Cooney, 1973).

In spite of the fact that bacteria tend to have a slightly higher protein content than yeasts, they are considered to be less attractive as sources of SCP. This is mainly due to the intrinsically higher nucleic acid content of bacteria, and the difficulty of recovering the smaller bacterial cells.

More attention has been focused on yeasts than any other group of heterotrophic microorganisms as sources of microbial food. The main carbohydrate substrate for the food and feed yeasts currently being produced is molasses, the waste product of sugar refining. Sulphite waste liquor produced in the manufacture of wood pulp has been widely used for yeast production in both Europe and North America. However, the recent changes from the sulphite wood pulping system to the sulphate or Kraft process has led to closure of many of the yeast production facilities (Sobkowicz, 1976).

Whey, a by-product of cheese making, is another potentially attractive material for microbial protein production, as it contains 4-5% lactose (Marth, 1970; Muller, 1969). Yeasts such as *Saccharomyces fragilis* (Wasserman, 1960a and 1960b), *Trichosporon cutaneum* (Atkin *et al*, 1963), *Torula cremoris* (Graham *et al*, 1953), *Kluyveromyces fragilis* (Hernandez *et al*, 1978) and *Rhodotorula gracilis* (Mickle *et al*, 1974) have been successfully cultivated on whey. Commercial production of SCP from whey in France and America has recently been reported (Kosikowski, 1979).

Many other carbohydrate containing materials, especially those which result from agricultural and food processing operations, have been investigated as food or feed yeast substrates. The yeast

normally chosen for growth of these carbohydrates wastes is *Candida utilis*. The advantages of this yeast are its ability to utilise ammonium and nitrate nitrogen, to assimilate pentose and hexose sugars, and the fact that it is universally accepted as feed for animals. *Candida utilis* has been cultivated successfully on the effluent from the wet carbonisation of peat (Kuster, 1969; McLoughlin, 1971; McLoughlin & Kuster, 1972a, 1972b and 1972c), sauerkraut waste (Hang *et al*, 1972, 1973a and 1973b), corn steep liquor (Salah Foda *et al*, 1973), wood hydrolysate (Harris *et al*, 1951) and coffee bean pulp juice (Staudinger, 1968).

Other yeasts have been investigated as sources of SCP. *Geotrichum candidum* has been grown on sauerkraut waste (Hang *et al*, 1974), *Candida scottii* on plant hydrolysate medium (Semushina *et al*, 1974), *Candida tropicalis* on straw hydrolysate (Volfova & Kyslikova, 1979) and *Saccharomyces fragilis* on coconut water waste (Smith & Bull, 1976a and 1976b) and lemonade-processing waste water (Hang, 1980).

A commercial plant has been operating in Sweden since 1974 in which the solid and liquid wastes of potato products are converted to SCP. In this process, which has been given the name "Symba", symbiotic culture of two yeasts, *Endomycopsis fibuliger* and *Candida utilis* is employed. The amylases produced by *Endomycopsis fibuliger* break down the starch to glucose, which is readily utilised by the faster growing *Candida utilis*. The product is claimed to be 90% *Candida utilis* (Skogman, 1976).

In the early 1960's the Societe Francaise de Petrole BP, while conducting research into the dewaxing of petroleum fractions, struck on the possibility of cultivating feed yeasts on hydrocarbons (Champagnat *et al*, 1963). Since then numerous patents have been issued for the production of SCP from petroleum, and countries in both East and West have constructed factories (Dimmling & Seipenbusch, 1978). Processes employing both yeasts and bacteria have been developed (Wang, 1968; Humphrey, 1967).

The production of SCP from hydrocarbons has prompted a great deal of microbiological and biochemical work on the mechanisms of substrate utilisation (Gradova *et al*, 1969a; Golobov *et al*, 1969; Klug & Markovetz, 1971; Lebault & Azoulay, 1971; Nishio & Kamikubo, 1971). Special interest has been shown in the effect hydrocarbon substrates have on the lipid composition of microbial cells (Alentyva *et al*, 1969; Dunlap & Perry, 1968; Gradova *et al*, 1969b; Mishina *et al*, 1973).

In order to produce SCP from hydrocarbons on a commercial scale, two major technological difficulties have to be overcome. Oxygen requirements are 2.5 to 3 times that of yeast grown on carbohydrates, and this inevitably creates problems of mass transfer efficiency. Considerable heat is produced during microbial growth on hydrocarbons, and this necessitates mechanical refrigeration to maintain an optimum temperature level (Wang, 1968). The use of thermophilic microorganisms could alleviate the cooling problem, and may help to maintain aseptic conditions (Klug & Markovetz, 1967; Matales *et al*, 1967). The 1973 and subsequent oil crises increased the price of petro-chemical raw materials, halted the construction of several proposed SCP plants and caused the closure of others.

Fungi have been reported to be more slow growing and to yield biomass with a lower protein content than bacteria and yeasts (Worgan, 1974). Recently, however, several fungal species have been found to grow as rapidly and give protein yields equivalent to those of food yeasts. Fungi are capable of utilising a wide range of organic substrates. They also have the advantage that they are normally harvested by simple filtration methods which are not applicable to bacteria and yeasts.

Aspergillus oryzae, Fusarium semitectum and *Trichoderma viride* have been grown on citrus pulp, soya bean and cheese wheys, olive and palm oil waste liquors and starch processing effluents. In several of these examples, the reduction of the Biological Oxygen Demand (BOD) of the waste is an essential part of the process (Worgan, 1976).

Several industrial processes have been established to produce fungal protein from carbohydrate waste materials. Finland's paper industry has been producing pilot quantities of Pekilo protein from sulphite waste liquor since 1971. The microorganism employed in the process is a mycelial fungus of the genus *Paecilomyces*. The crude protein content of the product is reported to be 60% (Romantschuk & Lehtomaki, 1978). A simple robust procedure has been developed by Tate & Lyle for the production of fungal protein from the sugars extracted from carob pods or papaya cullage. The microorganisms include *Aspergillus niger* and a *Fusarium* species. The cultivation is not undertaken aseptically, as it is considered that the organism has significant competitive advantages over potential contaminants

(Imrie & Vlitos, 1975; Sekeri-Pataryas *et al*, 1973; Tate & Lyle Ltd, 1975). Since 1967, Rank Hovis McDougall have been working on the production of microfungal protein from carbohydrate wastes (Spicer, 1970 and 1973). They have concluded that the microfungal protein of *Fusarium graminearum* grown on carbohydrate wastes is a potentially safe and nutritious food for humans (Duthie, 1975). The monosaccharide and disaccharide breakdown products of the cellulosic components of vegetable materials (Worgan, 1978) and sawdust (Fuska & Kollarova, 1977) have also been successfully used as carbon sources for the production of microfungal protein.

The use of homogenised mycelium for inoculation, and the application of submerged culture techniques for growth, has made the commercial production of the higher fungi feasible. These micro-organisms are rich in protein and B group vitamins. This topic has been extensively reviewed by Worgan (1968). Both *Morchella* species and *Agaricus* species have been grown on food processing wastes, sulphite waste liquors and molasses (Block *et al*, 1953; Litchfield, 1964; Reusser *et al*, 1958).

Although in principle all types of bacteria, fungi and algae can be utilised for the production of SCP, nutritional value and safety of the product as food or feed are important criteria for the selection of suitable microbial species. Primarily the amino acid pattern of a protein determines its value as a source of nitrogen for the growth and maintenance of animals and humans. The Food and Agriculture Organication (FAO) and World Health Organisation (WHO) have produced definitions and standards for protein and reference amino acid requirements (FAO/WHO, 1973). Table 1 shows the essential amino acid pattern of selected SCP sources and some conventional protein sources as compared to the FAO reference amino acid pattern. In general, sources of SCP are deficient in methionine and cystine but they have fairly large quantities of lysine, an amino acid which tends to be lacking in cereals (Tsien *et al*, 1957).

Chemical analysis of the amino acid composition of SCP sources, however, does not always reflect the pattern of physiologically available amino acids. The cell wall prevents maximum utilisation of cytoplasmic protein. Methods of isolating the protein from other cellular components have been investigated. Ball-milling (Hedenskog, *et al*, 1970), homogenisation (Tannenbaum & Miller, 1967) and solvent

TABLE 1 The essential amino acid patterns of *Candida tropicalis*, "Symba" yeast, soya bean meal and fish meal as compared to the FAO/WHO reference pattern of essential amino acids (1973).

Amino acid	*Candida tropicalis*[a] grown on n-alkanes	"Symba" yeast grown on potato waste[b]	Fish meal[a]	Soya bean[a] meal	FAO/WHO pattern
Isoleucine	5.3	4.3	4.8	4.8	4.0
Leucine	7.8	7.5	7.5	6.1	7.0
Phenylalanine	2.8	5.4	4.4	4.9	6.0
Tyrosine	4.0	4.8	3.5	3.5	
Threonine	5.4	5.4	4.5	4.0	4.0
Tryptophan	1.3	1.3	1.3	1.3	1.0
Valine	5.8	4.2	5.6	5.0	5.0
Lysine	7.8	6.3	7.4	6.1	5.5
Cystine + Methionine	2.5	2.5	3.9	2.9	3.5

a. Taken from Senez (1972)

b. Taken from Skogman (1976)

extraction (Fijimak *et al*, 1973) have been employed for this purpose. Even simple heating increases the permeability of yeast cell walls to digestive enzymes (Hedenskog, 1978). There has also been research into the production of a genetic algal mutant lacking a cell wall (Davies, 1973), and a novel urea-soaking method for wall removal has been reported (Mitsuda *et al*, 1969).

Determination of the nutritional value of SCP by chemical analysis must be complemented by other methods, such as animal assay. Nutritional trials have been reported for yeasts (Yanez *et al*, 1972), bacteria (Kaufman *et al*, 1957), algae (Omstedt *et al*, 1973) and fungi (Duthie, 1975). The recent interest in hydrocarbon grown yeasts has led to a large volume of work being presented on the nutritional quality of these products. Feeding trials using chickens (Weerden *et al*, 1970; Woodham & Deans, 1973), calves (Shacklady & Gatumel, 1972) and pigs (Shacklady, 1969 and 1970) have indicated that hydrocarbon grown yeast is a suitable protein source for any species of animal (Shacklady, 1975).

It has long been recognised that due to their high purine content, the use of microorganisms as sources of protein for human consumption greatly increases the amount of uric acid in the bloodstream. This compound has a low solubility at the pH of body fluids, and is relatively poorly excreted by the kidneys. Elevation of blood uric acid content may lead to the formation of crystals in the joints, and with excessive renal clearance loads, stones may be deposited in the urinary tract (Waslien *et al*, 1968 and 1970).

Based mainly on information obtained from studies using graded ribonucleic acid (RNA) feeding, a maximum intake of 2 g per day is suggested for most normal human subjects (Edozien *et al*, 1970; Waslien *et al*, 1970). The RNA content can, where necessary, be reduced to a level which would make all SCP sources acceptable in human nutrition. Various methods for the reduction of the nucleic acid content of microorganisms have been described (Sinskey & Tannenbaum, 1975). When SCP is used as animal feed, nucleic acid content is not a problem, because all animals apart from the higher apes have the enzyme uricase, which catalyses the oxidation of uric acid to the more soluble allantoin (Edozien *et al*, 1970).

The establishment of the nutritional value of an SCP product has to be complemented by conclusive evidence that it is toxicologically safe. Several areas of production can lead to toxicity of the final product. Novel protein sources are often derived from groups of

organisms which harbour toxin-producing species (deGroot, 1974). There is a constant danger that the cells may accumulate harmful compounds derived from the substrate. This is particularly important when the substrate is unrefined hydrocarbons, and solvent extraction of the cells is often necessary (Gaskell, 1969; Walker, 1972). SCP materials are often submitted to treatment by heat, solvents or alkali, each of which may impart undesirable or even toxic properties to the resulting product (de Groot, 1974). It is therefore necessary to conduct extensive toxicological trials to evaluate the safety of SCP before it is used as a food or feed.

Extensive toxicity trails have been reported for hydrocarbon grown yeasts, and these have indicated their safety as an animal feed supplements (Engel, 1972; de Groot *et al*, 1970a, 1970b and 1971). The results of initial work into the safety of a micro-fungal product from *Fusarium graminearum* has indicated its utility for human consumption (Duthie, 1975). It has been suggested that the extent to which a toxicological evaluation has to be applied depends on the degree of novelty of the SCP product (van der Wal, 1976).

In order to standardise the nutritional and toxicological testing of SCP on an international basis, guidelines have been produced by the Protein and Calorie Advisory Group of the United Nations. The most notable of these are the PAG Guideline No 6 (1974) on the requirements for preclinical testing, PAG Guideline No 7 (1974) on food specifications and human testing and PAG Guideline No 15 (1974) on animal feeds.

It must not be overlooked that apart from containing a large quantity of protein, SCP is a rich source of B group vitamins, and can provide a large amount of dietary lipid. With increasing technological developments, it now becomes practicable to consider the production of a "single cell oil". Most substrates currently used for SCP production, including hydrocarbons, starch and lactose, can be used by fat accumulating yeasts and fungi such as *Lipomyces starkeyi*, *Candida lipolytica* and *Aspergillus terreus* (Ratledge, 1976; Whitworth and Ratledge, 1974).

Several processes for the production of SCP have been operated commercially and, as indicated previously, in recent years many plants utilising hydrocarbon substrates have been built. However, the knowledge that world supplies of fossil fuels have been severely depleted, has focused scientific interest on cellulose, which is the most abundant natural carbon source (Birch *et al*, 1976; Gaden

et al, 1976; Wilke, 1975).

Less than a quarter of current agricultural production is actually consumed as food or feed (Worgan, 1973). The quantity of cellulose involved in crop residues combined with the waste of forest industries has inspired various attempts at cellulose conversion. The most popular solution is to convert cellulosic wastes to food, or to upgrade their value as livestock feed (Han *et al*, 1971; Worgan, 1976). Another more fundamental approach is to regard the whole plant as a source of food or feed raw materials. This concept has been exploited by Pirie (1976) in the extraction of leaf protein accompanied by the microbial conversion of the remainder of the crop to SCP.

Cellulose is a carbohydrate polymer that occurs in the primary and secondary cell walls of plants. In the main the cellulose of agricultural and forestry by-products is intimately associated with the complex aromatic polymer lignin, and the pentose polymer hemicellulose (Gascoigne & Gascoigne, 1960). This ligno-cellulosic complex is resistant to rapid degradation by microorganisms due to the protective effect of the lignin polymer and the crystalline nature of the cellulose (Cowling, 1975).

Cellulose is a polymer, molecular weight 300,000-500,000, composed of glucose units linked by β 1-4 glycosidic bonds. It occurs naturally in close association with other polysaccharides. The polymeric matrix normally consists of highly ordered crystalline regions and more random amorphous areas (Gascoigne & Gascoigne, 1960). In crystalline cellulose the molecules have a thread-like arrangement, existing as fibrils, long bundles of molecules stabilised laterally by hydrogen bonding between hydroxyl groups of adjacent molecules. Molecular arrangement in the fibrillar bundles is so regular that cellulose has a crystalline X-ray diffraction pattern. The consequence of this high degree of order in native cellulose is that neither water nor enzymes can enter the structure (Reese *et al*, 1972).

Hemicelluloses appear to function as reserve materials in kernels and seeds, or as structural materials in seeds, fruits, husks, straw and wood. The structural hemicelluloses yield mainly pentoses on hydrolysis, of which xylose and to a lesser extent arabinose are most common. Some hemicelluloses contain uronic acid groups rather than neutral sugars as the dominant component (Gascoigne & Gascoigne, 1960; Adams, 1965). Hemicelluloses form a matrix around the cellulose and penetrate the spaces between the cellulose molecules in the

amorphous regions (Cowling, 1975).

Lignin surrounds cellulosic substances in a three-dimensional network (Bellamy, 1974); it is a complex aromatic polymer, probably very polydisperse *in situ*, but of extremely high molecular weight. It is formed by the polymerisation of oxidatively formed radicals of p-hydroxy cinnamyl alcohols, and is thus a polymer of phenyl-propane units (Kirk, 1975).

The biological utilisation of cellulose depends on its conversion to glucose, which is in turn converted to other useful products. In single conversion reactions such as acid or enzymic hydrolysis the monomer accumulates in large amounts. Many microorganisms, however, can utilise cellulose as a sole carbon source, in which case the production of glucose is one of a series of reactions and the glucose is a transitory intermediate which never accumulates, and is often scarcely detectable. Microorganisms capable of this reaction include some filamentous fungi, some aerobic bacteria from the soil, some anaerobic bacteria from the rumen and sewage, some actinomycetes from compost and wood rotting basidiomycetes (Reese *et al*, 1972).

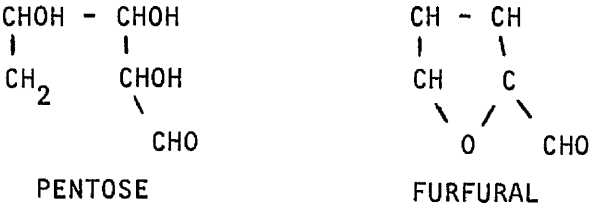
Acid hydrolysis of polysaccharides results in the formation of constituent monosaccharide units. Partial acid hydrolysis yields di-, tri- and oligo-saccharides. Care is required under acid conditions, because it is possible for the liberated monomers to recombine in a manner different from that in the original polymer. Extremely dilute aqueous solutions of acids catalyse interchange between various anomeric and ring forms of the monosaccharide. In addition to the formation of 1,6-anhydro compounds the action of hot aqueous acids on aldoses can yield di-, tri- and oligo-saccharides by elimination of water between anomeric hydroxyl groups (Guthrie & Honeyman, 1964).

The chemical action of acids on cellulose is the catalysis of the cleavage of glycosidic bonds, leading to a reduction in molecular weight, production of reducing sugars, and a rapid fall in tensile strength. There is evidence to suggest that the reaction is confined to the amorphous regions of the cellulose matrix, and that hydrolysis does not occur to any significant extent on the surfaces of the crystals, even although they are accessible (Mann, 1963). A 50% weight/weight (w/w) concentration of sulphuric acid (H_2SO_4) causes swelling of the cellulose matrix, while above a concentration of 78% w/w carbonisation and solution occur (Gascoigne & Gascoigne, 1960).

Hemicelluloses such as xylan are hydrolysed by dilute H_2SO_4,

which can then dehydrate the liberated pentose (Figure 1).

Figure 1 Dehydration of pentose to furfural taken from Fieser & Fieser, 1956.



The wide variety of naturally occurring polysaccharides containing glycosidic bonds of varying acid liabilities has precluded an overall adequate procedure being developed for their complete acid hydrolysis. Reasonably satisfactory hydrolytic conditions may be devised for several general types of polysaccharides, depending on the composition of individual polysaccharides, ring formation of component monosaccharides and the configuration of glycosidic bonds (i.e. β or α) (Saeman, 1945; Kobayashi, 1971).

On acid hydrolysis, polysaccharides containing uronic acid residues yield neutral sugars, small quantities of uronic acid monomers and comparatively resistant aldobioruonic acids. The latter can only be hydrolysed by such drastic treatment that partial degradation of any pentose will almost certainly result (Kikuchi & Yokotsuka, 1973). In addition, on heating with dilute mineral acids, uronic acids decarboxylate to give decomposition products of unknown composition (Kobayashi, 1971). Stamm (1964) has investigated the hydrolysis of the mixed polysaccharides which occur in woody tissue. He points out that,although concentrated acids at relatively low temperatures cause quite rapid and complete hydrolysis of cellulose with little decomposition of the monomers produced, the difficulty of acid recovery has limited their use.

Most research has focused on hydrolysis in low expendable concentrations of acid catalyst at temperatures of above 100°C. In this work measurements of the rate of hydrolysis of wood with 0.4-1.6% H_2SO_4 at 160-195°C have been tabulated (Stamm, 1964). When cellulosic materials are hydrolysed with dilute H_2SO_4, the monomers formed are subject to decomposition. Stamm (1964) reports

the hydrolysis rate constants and the half-lifes of the five main sugars obtained from wood polysaccharides. These are D-glucose, D-galactose, D-mannose, D-arabinose and D-xylose (see Table 2).

TABLE 2 Decomposition of wood sugars in 0.8 per cent H_2SO_4 (0.08 mole per litre) at 180°C, taken from Stamm, 1964.

Sugar	Hydrolysis rate constant (min^{-1})	Half-life (in min)
D-glucose	0.0242	28.6
D-galactose	0.0263	26.4
D-mannose	0.0358	19.4
D-arabinose	0.0421	16.4
D-xylose	0.0721	9.6

The data presented in Table 2 demonstrates that hydrolysis of wood by H_2SO_4 produces five component sugars, of which xylose monomers are formed fastest and decompose most rapidly. The reverse is true of glucose. Information has also been produced by Stamm (1964) to show that within limits in weak solutions of H_2SO_4, phosphoric acid (H_3PO_4) and hydrochloric acid (HCl) more favourable ratios for the rates of monomer formation to the rates of decomposition are achieved as the acid concentration and the temperature increase. Thus, as Timmel (1957) points out, any quantitative values for monosaccharides produced by acid hydrolysis of polymers must be corrected to take account of monomer degradation during hydrolysis.

Investigations into methods of wood saccharification have provided much valuable information on the mechanisms and rates of hydrolysis of cellulosic compounds. The hydrolysis rate of wood hemicellulose has been shown to be extremely large compared to the rate of cellulose hydrolysis (Saeman, 1945). This means that the hemicellulosic fraction of wood can be broken down by mild acid conditions which will leave the cellulose almost intact. This is termed the pre-hydrolysis stage of the wood saccharification process (Kobayashi & Sakai, 1956). The cellulose/lignin residue from the pre-hydrolysis stage is treated with concentrated H_2SO_4 at temperatures below 100°C, which solubilises the cellulose and hydrolyses it to short chain glucose polymers. The acid is then diluted and the hydrolysis of the soluble glucose polymers takes place in the post-hydrolysis process (Kobayashi & Sakai, 1958a and 1958b).

This procedure has the disadvantages that a large amount of H_2SO_4 is consumed during the main hydrolysis procedure (Sakai, 1965), and that large amounts of dilute acid are produced at the pre-hydrolysis and post-hydrolysis stages. A new wood saccharification process using medium strength acid in the pre-hydrolysis stage has been developed to overcome this problem (Kobayashi *et al*, 1962). In this case the acid impregnated residue from pre-hydrolysis is dried, which concentrates the acid, and effects the main-hydrolysis of cellulose. The recovery of used H_2SO_4 by a dialysis method using an ion-exchange membrane can be carried out successfully from a water extract of the main-hydrolysis products, before or after their post-hydrolysis. This recovered acid is recycled to the pre-hydrolysis stage (Kobayash *et al*, 1962).

Hydrolysis of wood can also be carried out in dilute expendable acid. In order to make reaction times reasonable and yields good, elevated temperatures above 100°C have to be employed. This necessitates the introduction of pressure equipment (Stamm, 1964).

The composition of the cellulosic faction of wood varies considerably with species. In general, hardwoods contain more hemicellulose than softwoods. Thus, the choice of a suitable hydrolysis process is governed by the composition of the cellulosic fractions of the wood, the hydrolysis rates of these fractions and the decomposition rates of the resulting monomers.

Porteus (1974) has investigated a weak acid (1% H_2SO_4) high temperature (230°C) treatment of domestic refuse. This batch isothermal process of 1-2 min duration results in decomposition of 66% of the monosaccharides released. In an industrial situation a steady state plug flow reactor would control reaction time to 20 sec, and thus undegraded sugar yields should increase.

For conditions of enzymic and acid hydrolysis which produce equal losses in the weight of cellulose, the residue from enzymic treatment retains a much higher degree of polymerisation than the residue from acid treatment. This indicates that regions of the cellulose accessible to the large enzymic molecules are rapidly and completely reduced to soluble products. However, when all readily available polymer is dissolved, hydrolysis as evidenced by glucose production continues only at the surfaces of the crystalline cellulose. Acid treatment results in a more extensive breakdown of

the cellulose structure, as the active catalyst can penetrate the crystalline cellulose structure, and randomly hydrolyse the β 1-4, β 1-6, β 1-2 or β 1-3 glycosidic bonds at points which the larger enzyme molecules cannot reach (Ghose & Pathak, 1973; Walseth, 1952). The action of enzymes is much more specific. Cellulase enzymes so far isolated and identified, split the β 1-4 glycosidic bonds of cellulose exclusively. This specificity of cellulases extends even beyond this, for if the glucose units of the chain are substituted or modified in any way the action of the enzyme is impaired (Reese, 1956).

Until recently, enzymic hydrolysis of cellulose was too slow and inefficient to be considered for commercial exploitation, recent developments have changed the outlook. The introduction of methods of substrate pre-treatment and the production of more active enzyme preparations are the two important factors contributing to the success of enzymic cellulose hydrolysis (Reese *et al*, 1972).

A significant contribution in this field has been made by a team led by Mandels at the US Army Natick Laboratories (Gaden *et al*, 1976; Mandels *et al*, 1974). The achievement of this group has been the development of mutant strains of the fungus *Trichoderma viride*, which produce cellulase enzymes of high stability and potency (Mandels *et al*, 1971a; Mandels, 1975).

It is now well established that cellulase is a multi-component enzyme system composed of at least three specific types of enzyme (Wood, 1975). Fourteen amino acids have been isolated from crude cellulase (Pathak & Ghose, 1973). A C1 component initiates the attack on crystalline cellulose, and it is believed to de-aggregate the cellulose chains in preparation for attack by the hydrolytic (CX) enzymes of the cellulase complex. The CX components, which are the β-(1-4) gluconases, can hydrolyse the soluble derivatives of cellulose or swollen or partially degraded cellulose. Highly ordered substrates are not attacked by β-(1-4) gluconases. Cellobiose and cellotriose are the major products of CX hydrolysis. The third type of enzymes are the β-glucosidases which hydrolyse cellobiose or short chain cello - oligosaccharides to glucose.

Most enzyme preparations from culture filtrates of cellulolytic organisms contain CX and β-glucosidase enzymes, but few contain appreciable quantities of C1 enzymes (Wood, 1975). *Trichoderma viride*

is one of the few cellulolytic fungi which has been found to produce significant qualities of C1 cellulase (Mandels, 1975).

It has been found that cellulose adsorbs the cellulase complex, at pH 4-5 and 25-50°C, conditions which are optimum for enzyme action. The cellulose is digested, and the released enzyme is re-adsorbed on excess or freshly added cellulose with retention of activity. Sugars can be separated from the enzyme-cellulose complex by simple filtration or centrifugation (Mandels *et al*, 1971b).

The rate of hydrolysis by *Trichoderma viride* cellulase (Mandels *et al*, 1971b) and *Aspergillus niger* cellulase (Walseth, 1952) is much lower than that predicted by Michaelis-Menton kinetics. From the evidence available it is proposed that a multi-substrate model, with substrate and product inhibition functions, would be the most reasonable explanation of thekinetics of the enzymic hydrolysis of cellulose. This indicates that the reaction rate is mainly limited by the varying degrees of crystallinity within the cellulose structure, and that the concentration of cellulose or the products of hydrolysis have a secondary effect. The nature of the enzyme system used, the water content of the sample and the presence of other substances such as lignin also effect the efficiency of cellulose hydrolysis (Howell & Stuck, 1975).

A survey of the literature reveals that the reactivity of waste cellulose can be modified by a wide variety of physical and chemical treatments (Dunlap, 1969; Gajzago *et al*, 1974; Mandels *et al*, 1974; Millett & Baker, 1975; Millett *et al*, 1976). The two major deterrents to the effective utilisation of ligno-cellulosic residues for chemical, enzymic or microbiological conversion processes are the crystalline structure of cellulose and the presence of lignin. Lignin restricts enzymic and microbial access to the cellulose; whereas cellulose crystallinity affects the rate of all three modes of attack. Where lignin is the major barrier to the effective utilisation of cellulose, delignification is the most universally applicable pre-treatment. This normally involves treatment with chlorine dioxide or sulphur dioxide which is a costly process (Millet & Baker, 1975). Research has shown that a less harsh treatment of partial delignification holds some promise (Millett *et al*, 1976). Methods of decreasing cellulose crystallinity are more widely employed. These include irradiation with gamma rays, steaming (Millett & Baker, 1975), photochemical treatment (Rogers *et al*, 1972) or grinding which decreases particle size and increases surface area (Ghose & Kostick, 1969; Ghose, 1969b; Katz & Reese, 1968). However, the most commonly used method of

increasing the susceptibility of cellulose to enzymic attack is the swelling of the crystalline matrix in aqueous ammonia or sodium hydroxide. Alkaline treatment is widely used to increase the digestibility of ruminant feedstuffs by making the cellulose more available to the organisms of the rumen (Wilson & Pidgen, 1964). Pre-treatments with sodium hydroxide (NaOH) have been used successfully to increase the reducing sugar yields of both enzymic (Gajzago *et al*, 1973) and acid (National Research and Development Corporation, 1971) hydrolysis of cellulosic wastes. Soaking sugar cane bagasse in 4% NaOH at 100°C for 15 min has been shown to increase cellulose utilisation by a *Cellulomonas* species from 29.4% to 73% (Han & Callihan, 1974).

Extensive investigations into the efficiency of enzymic conversion of chemically pre-treated cellulosic waste has been undertaken (Gajzago *et al*, 1973 and 1974; Vamos-Vigyazo *et al*, 1972). A one-step procedure using dilute H_2SO_4 at 100°C for 2 h has been shown to be more efficient with corn cobs than NaOH soaking (Gajzago *et al*, 1973).

While intracrystalline swelling techniques enhance hydrolytic action, their economic feasibility is questionable. By far the most successful methods for effecting drastic alteration of cellulose crystallinity are the physical treatments of electron irradiation and vibratory ball-milling (Millett *et al*, 1976). Ball-milling of newspaper has so far been shown to be the most efficient pre-treatment (Mandels *et al*, 1974). This technique has resulted in 67% saccharification of available cellulose (Brown & Fitzpatrick, 1976). This method is expensive and studies to find new physical and chemical pre-treatment methods are an important priority (Andren *et al*, 1976).

The knowledge that ruminants naturally convert cellulose to human food by the process of anaerobic microbial digestion, has led to the direct use of mesophilic anaerobic microorganisms for the production of SCP from cellulose. The anaerobic attack of cellulose by mixed bacterial cultures produces a mixture of acetic acid, butyric acid, lactic acid, formic acid, ethanol and glucose. These products contain 60-90% of the free energy of cellulose and are all available as carbon sources for the aerobic growth of yeast (Hall, 1965). Cellulase production by a thermophilic *Clostridium* species has been investigated (Lee & Blackburn, 1975).

The use of mesophilic aerobic microorganisms for the bio-conversion of waste cellulosics has been attempted by numerous investigators. Moulds, yeasts, actinomycetes and bacteria have been used. A mixed culture of two unidentified gram +ve rods and one gram -ve rod have been found to grow on waste paper (Paredes-Lopez and Gonzales, 1973). This type of process, in which unknown organisms are employed, can result in a product of variable toxicity and nutritional quality. Thus, most workers have used pure cultures of identified organisms in order to minimise such problems.

Studies on the utilisation of sugar cane bagasse have been carried out by a team at the Louisiana State University (Callihan & Dunlap, 1969; Dunlap, 1969; Dunlap & Callihan, 1969; Han *et al*, 1971; Han & Callihan, 1974; Srinivasan & Han, 1969). The organism used in this work, a species of the genus *Cellulomonas*, cannot utilise lignin or ligno-cellulose complexes. Prior to microbial growth, the cellulose of the bagasse is swollen by soaking in hot NaOH. An increased protein yield has been observed when a mixed culture of *Alcaligenes faecalis* and the *Cellulomonas* species is used (Callihan & Dunlap, 1969; Dunlap, 1975). It is suggested that in this symbiotic culture, *Alcaligenes faecalis* consumes a hydrolysis product which had previously inhibited the action of the cellulase enzymes of the *Cellulomonas* species. This principle is the basis of a Danish process for the utilisation of NaOH treated barley straw by a mixed culture of the cellulolytic fungus *Trichoderma viride* and the yeast *Saccaromyces cerevisiae* (Peitersen, 1975b). In comparison with fermentations using *Trichoderma viride* alone (Peitersen, 1975a), the production time for maximum yields of cellulases and cellular protein is reduced by several days. The amino acid pattern of the mixed fermentation product resembles that of a pure culture of *Trichoderma viride* (Peitersen, 1975b). The results of these experiments indicate that the use of symbiotic culture merits more attention.

Extensive screening of cellulolytic bacteria and fungi has led to the selection of the fungus *Myrothecium verrucaria* as the organism capable of producing maximum protein yields from ball-milled newspaper (Updegraff, 1971). It has been found that the growth of *Aspergillus oryzae* enriches the protein content of surplus barley, but shortcomings of this organism in feeding trials have resulted in the choice of *Fusarium semitectum* as an alternative organism (Smith *et al*, 1975).

Diplodia gossypina, a fungus which causes ball-rot in cotton, has been used to convert cotton seed waste to ruminant feed. The results of feeding trials, however, have indicated adverse physiological effects (Lynch *et al*, 1977). The cellulolytic fungi *Chaetomium globosum* and *Myrothecium verrucaria* have been reported to effectively utilise partially delignified wheat straw and groundnut hulls as carbon sources for SCP (Singh *et al*, 1976).

Romanelli *et al* (1975) have shown that thermophilic cellulolytic fungal species, such as *Sporotrichum thermophile*, degrade pure cellulose efficiently. Although this work has concentrated on establishing the optimum conditions for cellulase production on pure cellulose, it is thought that this group of fungi could have an important role to fulfil in the upgrading of cellulosic wastes. Some thermophilic actinomycetes have also been shown to be active cellulose decomposers. *Thermomonospora fusca* has been used to convert pulping fines, a waste product of the paper industry, to microbial protein (Crawford & McCoy, 1973).

The physical association of cellulose with lignin is so intimate that efficient enzymic hydrolysis of cellulose is impossible unless the lignin is degraded first. It has been stated previously that chemical delignification is expensive (Millett & Baker, 1975). There is, therefore, growing recognition that it is desirable to use micro-organisms which produce both lignin-degrading and cellulose-degrading enzymes. Consequently, bioconversions using wood-destroying fungi of the white-rot type are currently under investigation (Kirk, 1975). The biodegradation of lignin requires a complex extracellular system of enzymes and co-enzymes and the functions of the components are yet to be fully elucidated (Ander & Eriksson, 1976).

Workers in Sweden have investigated the possibility of growing a thermotolerant white-rot fungus on various agricultural wastes including cereal flours (von Hofsten & von Hofsten, 1974; von Hofsten 1976; von Hofsten & Ryden, 1975). The organism occurs naturally in woodchip piles in Sweden, and has been shown to have the ultrastructural characteristics of a basidiomycete. It has been given the name *Sporotrichum pulverulentum* (von Hofsten, 1976), and is described as an imperfect state of *Phanerochaete chryosporium* (Burdsall & Eslyn, 1974). The combination of another white-rot fungus, *Fomes lividus*, and sodium hydroxide treatment has been successfully used to modify beech, oak and poplar sawdusts, in an attempt to produce useful animal feed

components (Hartley *et al*, 1974).

Prior to microbial utilisation, most cellulosic wastes so far discussed require supplementation of the nitrogen content. This is not true of animal excreta, which contain large amounts of organic and ammonium nitrogen. Upgrading of farm animal excreta is thus an attractive proposition.

Cattle feedlot waste has been found to contain all the necessary nutrients for batch fermentation with the fungus *Trichoderma viride*. The organism utilises two-thirds of the carbohydrate of feedlot waste while elaborating cellulase in quantities comparable to commercial preparations. The product is essentially odour-free (Griffin *et al*, 1974). A recent study indicated the feasibility of combining disposal of cow manure wastes with the production of the blue-green alga *Spirulina maxima*, which can be used as an alternative protein source (Oron *et al*, 1979).

Of all the intensively produced livestock in this country, pigs probably present the most significant waste disposal problem. This is mainly due to the fact that small areas of arable land are normally associated with intensive pig units. The amount of waste exceeds the soil's ability to recycle it efficiently, and alternative methods of treatment must be devised. The use of microorganisms to achieve quick recycling is thought to be a useful way of alleviating the pollution problem. This can also provide a cheap source of protein to be incorporated into animal rations. Pig waste contains large amounts of undigested cellulosic material of plant origin, and large amounts of nitrogen in the form of ammonia (Evans *et al*, 1978).

Investigations at the Biodeterioration Information Centre have shown that the mixed indigenous flora of thermophilic fungi can be encouraged to utilise the cellulose and nitrogen of pig waste. The cellulosic fraction of pig waste is supplemented by the addition of waste straw. In large scale experiments the slurry/straw mixture has been placed in a tower on metal slats and heated by heating coils to 50°C in 24 h (Seal & Eggins, 1976). It is envisaged that the liquid run off, which has considerably lowered polluting power, can be sprayed on the land (Seal, 1973). After 14 days the solid product has been enhanced in protein from 5% to between 10 and 13% and the cellulose content reduced by 30% (Seal & Eggins, 1976). This process is reported to kill *Salmonella* species but tapeworm and roundworm cysts

are likely to survive. Fungal pathogens, such as *Aspergillus fumigatus* and *Mucor pusillus* are able to survive the process, and are only excluded by careful pH control (Seal, 1973). Protein production and waste treatment are also the aim of a project in which pig slurry is inoculated with the mesophilic fungi *Leptomitis lacteus* or *Mucor racemosus*. *Mucor racemosus* has been found to grow ten times more quickly than *Leptomitis lacteus* on pig slurry and straw mixtures. In this process the inoculated fungus is stated to outgrow the indigenous flora. No results on pathogen survival have been reported (McGill, 1973). More recently, *Candida ingens*, a pellicle forming yeast utilising volatile fatty acids has been grown on the non-sterile supernatants from anaerobically fermented pig waste. Once again, control of pH is used to limit the growth of other competing organisms, however, contaminants are reported to grow when the pH is not closely monitored (Henry & Thomson, 1979).

None of these projects produce an end-product of known microbial composition. In each case the mixed indigenous flora of the slurry is able to grow. The control of pH and temperature is used to establish the dominance of desirable organisms. Thus, the composition, nutritional quality and pathogenicity of the product can vary from batch to batch. It has already been discussed that this is considered to be an unacceptable method of producing SCP (van der Wal, 1976). It is therefore essential that any method of producing SCP from pig excreta includes some method of sterilising the medium prior to inoculation of a pure culture of the selected microorganism. In the process described by Seal & Eggins (1976) only 30% of the cellulose is broken down by microbial action. Thus, it would be advantageous to combine a method of sterilisation, and a method of pre-treatment which would render the cellulosic constituents more available to microorganisms.

In considering the available methods, it has to be remembered that apart from α-cellulose the solids of pig slurry are likely to contain significant quantities of hemicelluloses, which are also useful carbon and energy sources for microbial growth. These hemicelluloses are much more labile than cellulose (Saeman, 1945). A method of pre-treatment must be selected to conserve this source of microbial nutrition, but also to have some disruptive effect on the crystalline structure of the cellulose. Very dilute H_2SO_4 at temperatures of between 150°C

and 175°C has been successfully used to hydrolyse the hemicelluloses of wood (Kobayashi & Sakai, 1956). It has also been shown that dilute H_2SO_4 at 100°C is an efficient method of increasing the availability of corn cob hemicellulose. It is therefore proposed that pig slurry be subjected to mild acid treatment. In this way it is postulated that a sterile liquid containing considerable amounts of ammonium nitrogen and the mono-, di-, tri- and oligo-saccharide breakdown products of hemicellulose will be produced. Microorganisms such as yeasts can be cultivated on this type of medium. It is also hoped that the solid residue from the acid treatment can be used for the cultivation of a cellulolytic fungus.

SCOPE OF WORK

It is probable that modern farming methods employing intensive husbandry techniques are likely to increase in scale in the future. Consequently, the attendant problem of disposing of animal waste is also likely to increase. The approach most commonly adopted at present is to devise a system whereby the waste material can be disposed of without causing any adverse environmental effects.

A new concept of recycling waste from non-ruminant animals has recently been investigated (Smith, 1973). Alternatively, the waste material can be utilised to produce an end-product which has a commercial value. This approach has tended to receive less attention, and has only recently been investigated to any extent (McGill, 1973; Seal, 1973). It is recognised that the economics of any system evolved for utilising animal wastes may override any advantages gained. It is considered, however, that before the economic factors are investigated the technical feasibility of waste utilisation must be established. In addition, the simple financial aspect of the problem may be of less importance than the development of a system which prevents the dissipation of material resources.

Accordingly, a series of preliminary investigations were conducted to establish the feasibility of producing Single Cell Protein (SCP) from pig slurry. The choice of end-product was made because current interest in the production of SCP from other more expensive raw materials indicates that in the future SCP may be more widely used than at present. Pig slurry was selected as the raw material for this study, because it was considered that this waste was likely to create a greater disposal problem in the immediate future than any other animal waste.

Much of the carbohydrate in pig slurry is present in the form of the cellulosic constituents of plant tissue, and is relatively resistant to degradation by microorganisms. Therefore some form of pre-treatment is required to render the cellulosic materials more available to microorganisms. It is also considered essential that the end-product is non-toxic and of constant composition. Thus, the raw pig slurry was treated with dilute H_2SO_4 at temperatures below 100^{o}C to increase the availability of the component

carbohydrates and sterilise the resulting growth medium.

The first part of the experimental section reports on the qualitative analysis by Thin-layer Chromatography (TLC) and paper chromatography of the soluble carbohydrates released by mild H_2SO_4 hydrolysis of pig slurry. The development of a High Pressure Liquid Chromatographic (HPLC) technique for quantifying the monosaccharides released by hydrolysis is described. This analysis is combined with measurement of the residual cellulosic fractions to determine the success of the mild acid hydrolysis systems tested. Combinations of acid concentrations between 1% and 10% w/v and temperatures of 80°C, 90°C and 100°C were investigated, with the aim of maximising the release of soluble carbohydrates from the cellulosic constituents of pig slurry.

The second Section describes the growth of several carefully chosen yeast species on the liquid hydrolysates selected from the results of Section 1. The detection of a substance inhibitory to yeast growth is reported, and an investigation into the source and nature of this compound is described. Methods of overcoming the inhibitory effect were investigated and the yeast species best able to grow in the presence of the inhibitor was selected. The classification of this yeast species is described.

The liquid remaining after yeast growth contains large amounts of residual ammonia. Section 3 describes an investigation in which this liquid was added back to the solid cellulosic residue of hydrolysis, in order to promote the growth of a cellulolytic white-rot fungus, *Sporotrichum pulverulentum*. The results of growth trials with this organism are reported.

In Section 4, amino acid analysis is used to assess the nutritional potential of the products of microbial growth on the liquid and solid residues of acid hydrolysed slurry. Finally, in Section 5, the measurement of chemical parameters of the residual liquid after fungal cultivation is reported.

EXPERIMENTS METHODS AND RESULTS

1. STUDIES ON THE EFFECT OF SULPHURIC ACID HYDROLYSIS ON THE CELLULOSIC CONSTITUENTS OF PIG SLURRY AND THE ANALYSIS OF LOW MOLECULAR WEIGHT CARBOHYDRATES PRODUCED BY THIS TREATMENT

2. STUDIES ON THE GROWTH OF SELECTED YEAST SPECIES ON PIG SLURRY HYDROLYSATE

3. STUDIES ON THE GROWTH OF THE CELLULOLYTIC FUNGAL SPECIES *SPOROTRICHUM PULVERULENTUM* ON HYDROLYSED PIG SLURRY

4. ASSESSMENT OF THE PROTEIN CONTENT, PROTEIN QUALITY AND HEAVY METAL CONTENT OF THE PRODUCTS OF MICROBIAL GROWTH ON ACID HYDROLYSED PIG SLURRY

5. CHEMICAL ANALYSIS OF THE RESIDUAL LIQUID FRACTIONS PRODUCED WHEN MICROORGANISMS ARE GROWN ON ACID HYDROLYSED PIG SLURRY

1. STUDIES ON THE EFFECT OF SULPHURIC ACID HYDROLYSIS ON THE CELLULOSIC CONSTITUENTS OF PIG SLURRY AND THE ANALYSIS OF LOW MOLECULAR WEIGHT CARBOHYDRATES PRODUCED BY THIS TREATMENT

INTRODUCTION

Most of the information on the breakdown of cellulose to glucose by H_2SO_4 hydrolysis has been obtained from work with wood as the raw material. The acid hydrolysis of wood cellulose has been approached in two ways. Some workers developed processes utilising high acid concentrations at temperatures below 100°C (Kobayashi *et al*, 1962), others advocated the use of low acid concentrations at high temperatures (Saeman, 1945). The latter approach has gained favour with those working on the acid hydrolysis of waste cellulosic materials (Porteus, 1974), the attraction of this system being the use of dilute expendable amounts of acid.

In most naturally occurring cellulosic materials the cellulose is closely associated with readily hydrolysed hemicellulosic constituents. On hydrolysis these hemicelluloses yield several different monosaccharides including large quantities of the pentoses, xylose and arabinose. It has been shown that during wood hydrolysis in dilute acid at high temperatures, xylose and arabinose are much more labile than glucose (Saeman, 1945). Thus, in hydrolysis systems which are designed to maximise the yield of glucose from wood, the monomers produced from the hemicellulose fractions are rapidly decomposed to furfural (Saeman, 1945), and then further broken down to produce aromatic phenolic and enolic compounds (Anderegg & Neukom, 1974; Popoff & Theander, 1972). The hydrolytic conditions used on wood are generally designed to maximise the yield of glucose, which is a valuable substrate for the production of ethyl alcohol, without regard to the destruction of the pentoses. However, the realisation that furfural can be used in polymerisation reactions to produce valuable products, such as resins (Fieser & Fieser, 1956), led to the controlled production of furfural from pentoses. This resulted in the development of a process which maximised the yield of both undegraded pentoses and undegraded glucose from wood (Kobayashi & Sakai 1956). In this process the hemicelluloses are broken down to

pentoses by mild acid treatment in a pre-hydrolysis step, which leaves the cellulose intact to be treated separately.

The hydrolysis of pig excreta to produce monosaccharides for the production of SCP presents a similar problem to that presented by hydrolysis of wood. Pigs are monogastric animals, which are fed a diet based mainly on barley (Table 3). Their excreta therefore contains a large amount of undigested cellulosic material, which is a mixture of hemicellulose and cellulose. Pentoses are valuable carbon sources for microorganisms. Thus, the initial aim of this project was to maximise the yield of utilisable carbohydrate by the hydrolysis of the hemicellulosic fraction of pig slurry. It was postulated that this can be achieved using a mild acid treatment system, and that residual undegraded cellulose could be utilised in a second process.

A system using dilute expendable concentrations of acid is proposed to break down the hemicellulosic fraction of pig slurry. It was pointed out by Stamm (1964) that at temperatures over 100°C, the higher the temperature the more favourable the rate of monomer formation to monomer destruction. At temperatures above 100°C the hydrolysis equipment must be pressurised and the reaction times are often only a few seconds. On the industrial scale a steady state plug flow reactor (Porteus, 1974) can be used to achieve short contact times, and the slurry of cellulosic material must be of a consistency that can be easily pumped and mixed. With pig slurry this may necessitate dilution to an extent that renders this type of system impracticable. In the laboratory it is virtually impossible to achieve such short reaction times. A cylindrical steel bomb batch reactor has been described (Porteus, 1974), but the complication of heating and cooling times make it impossible to obtain results that can be compared to those produced using a continuous industrial system. Furthermore, it is unlikely that an economically priced on farm system using such sophisticated equipment could be developed. In view of these potential difficulties, it was decided to employ hydrolysis temperatures of 100°C and below in this work. Temperatures of 80°C, 90°C and 100°C at acid concentrations of 2.5%, 5% and 10% and of 90°C and 100°C at acid concentrations of 1% were investigated. It was accepted

TABLE 3 The composition of the pig rearing ration used for feeding fattening gilts

Barley Meal	635 kilograms (kg)
Fine Thirds	254 kg
White Fish Meal	76.2 kg
Extracted Soyabean Meal	50.8 kg
NAAS Vitamin Mineral Supplement	18.1 kg
Copper Sulphate	0.45 kg
Zinc Carbonate	0.23 kg

that, at these lower temperatures, the monomer yield might be lower than those which could be attained using temperatures above 100°C. The success of any particular hydrolysis system can be monitored by measuring the yield of soluble carbohydrates. Thus, a method of quantifying the carbohydrates in the hydrolysate had to be developed. This necessitated the prior qualitative assessment of the carbohydrates present in the hydrolysate by such methods as paper and thin-layer chromatography.

Although quantitative analysis of the carbohydrates released by acid hydrolysis of the hemicellulosic constituents of pig slurry provides relevant information on the success of a particular treatment system, it was decided that these results would be complemented by an estimation of the cellulosic materials present in the slurry pre- and post-hydrolysis. Using both sets of analyses it was hoped to establish the conditions which produce maximum degradation of the hemicelluloses with minimum destruction of the monosaccharides released by hydrolysis.

Qualitative analysis of the low molecular weight carbohydrates present in acid hydrolysed pig slurry

THIN-LAYER CHROMATOGRAPHY (TLC)

Thin-Layer Chromatography (TLC) affords a simple, rapid and sensitive method for the qualitative analysis of low molecular weight sugars. Carbohydrates being strongly hydrophilic, require polar solvent systems. These solvents have relatively slow migration rates requiring from 0.5 to 3 h for one ascent of the plate (Lewis & Smith, 1969). It has been established that impregnating silica gel and Keiselguhr layers with salts such as sodium acetate enhances the resolution of simple sugar mixtures (Stahl & Kaltenbach, 1961).

Separations of all the more common sugars have been attempted using known absorbents impregnated with several different organic salts (Lato *et al*, 1968, Ovodov *et al*, 1967). It has been found to be impossible to produce one solvent system which will clearly separate all common simple sugars (Lato *et al*, 1968).

Materials and Methods

Preparation of Sample for TLC

One hundred millilitres (100 ml) of pig slurry[1] (concentration of solids approx 100 $g.l^{-1}$) were hydrolysed with 10 ml of concentrated H_2SO_4[2] at 100°C for 2 h. The fibrous solids were removed by filtration using glass fibre paper GF/B (Whatman Lab Sales Ltd., Springfield Mill, Maidstone, Kent). The filtrate was neutralised by the addition of Calcium hydroxide ($Ca(OH)_2$) and the resulting precipitate of Calcium sulphate ($CaSO_4$) separated from the neutral liquid by filtration through Whatman's No. 1 filter paper. Freeze-drying of the filtrate and resuspension in methanol removed the ionic salts, as they remained undissolved and were easily removed by filtration. The carbohydrates dissolved in the methanol and the resulting clear dark brown liquid was used as the sample for TLC.

It was possible that this liquid contained other non-ionic compounds, e.g. proteinaceous materials, which may interfere with the TLC of sugars. For this reason, a portion of the liquid was clarified with charcoal, which removes high molecular weight organic compounds. This treatment rendered the resulting liquid completely colourless. The charcoal treated sample and the untreated sample were compared by TLC.

Reference Standards

A set of reference sugar standards which included arabinose, glucose, galactose, glucuronic acid, ribose, xylose, fructose, melibiose, maltose and sucrose was obtained from Shandon Scientific Company Ltd, 65 Pound Lane, Willesden, London). The concentration of these standards was 2.5 grammes of sugar/litre ($g.l^{-1}$) in a 10% v/v solution of isopropanol in water. Solutions of mannose and galacturonic acid were prepared to these specifications.

Preparation of TLC Plates

In preliminary experiments various absorbents were tested. Of these, silica gel G (Merck, Darmstadt, Germany) impregnated with 0.02 M sodium acetate (Lewis & Smith, 1969) and silica gel G impregnated with 0.2 M sodium dihydrogen phosphate (Ovodov *et al*,

1. *Unless otherwise stated, the slurry used throughout this work was obtained from fattening gilts fed a diet based on barley (Table 3).*

2. *Unless otherwise stated, all chemicals used were of analar grade supplied by BDH Chemicals Ltd, Poole, Dorset, BH12 4NN.*

1967) proved most useful in the qualitative assessment of the sugars present in pig slurry hydrolysate. 20 x 20 centimetre (cm) plates were prepared according to the method of Lewis & Smith (1969) at the normal thickness of 250 μ.

Solvents

Several of the solvents listed by Lewis & Smith (1969) for the separation of sugars were tested on sodium acetate impregnated silica gel G. Chloroform-methanol (60:40 by volume) and acetone-water (90:10 by volume) proved to be the most useful of all solvent systems employed. Efficient separation of the monosaccharide components of hydrolysed pig slurry was also achieved using silica gel G impregnated with sodium dihydrogen phosphate and the n-butanol-acetone-water (40:50:10 by volume) solvent system developed by Ovodov *et al* (1967).

Spot application

Spots were applied to the plates using a multi-purpose template (Shandon Scientific Co Ltd) and precision microbulb pipettes ("microcaps") (Drummond Scientific Company). Sugar standards were applied in 2 μl amounts. Satisfactory separation and visualisation of component carbohydrates was achieved when 5 μl quantities of sample or charcoal-treated sample were used. All spots were 2.5 mm in diameter.

Development of plates

Plates were developed by the standard ascending technique in a universal TLC chromotank at a temperature of 20^{o}C (Shandon Scientific Company Ltd), until the solvent moved 15-16 cm up the plate (Stahl, 1969).

Visualisation

The anisaldehyde-sulphuric acid reagent and the aniline-diphenylamine reagent of Lewis & Smith (1967)were successfully used to visualise the carbohydrates. These reagents produce characteristic colours for different sugars which aid identification. Changes in TLC conditions or development temperatures may alter the colours produced by aniline-diphenylamine reagent. Such changes were shown to be of advantage in the identification of the carbohydrate components of pig slurry, and consequently results reported are for this reagent.

hRf values in TLC

The migration distances of substances on thin-layer chromatographs are generally fixed as Rf values. This is the distance of the spot

centre from the origin divided by the distance that the solvent front has moved from the start. In this work the Rf values were multiplied by 10.0 and are termed hRf values.

Results and Discussion

Chromatograph 1 was produced on silica gel G impregnated with 0.02 M sodium acetate and developed in chloroform:methanol (60:40 by volume). Visualisation was achieved with analine diphenylamine spray. All the standards listed previously, the sample and the charcoal-treated sample were chromatographed on this plate.

It can be seen from this chromatograph that both the sample and the charcoal treated sample separate into 5 distinct components. The presence of contaminating materials does not appear to interfere with the separation of the sample components, and charcoal-treatment confers no obvious advantages to the separation procedure. Visual assessment indicated that the spots produced in the untreated sample were of similar intensity to those produced after charcoal-treatment.

The hRf values and colour development of the sugar standards and the carbohydrate components of the sample and charcoal-treated sample are presented in Table 4.

By comparing the colour development and hRf values of the sample components and the standard carbohydrates, it is apparent that melibiose, maltose, deoxyribose, fructose and sucrose were absent from the sample. In fact, the sample did not appear to contain detectable quantities of any di- or oligosaccharides. This system detects, but does not separate, the uronic acids of glucose and galactose. Component 5 of the sample could correspond to either or both galacturonic acid and glucuronic acid. Sample components 2 and 1 correspond to the pentose sugars, arabinose and xylose, respectively in both hRf value and colour. However, sample components 4 and 3 at hRfs 21 and 26 cannot be completely identified by this chromatographic system. Component 4 coincides with both ribose and galactose in hRf value. The development of different colours helps to separate these two sugars, as ribose produces a grey colour and galactose is green on this chromatograph. Sample component 4 develops an intense shade of green, the same as that of galactose. Component 3 at hRf 26 has the same hRf as mannose and is very close to the hRf of glucose which is 28. Both these sugars and the sample spot show up as a

Chromatograph 1 Thin-layer chromatography of carbohydrates on sodium acetate impregnated silica gel G run in chloroform and methanol solvent and visualised with analine diphenylamine spray.

A	Arabinose
Ga	Galactose
GaA	Galacturonic acid
Gl	Glucose
GlA	Glucuronic acid
M	Mannose
R	Ribose
X	Xylose
Me	Melibiose
Ma	Maltose
D	Deoxyribose
F	Fructose
S	Sucrose
Sa	Hydrolysed slurry
CSa	Charcoal treated hydrolysed slurry

grey

green

brown

grey/green

blue

orange

Chromatograph 1

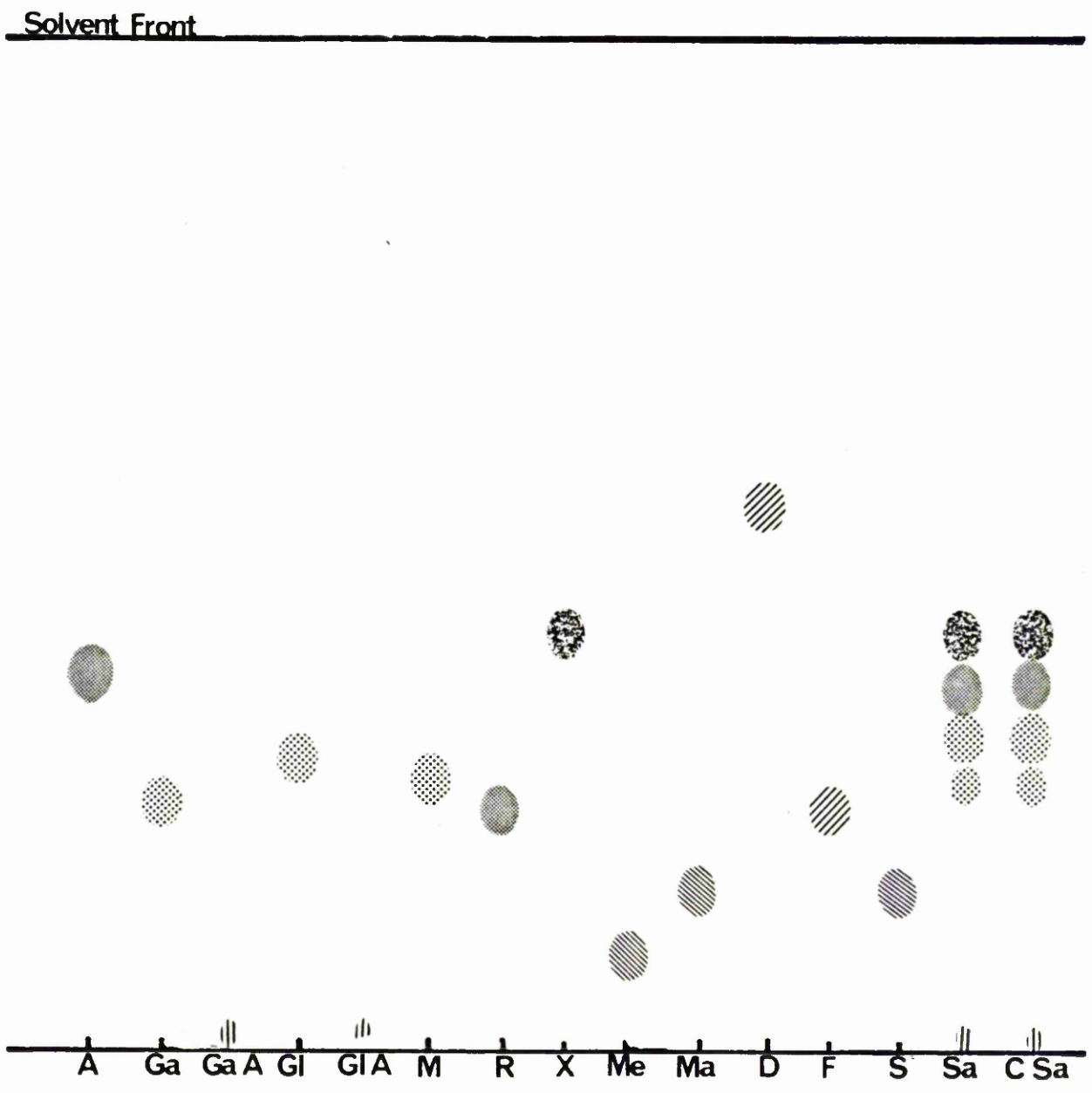


TABLE 4 hRf values and colour development of carbohydrates on sodium acetate impregnated silica gel G run in chloroform-methanol solvent and visualised with aniline diphenylamine spray.

Carbohydrate		hRf	Colour
Arabinose		36	Grey
Galactose		21	Green
Galacturonic acid		1	Brown
Glucose		28	Green
Glucuronic acid		1	Brown
Mannose		26	Green
Ribose		21	Grey
Xylose		41	Grey/Green
Melibiose		8	Blue
Maltose		13	Blue
Deoxyribose		63	Orange
Fructose		26	Orange
Sucrose		17	Green
Sample component	5	1	Brown
	4	21	Green
	3	26	Green
	2	36	Grey
	1	40	Grey/Green
Charcoal treated sample component	5	1	Brown
	4	21	Green
	3	26	Green
	2	36	Grey
	1	40	Grey/Green

green colour on the chromatograph, and thus the sample could contain either or both of these sugars.

Chromatograph 2 was developed in the acetone-water (90:10 by volume) solvent system. Apart from this variation, the chromatographic procedures were the same as those used to produce Chromatograph 1. The sugars which were demonstrated to be absent from the sample by Chromatograph 1 namely, melibiose, maltose, deoxyribose, fructose and sucrose, were not run on Chromatograph 2. The hRf values and the colours developed by the sugar standards and the sample components on Chromatograph 2 are given in Table 5.

The sample components 2 and 1 clearly correspond in colour and hRf value to arabinose and xylose respectively, as in Chromatograph 1. The uronic acids appear to be present in the sample, but again they are not differentiated. On this chromatograph ribose and galactose were completely separated. Sample component 4 has the same hRf value, 52, as galactose, whereas ribose runs at an hRf of 65. Once again it is impossible to distinguish glucose and mannose as both move similar distances up the plate and develop the same colour. For this reason sample component 3 cannot be identified, as it corresponds to both of these sugars in this TLC system.

Finally, Chromatograph 3 was produced on silica gel G impregnated with 0.2 M sodium dihydrogen phosphate and developed in n-butanol-acetone-water solvent (40:50:10 by volume) (Ovodov *et al*, 1967), and visualised with aniline diphenylamine spray. The best separations of the carbohydrate components of hydrolysed pig slurry are achieved using this TLC system. The sample separates into 6 distinct components on this chromatograph. The identity of the components already separated on chromatographs 1 and 2, namely arabinose, galactose and xylose, are confirmed by this chromatograph. The hRf values and colour developments for this system are presented in Table 6.

Unlike the previously reported TLC systems, glucuronic acid and galacturonic acid are separated by this procedure. Both uronic acids appear to be present in the sample in small amounts. Similarly, glucose and mannose are clearly distinguished by this system. The hRf of mannose is 27, whereas that of glucose is 20. Component 3 of the sample clearly corresponds to glucose and no trace of a sample component corresponding to mannose is observed. The sample spot with hRf 29 runs close to the hRf of mannose, but develops with

Chromatograph 2 Thin-layer chromatography of carbohydrates on sodium acetate impregnated silica gel G run in acetone-water solvent and visualised by aniline diphenylamine spray.

A	Arabinose
Ga	Galactose
GaA	Galacturonic acid
Gl	Glucose
GlA	Glucuronic acid
M	Mannose
R	Ribose
X	Xylose
Sa	Hydrolysed slurry

Green

Brown

Grey/Green

Blue

Chromatograph 2

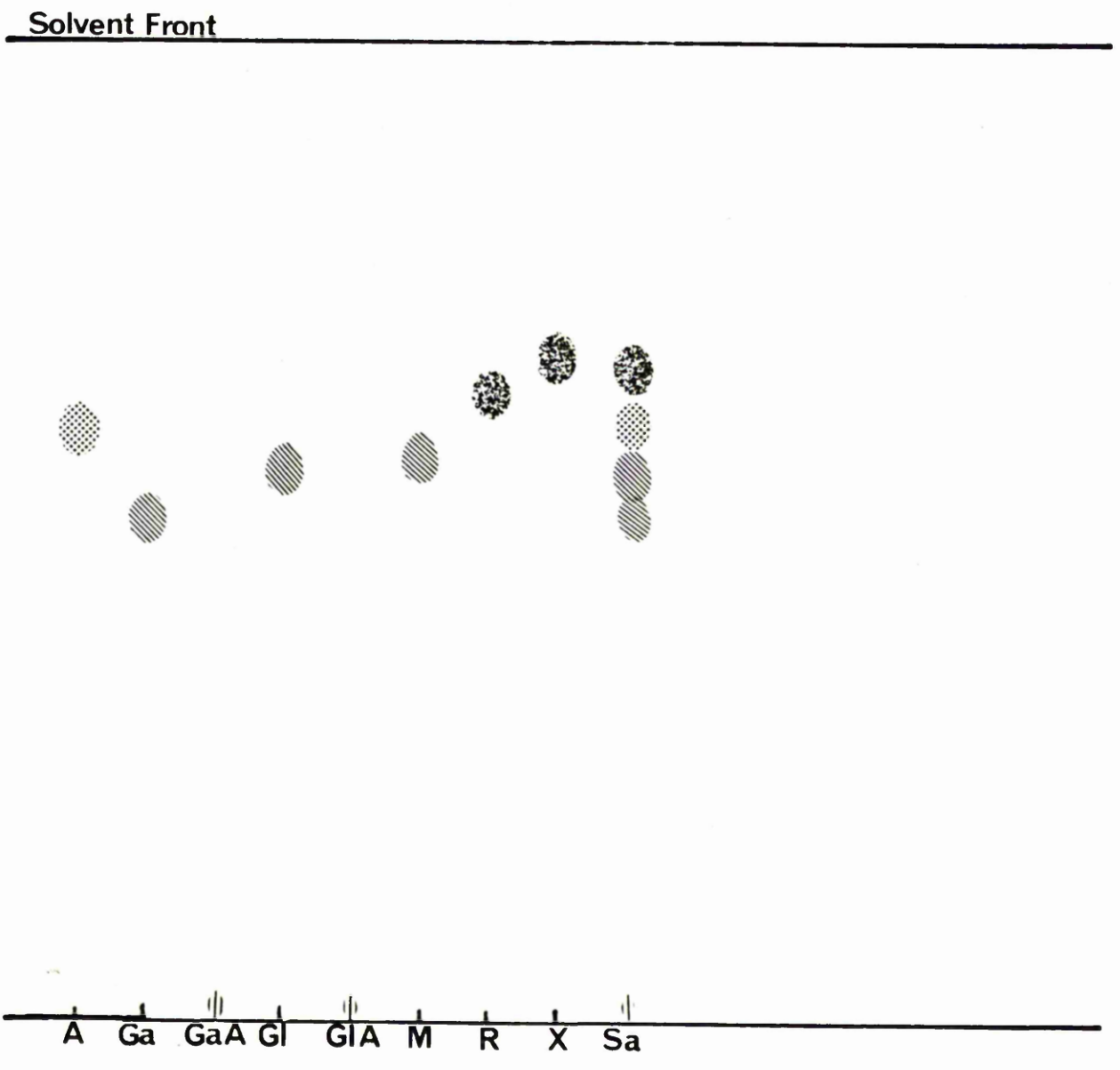


TABLE 5 hRf values and colour development of carbohydrates on sodium acetate impregnated silica gel G run in acetone-water solvent and visualised by aniline diphenylamine spray.

Carbohydrate	hRf value	Colour
Arabinose	61	Green
Galactose	52	Blue
Galacturonic acid	1	Brown
Glucose	57	Blue
Glucuronic acid	1	Brown
Mannose	58	Blue
Ribose	65	Grey/Green
Xylose	68	Grey/Green
Sample component 5	1	Brown
4	52	Blue
3	56	Blue
2	61	Green
1	57	Grey/Green

Chromatograph 3 Thin-layer chromatography of carbohydrates on sodium dihydrogen phosphate impregnated silica gel G run in n-butanol-acetone water solvent and visualised with analine diphenyl-amine spray.

A	Arabinose
Ga	Galactose
GaA	Galacturonic acid
Gl	Glucose
Gla	Glucuronic acid
M	Mannose
R	Ribose
X	Xylose
Sa	Hydrolysed slurry
▒	Green
▒	Blue
▒	Mauve

Chromatograph 3

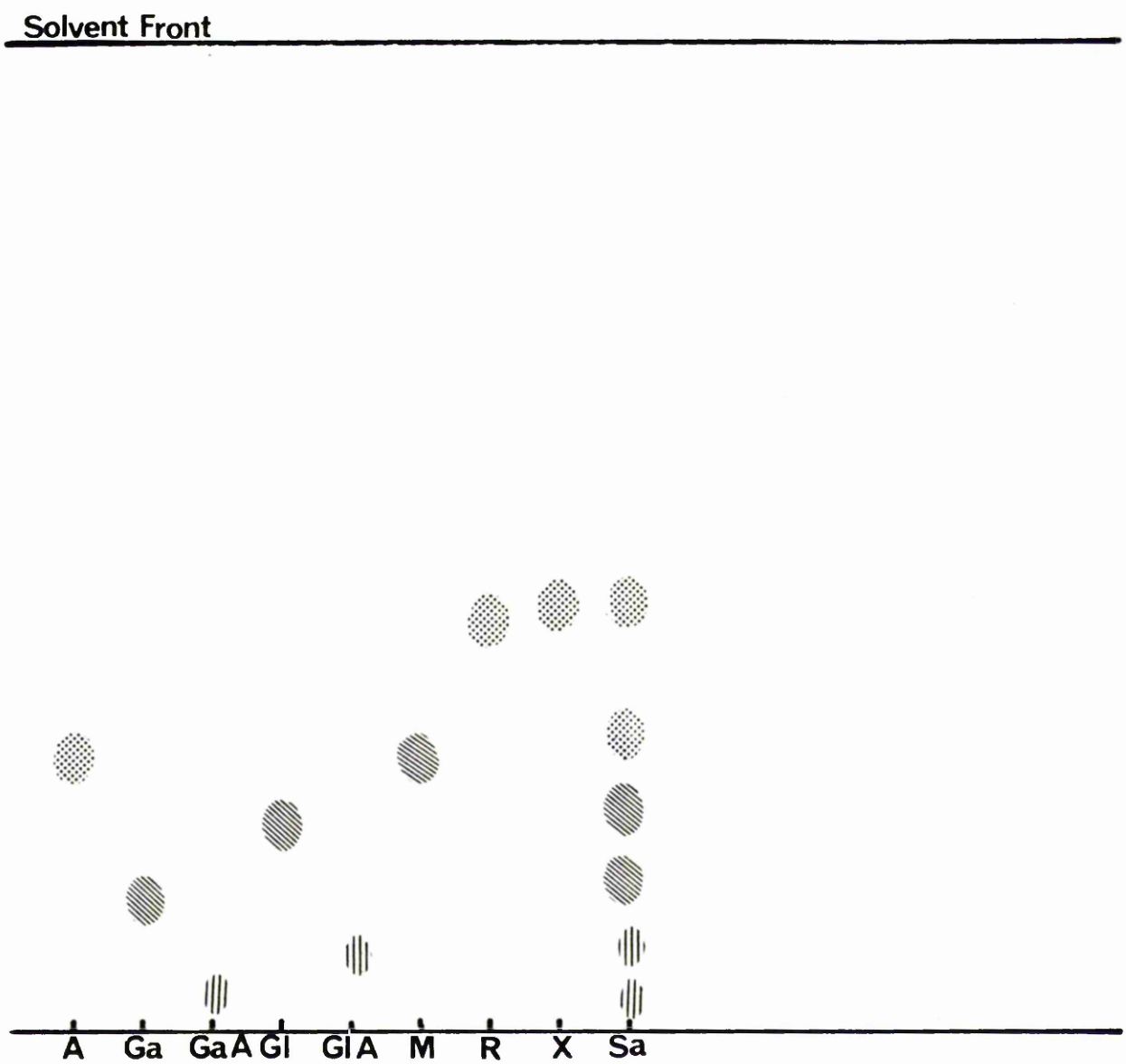


TABLE 6 hRf values and colour development of carbohydrates on sodium dihydrogen phosphate impregnated silica gel G, run in n-butanol acetone water solvent and visualised with aniline diphenylamine spray.

Carbohydrate	hRf value	Colour
Arabinose	29	Green
Galactose	14	Blue
Galacturonic acid	3	Mauve
Glucose	20	Blue
Glucuronic acid	7	Mauve
Mannose	27	Blue
Ribose	40	Green
Xylose	43	Green
Sample component 6	3	Mauve
5	6	Mauve
4	14	Blue
3	21	Blue
2	29	Green
1	42	Green

an intense green colour, which indicates that it corresponds to arabinose. Although differences in intensity of spots were difficult to assess visually, it appears that xylose and arabinose are present in slightly larger quantities than glucose. The spot corresponding to galactose is consistently fainter than those corresponding to the other three neutral sugars.

Conclusions

The results of TLC indicated that there are six detectable carbohydrates produced during the acid hydrolysis of pig slurry.

These carbohydrates are monosaccharides and no detectable quantities of di- or oligosaccharides are produced. Combining the results of the three TLC procedures, the six detectable carbohydrate components of hydrolysed pig slurry are identified as arabinose, galactose, glucose, xylose, glucuronic acid and galacturonic acid.

PAPER CHROMATOGRAPHY

Combining the results of three different TLC chromatographs enabled tentative identification of the monosaccharides present in hydrolysed pig slurry. Difficulties were encountered with TLC because no one procedure could concurrently separate arabinose, galactose, glucose, mannose, ribose and xylose.

Paper chromatography has the advantage that systems are available which will clearly separate the six sugars listed above on one chromatograph (Smith, 1960b). It has the disadvantage of being a time consuming, cumbersome and less sensitive technique than TLC, and is therefore less useful than TLC for preliminary investigations. It was considered that at this stage in the investigation, paper chromatography could be usefully employed to confirm the identity of the carbohydrate components of the hydrolysate.

Materials and Methods

Preparation of sample for paper chromatography - As described for TLC

References Standards - As used for TLC

Standard sugars applied to the paper chromatograph were arabinose, galactose, galacturonic acid, glucose, glucuronic acid.

Preparation of Paper

Whatman's No 1 filter paper (46 x 57 cm) was prepared for descending chromatography, as described by Smith (1960a).

Solvent

The ethyl acetate:pyridine:water (120:50:40 by volume) solvent described by Smith (1960b) was shown to efficiently separate both the carbohydrate components of pig slurry hydrolysate and the standards applied to the chromatograph.

Spot Application

Spots were applied using "microcaps" so that the diameter did not exceed 0.5 cm. It was established that 5 µl of standard sugar solutions and 10 µl of sample produced chromatographs with distinctly separated and clearly visualised spots.

Development of Chromatograph

The paper chromatograph was continuously developed for 10 h at 20°C in a model 500 "Chromotank" (Shandon Scientific Company Ltd) by the standard descending technique described by Smith (1960a).

Visualisation

Aniline reagent and aniline diphenylamine reagent (Smith, 1960b) were tested. Aniline reagent was found to cause significant background yellowing of the paper which made interpretation of results difficult. The time and temperature of heating proved to be critical with aniline diphenylamine reagent. Overheating led to lack of colour differentiation of spots, followed by complete disintegration of the paper. A two-stage visualisation system without the above problems was therefore adopted (Baird-Parker & Woodroffe, 1967). In this system a dried chromatograph is dipped in a 1% solution of p-amino benzoic acid in isobutyl alcohol and dried in a stream of air. The second stage involves dipping in a 0.5% solution of oxalic acid in glacial acetic acid, followed by heating at 105°C for 10 min. With this method pentoses show up as bright red spots and hexoses are yellow.

hRg Values in Paper Chromatography

In order to achieve separation of closely related sugars, it is necessary to run the solvent off the end of the paper to compensate for the low hRf values obtained with a finite solvent front. In such cases glucose is taken as a reference compound, and an arbitrary hRf value is defined so that the hRg for glucose in all solvents is 100. Thus, the hRg value of a carbohydrate is the distance it moves from the origin divided by the distance that glucose travels from the origin multiplied by 100.

Results and Discussion

The hRg values and colour development for the standard sugars tested and the components of pig slurry hydrolysate are presented in Table 7.

Paper chromatography separates the component monosaccharides of hydrolysed pig slurry very efficiently. The sample is split into 5 component carbohydrates as the uronic acids are not separated by this method, both running at an hRg of 3. From examination of hRg values and colour development, it can be seen that arabinose, galactose, glucose and xylose are the neutral sugars present in the sample. This confirms the identifications made using TLC. The spots corresponding to the pentose sugars were much more intense than those corresponding to the hexoses. The spot corresponding to galactose was particularly faint.

Conclusions

Hydrolysed pig slurry contains detectable quantities of arabinose, galactose, glucose, xylose and uronic acids.

Significant quantities of pentose sugars are produced by acid hydrolysis of pig slurry.

Qualitative assessment indicated that the uronic acids are present in barely detectable quantities, compared to the yields of neutral monosaccharides. Thus, it is considered that quantification of neutral sugars will give an adequate indication of the success of H_2SO_4 hydrolysis of pig slurry, and that quantification of the uronic acids is unnecessary.

Quantitative Analysis of the Low Molecular Weight Carbohydrates Present in Acid Hydrolysed Pig Slurry

There are many reported colorimetric methods of estimating "total carbohydrate". These include the use of orcinol/H_2SO_4 (Holden *et al*, 1950), anthrone/H_2SO_4 (Herbert *et al*, 1971), phenol/H_2SO_4 (Dubois *et al*, 1956), copper/arsenomolybdate (Nelson, 1944) and ferricyanide reagents (Guinn, 1967). All these techniques are limited in their usefulness for the quantification of monosaccharide mixtures because of the different sensitivities of individual carbohydrates to the reagents. This necessitates the separation of the components of a mixture before accurate quantitative analysis can be achieved by the above method.

TABLE 7 hRf values and colour development of sugars on Whatman's No. 1 paper run in ethyl acetate:pyridine:water solvent for 10 h and visualised by the method described by Baird-Parker & Woodroffe (1967).

SUGAR	hRg VALUE	COLOUR
Arabinose	143	Red
Galactose	18	Yellow
Galacturonic acid	3	Yellow
Glucose	100	Yellow
Glucuronic acid	3	Yellow
Mannose	115	Yellow
Ribose	230	Red
Xylose	169	Red
Sample component 1	3	Yellow
2	76	Yellow
3	99	Yellow
4	142	Red
5	168	Red

Enzymic assay is an accurate, reproducible and sensitive technique for the detection and quantification of monosaccharides. This technique has the advantage of specificity, but unfortunately enzymic analysis systems have only been developed for glucose and galactose (Herbert *et al*, 1971).

Several chromatographic methods are available for separating and quantifying carbohydrates. The inherent errors involved in the quantification of carbohydrates separated by either paper or thin-layer chromatography have been well documented (Court, 1968; Lehrfeld & Goodwin, 1969; Welch & Martin, 1972). Apart from these obvious disadvantages to quantification, the carbohydrates present in pig slurry hydrolysate have already been shown to be inseparable by monodimensional TLC (pages 32-34). Bidemensional TLC produces greater separation, but is not attractive for quantification because of the time necessary for development, and the fact that the spots tend to be diffuse. Paper chromatography produces well separated spots, but unfortunately the long development times make it less attractive for the quantification of large numbers of samples.

Another method of separation which was considered was column chromatography. The use of cellulose columns for carbohydrate separations has been reported (Nunn *et al*, 1971). However, in laboratory tests the resolution of the monosaccharide components of pig slurry hydrolysate was found to be worse than that obtained by TLC, and therefore it seemed unlikely that accurate quantification of individual monosaccharides could be achieved by this method. Gel filtration on columns of cross-linked dextran was also investigated. In this case separation is based on molecular size differences (Pharmacia, 1969) which, with monosaccharides, are insignificantly small and thus, as expected, laboratory tests showed no detectable separation of the monosaccharide components of pig slurry hydrolysate.

It was therefore concluded that to obtain rapid, accurate and reproducible quantification of monosaccharide mixtures, the more sophisticated techniques of Gas Liquid Chromatography (GLC) or High Pressure Liquid Chromatography (HPLC) would have to be employed. GLC of the volatile derivatives of carbohydrates has been widely and successfully used for quantification (Birch, 1973, Clamp, 1973; Grob, 1975; Herb, 1968; Reid *et al*, 1970; Shaw & Moss, 1969). HPLC

has certain important advantages over GLC. These are avoidance of conversion of the carbohydrates into volatile derivatives, and the avoidance of the multiple peaks which occur in GLC due to different stereometric forms of specific carbohydrates. In view of these disadvantages in the use of GLC, HPLC was adopted for the quantitative analysis of the carbohydrate components of pig slurry hydrolysate.

Recently, the upsurge in interest in the technique of HPLC has led to considerable advances in pump, column packing and detection technology (Done, 1979; Knox, 1973 and 1974; Majors, 1974; Mikes, 1979; Nikelly & Ventura, 1979; Snyder & Kirkland, 1979). HPLC with ion-exchange resins is now an established technique for carbohydrate analysis (Jonsson & Samuelson, 1967; Martinsson & Samuelson, 1970; Samuelson, 1969; Snyder & Kirkland, 1979; Walker & Saunders, 1970).

After considering the separation systems reviewed in the literature (Kennedy, 1973), a method developed by Hobbs & Lawrence (1972) was adopted. In this method a cation exchange resin charged with trimethylamine counterions is used to separate mono- and disaccharides on a column 0.40 centimetres (cm) in diameter by 100 cm in length. The eluant is an ethanol/water mixture. Although the mechanism is not clearly understood, separation is thought to be based on the fact that in the ion-exchange resin, the ratio of ethanol to water is greater inside the resin particle. The more water soluble monosaccharides such as glucose are therefore retained inside the resin longer than the less water soluble carbohydrates such as xylose. In addition, interacting forces between solutes and counterions are thought to have some effect on separation (Samuelson, 1969).

Hobbs & Lawrence (1972) used a modified moving wire/flame ionisation detector for the direct detection of carbohydrates in the eluant. Photometric, refractometric and fluorometric monitoring are also extensively used in HPLC monitoring (Knox, 1974). For neutral carbohydrates, automated spectrophotometric versions of the phenol-, orcinol-, anthrone-, and cysteine-H_2SO_4 colorimetric assays have also been used (Kennedy, 1973). The reproducibility and sensitivity of these assays, combined with the fact that a spectrophotometer was available in the department, led to the adoption of a colorimetric

technique for carbohydrate detection, in place of the moving wire/ flame ionisation detector of Hobbs & Lawrence (1972). The more common colorimetric methods involve the use of concentrated H_2SO_4, which can cause considerable damage to the spectrophotometer if any leaks develop in the system. In order to avoid this an extremely sensitive, non-corrosive technique using p-anisyl tetrazolium chloride as the developing agent was adopted (Mopper & Degens, 1972).

The use of alkaline p-anisyl tetrazolium chloride for the quantification of reducing sugars in batch systems has been recognised for several years (Cheronis & Zymaris, 1957). The alkaline solution of the quaternary ammonium salt is colourless, but on reduction, which takes place in the presence of the reducing group of carbohydrates, highly coloured substances called formazans are produced. Although the diformazan anion is water soluble, the reaction can carry on past this stage and an insoluble diformazan precipitate is formed. This insoluble precipitate has held up development of the reaction for automated systems. It has recently been discovered that ethanol is effective in preventing this precipitation (Mopper & Degens, 1972). This is fortunate, as ethanol is the eluant used in many of the HPLC systems developed for sugar analysis, and utilised in the separation system developed by Hobbs & Lawrence (1972). Thus, the separation system and the method of detection selected for this study appear to be compatible.

HIGH PRESSURE LIQUID CHROMATOGRAPHY (HPLC)

Materials and Methods

Apparatus

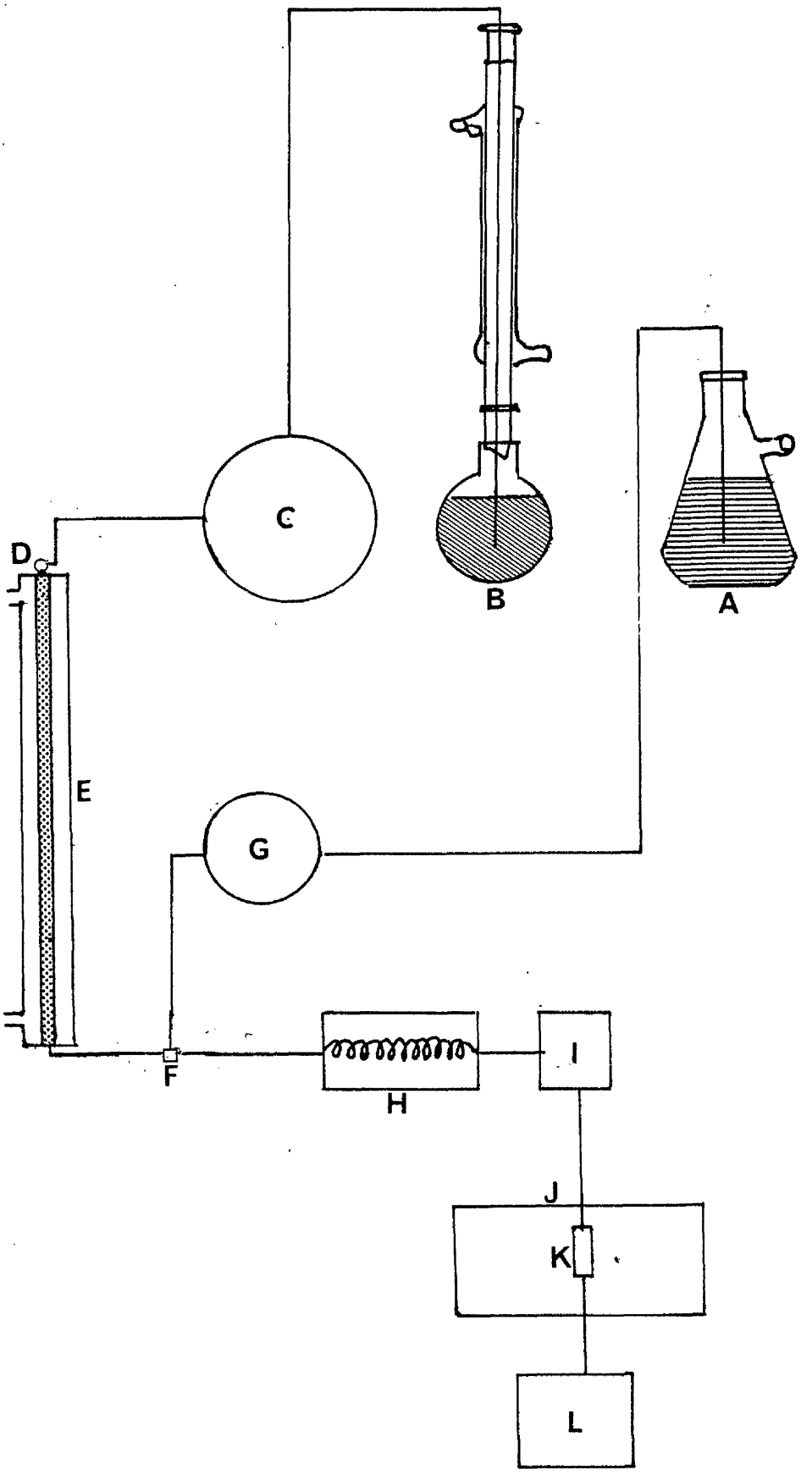
A liquid chromatographic system consisting of a constantly refluxing solvent reservoir, pump, thermostatically controlled column, and continuous spectrophotometric detection system was used (Figure 2).

A variable flow rate, reciprocating piston pump, model LD 711, supplied by James Jobling Ltd (Stone, Staffordshire) delivered the eluant to the column. This pump is fitted with an adjustable, high efficiency pulse dampener, which enables a constant flow rate to be maintained. It is capable of working up to a maximum pressure of 1200 pounds per square inch (psi) and automatically compensates for changes in column resistance caused by slight variations in eluant composition and column temperature.

Figure 2 A schematic representation of the High Pressure Liquid Chromatographic system used to quantify the monosaccharide components of pig slurry hydrolysate.

A	deareated p-anisyl tetrazolium chloride
B	refluxing 80% w/w ethanol
C	high pressure pump
D	injection port
E	water jacketed chromatography column
F	three-way connector
G	peristaltic pump
H	thermostatically controlled water bath
I	cold water
J	spectrophotometer
K	flow-through cell
L	chart recorder

Figure 2



The column and accessories were also supplied by James Jobling Limited. The 100 cm long column has an internal diameter of 0.4 cm, and is constructed of borosilicate glass, polytetrafluoroethylene (PTFE) and "delrin". It is capable of operating up to a pressure of 800 psi, and is so designed that only glass and PTFE come into contact with the mobile phase. The column was heated by means of a water jacket, through which water circulated from a thermostatically controlled water bath.

The eluant from the column passed through 0.58 millimetre (mm) internal diameter (ID) PTFE tubing to a three-way PTFE connector, where it was mixed with a 0.2% w/v solution of p-anisyl tetrazolium chloride. The dye was pumped into the system by an almost pulse-free, variable speed, peristaltic pump, model 203, supplied by Scientific Industries Incorporated (UK) Ltd, (PO Box 26, Loughborough, Leicestershire, LE11 1DB).

From the three-way capillary connector, the eluant and dye mixture passed to a thermostatically controlled water bath at 80°C. A 211 cm length of 0.58 cm ID tubing was coiled in the bath. At the combined eluant and dye flow rates this allowed the mixture to be heated to 80°C for exactly 1 min.

After heating the reaction mixture passed through a thermos flask of cold water. Cooling the dye had the effect of stopping the reaction and stabilising colour development.

The cool mixture then passed to the detection system. This consisted of a Pye Unicam SP 1800 spectrophotometer (Pye Unicam, York Street, Cambridge), with a 1mm pathlength flow-through tubular quartz cell in the second sample position. The absorbance of the liquid passing through the cell was read at a wavelength of 520 nanometres (nm) on the 0-0.5 scale, with a slit width of 0.26 mm. The plot of absorbance against time was recorded on a Pye Unicam AR 25 linear recorder.

Separation System

The column was packed with a strongly acidic cation exchange resin Aminex A6, which had a particle size of 17.5 micrometres (μm) $\pm$ 2 μm (Biorad Laboratories Ltd, Homesdale Road, Bromley, Kent). This resin was converted to the trimethylammonium form by repeated washing with a normal (N) solution of trimethylamine prior to packing. The column was packed by allowing a slurry of the resin in the developing solvent to settle under gravity, and then applying pressure to the settled

resin. A pressure slightly higher than the operating pressure was used to ensure that the column did not pack down during use, thereby avoiding the creation of a dead space at the top of the column.

Hobbs & Lawrence (1972) reported an efficient separation of a complex mixture of monosaccharides and disaccharides on a column of Aminex A6 resin charged with trimethylammonium counterions. In this work they used a column of 0.4 cm diameter and 100 cm length at a temperature of 65°C. The eluant was 85% weight/weight (w/w) ethanol flowing at a rate of 0.35 $ml.min^{-1}$. For the purpose of separating the much simpler monosaccharide mixture of pig slurry hydrolysate, namely xylose, arabinose, glucose and galactose, on a similar column at an eluant flow rate of 0.35 $ml.min^{-1}$ it was found that the eluant could be diluted to 80% w/w ethanol and the column temperature raised to 85°C. This had the effect of considerably decreasing the analysis time and sharpening up the peaks (Figure 3). Increasing the water content of the mobile phase had the effect of decreasing the differential migration (Equation 1) of the component carbohydrates (Table 8).

EQUATION 1

$$K^1 = \frac{t_r - t_0}{t_0}$$

K^1 is the differential migration

t_r is the time taken for carbohydrate component X to pass through the column

t_0 is the time taken by the solvent or non-retained molecules such as α-tocopherol to pass through the column

Elevating the column temperature from 65°C to 85°C increased the separation efficiency and further decreased the differential migration.

80% w/w ethanol at 85°C was one of the extremes of conditions studied by Hobbs & Lawrence (1972). There was no information in this work on the effect of higher column temperatures on the column packing material or on monosaccharides. Thus, as 80% ethanol at 85°C gave acceptable separations of xylose, arabinose, glucose and galactose, these conditions were adopted for the analysis of pig slurry hydrolysate.

95% w/w ethanol was diluted to the required 80% w/w level using glass distilled water. A constant check was kept on the concentration of the ethanol solution by measuring the specific gravity (0.846). It was shown to be essential to use analar grade ethanol, which is

Figure 3 The effect of changes in column temperature and solvent composition on the separation of monosaccharides by HPLC.

A	Eluant Composition	85% w/w
	Temperature	$65^{o}C$
B	Eluant composition	80% w/w
	Temperature	$85^{o}C$

Figure 3

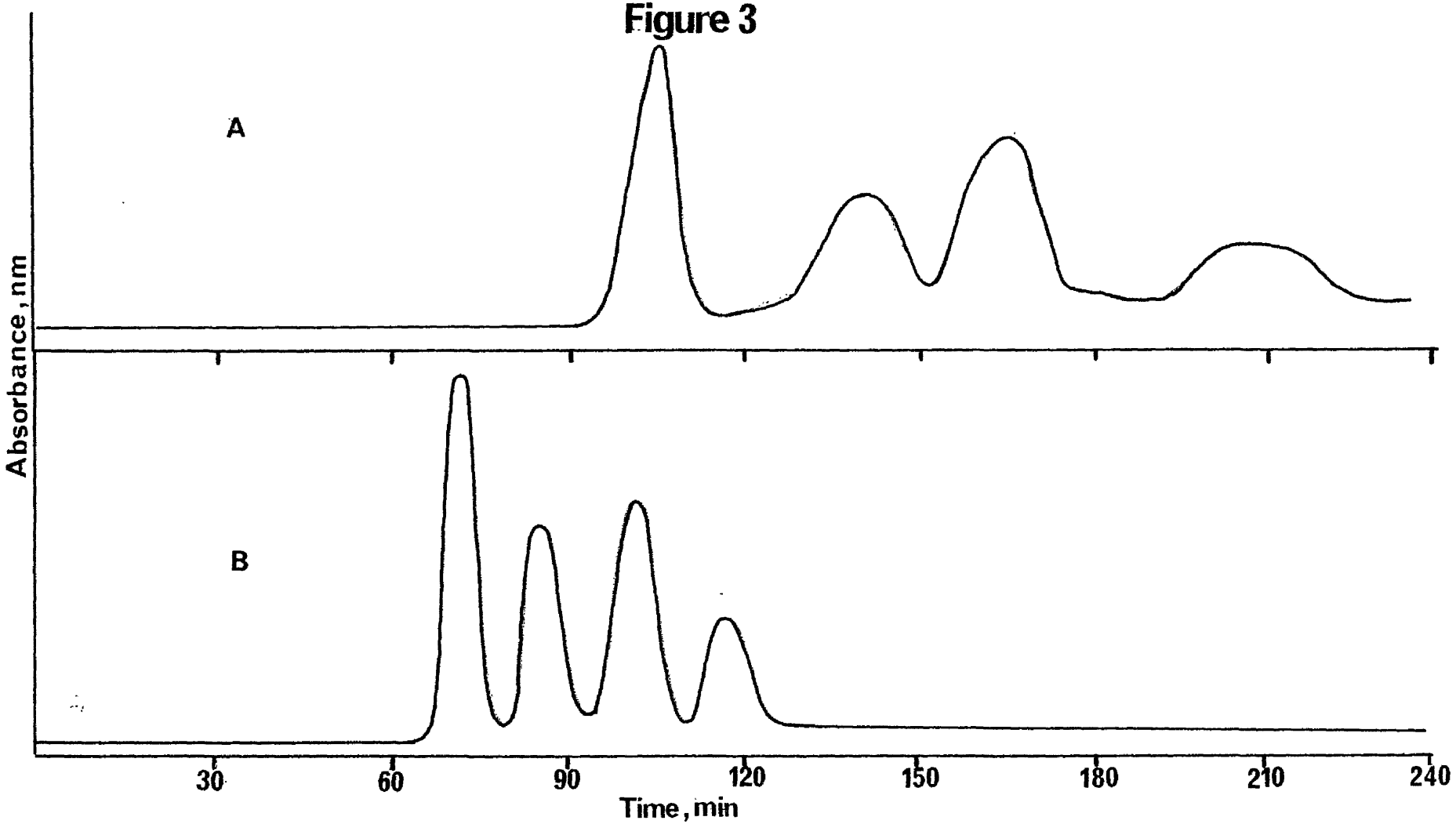


TABLE 8 The effect of solvent composition and temperature on the differential migrations of carbohydrates.

SOLVENT COMPOSTION W/W ETHANOL	TEMPERATURE ^{o}C	DIFFERENTIAL MIGRATION			
		XYLOSE	ARABINOSE	GLUCOSE	GALACTOSE
85%	65	6.6	9.1	10.9	13.8
80%	65	6.0	8.6	10.0	12.7
80%	85	4.9	6.2	8.0	9.3

TABLE 9 Resolution of monosaccharide peaks obtained with a column temperature of $85^{o}C$, eluant composition of 80% w/w ethanol, and flow rate of 0.35 $ml.min^{-1}$.

MONOSACCHARIDE PEAKS	RESOLUTION %
Xylose and Arabinose	135
Arabinose and Glucose	160
Glucose and Galactose	126

TABLE 10 Time taken for monosaccharides to pass through the HPLC system.

MONOSACCHARIDES	TIME (min)
Xylose	66-78
Arabinose	80-92
Galactose	109-125

completely free of metal ions, as such contaminating substances can seriously affect the charge on the column.

The eluant flow rate was another variable that was adjusted to reduce separation time. Unfortunately, increasing the eluant flow rate decreased the resolution of the peaks, as defined by Equation 2

EQUATION 2

$$R_s = \frac{t_2 - t_1}{0.5\ (tw_1 + tw_2)}$$

R_s is resolution

t_1 is the time taken by component X to pass through the column

t_2 is the time taken by component Y to pass through the column

tw_1 is the band width of component X

tw_2 is the band width of component Y

Snyder & Kirkland (1974) state that to obtain a precision of greater than $\pm$ 1% in the quantification of minor peaks by a peak area method, resolution has to be at least 125%. The resolution of the peaks obtained for xylose, arabinose, glucose, and galactose, using a column temperature of 85°C, eluant composition of 80% w/w and the flow rate of 0.35 $ml.min^{-1}$ recommended by Hobbs & Lawrence (1972) are shown in Table 9. From these results it can be seen that the least resolved components are glucose and galactose, which have a 126% resolution. Thus, any increase in flow rate above 0.35 $ml.min^{-1}$ will jeopardise accurate quantification of glucose and galactose.

It was concluded from these initial investigations that a separation system incorporating a column temperature of 85°C, an eluant composition of 80% w/w ethanol, an eluant flow rate of 0.35 $ml.min^{-1}$ efficiently separated xylose, arabinose, glucose and galactose.

Sample application

Samples were injected into the column through a silicon rubber septum, which was fitted into a standard Jobling glass-loaded PTFE injection head. Injections were made with a Hamilton HP 305 N high pressure syringe of 5 μl capacity, supplied by V A Howe and Co Ltd (88 Peterborough Road, London SW6). In order to establish a standard injection technique, 5 μl samples were always applied to the column, and therefore sample concentrations were altered accordingly.

Detection System

A 0.2% w/v solution of p-anisyl tetrazolium chloride was supplied by Eastman Organic Chemicals (Kodak Ltd, Acornfield, Kirkby, Liverpool) as agents for K & K Laboratories (Plainview, New York). The dye was prepared as a 0.2% w/v solution in 0.18 N NaOH (Mopper & Degans, 1972). Preliminary tests in the laboratory showed that there was no significant drop in dye sensitivity during a 10 h period, and therefore it would be sufficient to prepare fresh dye solution daily. Mopper & Degans (1972) reported that Beer's Law holds for sugar concentrations up to 50 μg, and that the limit of detectability is 10^{-10} moles.

The eluant and dye mixture was heated at 80°C for exactly 1 min. Cheronis & Zymaris (1957) demonstrated that severely overheating this dye could affect the accuracy of quantification, however heating at 95°C for 1 to 1.5 min had negligible effect.

It was observed that with only the ethanol eluant passing through the flow-through cell of the detection system, a large amount of baseline noise appeared on the recorder. This was not acceptable for accurate quantification, and was traced to the fact that the spectrophotometer was working at negative absorbance on the limit of its specifications. When the eluant and dye mixture passed through the system, however, the absorbance was raised slightly above this critical value, and the baseline noise virtually disappeared.

In order to prevent the formation of air bubbles in the low pressure lines of the detection system, it was necessary to degas the eluant and dye solutions prior to use. Initially, this was achieved by evacuation with a vacuum pump prior to each 8 h run. During the first exploratory experiments, however, considerable interference in the absorption readings on the spectrophotometer were observed. On investigation, this was found to be caused by a continual stream of small air bubbles in the eluant/dye mixture. Constant refluxing of the aqueous ethanol prior to entry into the delivery system was found to overcome this problem. Evacuation at the beginning of a run was still found to be adequate to remove air from the dye solution.

Finally, it was found that using this HPLC system non-retained molecules such as d-tocophenol pass through the separation system and detection system in 14.5 min. This is equivalent to the

passage of 50.75 ml of eluant which is termed the "dead volume" of the system. The times taken for xylose, arabinose, glucose and galactose to pass through the HPLC system were also recorded and are shown in Table 10.

Assessment of HPLC as a Method for the Quantification of Xylose, Arabinose, Glucose and Galactose

A standard calibration curve relating peak area to sample weight was prepared for each monosaccharide to be quantified. In order to produce statistically significant results, each time an individual monosaccharide was analysed, standard 10 μg, 20 μg, 40 μg and 50 μg amounts were injected in triplicate. This procedure was repeated on three separate days, so that the significance of slight variations in conditions such as column temperature, eluant composition, eluant flow rate, dye flow rate, dye concentration and dye development temperature could be evaluated.

Peak area was calculated by multiplying peak height by width at half height. Linear Regression Analysis (Davies & Goldsmith, 1972) was performed on the results for the four monosaccharides tested. The results of the statistical analysis are shown in Table 11.

These results indicate that the weight of monosaccharide in the sample can be predicted with 95% confidence to be within $\pm$ 2.0 μg of the estimated value for xylose, $\pm$ 1.9 μg for arabinose, $\pm$ 1.5 μg for glucose and $\pm$ 2.6 μg for galactose. From this analysis standard calibration curves were produced for xylose (Figure 4), arabinose (Figure 5), glucose (Figure 6) and galactose (Figure 7).

Preparation of Pig Slurry Hydrolysate for HPLC

A brown acid liquid containing a substantial amount of soluble inorganic salt was produced by the H_2SO_4 hydrolysis of pig slurry. This liquid was separated from the residual solid by centrifugation.

In order to assess the yield of utilisable carbohydrate produced by a particular hydrolysis system, the liquid was analysed by HPLC. Successful analysis of the carbohydrate components of this liquid could only be achieved if it was first neutralised, desalted and decolourised. The major requirement of any purification system is that no significant loss of carbohydrate occurs at any stage in the process. Thus, the destructive effect of various neutralisation,

TABLE 11 Statistical analysis of standard calibration curves relating peak area to weight obtained by HPLC of monosaccharides

	XYLOSE	ARABINOSE	GLUCOSE	GALACTOSE
Intercept	+12	+7.4	+11.5	-1.5
Standard error in intercept	4.30	2.96	2.94	3.65
95% confidence limits on intercept	$\pm$8.7	$\pm$6.0	$\pm$6.0	$\pm$7.4
Slope	8.93	6.6	8.3	5.9
Standard error on slope	0.13	0.087	0.087	0.105
95% confidence limits on slope	$\pm$6.026	$\pm$0.018	$\pm$0.020	$\pm$0.021

Standard calibration curves relating weight of carbohydrate to peak area obtained during HPLC analysis

Figure 4: Xylose

Figure 5: Arabinose

Figure 6: Glucose

Figure 7: Galactose

——— standard curve

– – – 95% confidence limits on standard curve

Figure 4

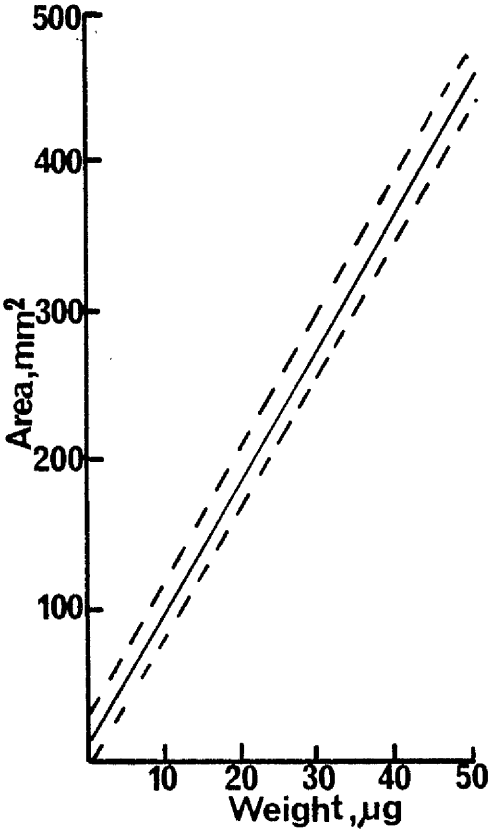


Figure 5

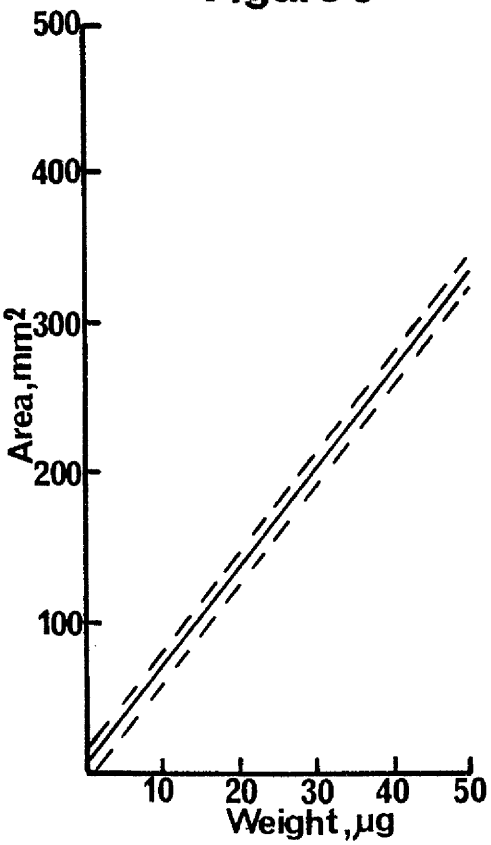


Figure 6

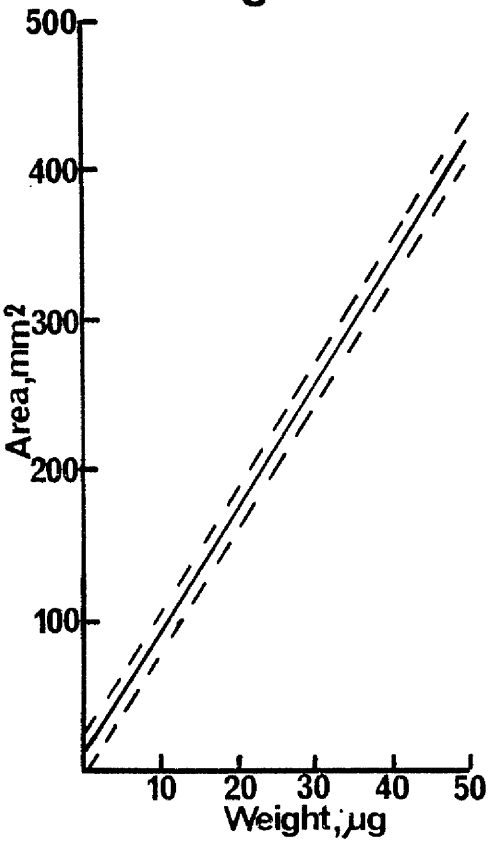
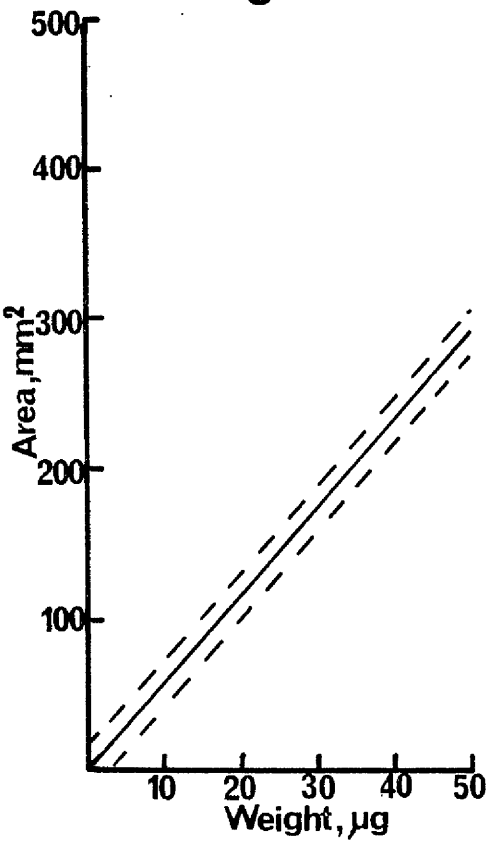


Figure 7



desalting and decolourising procedures were tested in the laboratory using a standard solution containing 1% w/v arabinose, 1% w/v galactose, 1% w/v glucose and 1% w/v xylose. This standard mixture was consistent in basic composition for all procedures tested. During the investigation of desalting and neutralisation, other components such as salt or acid had to be added. The quantities of monosaccharide remaining after decolourising, desalting or neutralising were measured by HPLC.

Decolourising: The hydrolysate produced by acid treatment of pig slurry is intensely brown in colour. It was thought that this colour was mainly attributable to bile pigments in the raw slurry and to the breakdown products of carbohydrates produced during acid hydrolysis. Prior to HPLC analysis these coloured organic compounds and other non-coloured large molecular weight organic compounds had to be removed in order to prevent the progressive contamination of the column.

The simplest method of removing colour is the addition of activated charcoal (Bevenue, 1949). It was shown that mixing 100 ml of hydrolysate with 2 g of activated charcoal removed all the coloured compounds. The charcoal was allowed to settle out prior to HPLC analysis. The results of chromatography indicated that activated charcoal did not absorb monosaccharides during the decolourising procedure described.

Desalting: Before a sample could be analysed by HPLC the inorganic salts, which interfere with the charge on the ion-exchange resin, had to be removed. Several methods of desalting biological fluids were considered. These were mixed bed cation and anion exchange resins, ion-retardation resin, ultrafiltration, gel filtration and drying prior to redissolving the carbohydrates in solvents such as methanol or ethanol in which inorganic salts are insoluble.

Examination of the specifications for ultrafiltration and gel filtration indicated that neither method could successfully desalt a solution of monosaccharides. The smallest available pore size for commercial ultrafiltration equipment allowed the passage of components with a molecular weight (MW) of 500 or below. Thus, both salts and monosaccharides passed through the membrane. "Desalting" is one of the major applications of gel filtration using cross-linked dextran.

In this context the term "desalting" refers not only to the removal of salts but also to the removal of other low molecular weight compounds from solutions of polymers. It was shown on columns of Sephadex G10 and G15 (Pharmacia (GB) Ltd, Sinclair House, West Ealing, London W13) that satisfactory separation of salts and monosaccharides was impossible by this method.

Columns of ion-retardation resin have been reported to remove sodium chloride from solutions of amino acids (Heathcote *et al*, 1971) and sugars (Biorad, 1973) with reproducible and quantitative recovery. Retardation of charged molecules is due to the unique structure of the resin with its adjacent anion and cation exchange sites. These attract the mobile ions and associate weakly with them retarding their progress through the column. Any uncharged molecules pass straight through the column in the eluant,which is normally water.

In order to test the ability of this method to desalt pig slurry hydrolysate, a test solution containing 1% $(NH_4)_2SO_4$ and 1% xylose, 1% arabinose, 1% glucose and 1% galactose was prepared. $(NH_4)SO_4$ was considered to be one of the major salts present in H_2SO_4 hydrolysed pig slurry. The ion-retardation resin Ag11A8 was supplied by Biorad Laboratories Ltd. Several combinations of sample loading rate, column length, column diameter and eluant flow rate were investigated. On collection in a fraction collector, the eluant was tested for the presence of salt and sugar. The presence of ammonium (NH_4^+) ion was detected by Nessler's reagent, sulphate (SO_4^{2-}) ions by calcium chloride, and monosaccharides by phenol/ H_2SO_4 reagent. The complete separation of monosaccharides and ammonium sulphate was not achieved in laboratory tests. This result is explained by the fact that Ag11A8 has been shown to have a greater affinity for chloride than sulphate (Hatch *et al*, 1957). Thus ammonium sulphate passed through the column relatively quickly, and was difficult to separate from non-electrolytes such as sugars.

Williams & Wright (1959) have reported that mixed beds of weakly basic anion exchange and strong cation exchange resins efficiently desalt solutions of salts and sugars. This was tested in the laboratory using a weakly basic anion exchanger Ag 3 X 4A (Biorad Laboratories Ltd) and strong cation exchanger Amberlite IRC (BDH Laboratory Chemicals Division) in the standard $(NH_4)_2SO_4$/monosaccharide saccharide solution. NH_4^+ and SO_4^{2-} ions were shown to be absent from

the desalted solution. However, HPLC analysis indicated that this procedure had a significant destructive effect on arabinose, galactose, glucose and xylose. The pentoses were apparently more labile than the hexoses.

The final method of desalting to be investigated was the drying of the sample, followed by resuspension in a polar solvent such as ethanol. Such solvents solubilise monosaccharides but do not solubilise inorganic salts which remain in suspension, and can be removed by simple decanting after settling or centrifugation. It is known that heating in the presence of ammonia decomposes monosaccharides. Thus, a freeze-drying procedure was adopted to avoid this problem. Samples of the standard $(NH_4)_2SO_4$/monosaccharide solution were dried to a pressure of 0.05 Torr on an Edwards Model EF03 Freeze Dryer (Edwards High Vacuum (Plant) Ltd, Manor Royal, Crawley, Sussex). The sugars were redissolved in ethanol by shaking the suspension on a wrist-shaker for 30 min. The recovery of monosaccharides was shown to be poor by HPLC analysis. However, when the slightly more polar methanol was used the monosaccharides were quantitatively recovered. In qualitative tests both ammonia and sulphate ions were shown to be absent from the methanolic solution of carbohydrates. The complete absence of salt was confirmed using a Conductivity Bridge. This procedure was adopted to desalt pig slurry hydrolysate prior to HPLC analysis.

Neutralisation: The method adopted for the neutralisation of acid hydrolysed slurry had to be able to neutralise concentrations of acid of between 1% and 10% without causing significant destruction of the carbohydrates. Several neutralising agents were tested. These were weakly basic anion exchange resin Ag 3 X 4A charged in both the hydroxyl (OH^-) and carbonate (CO_3^{2-}) form, ion-retardation resin Ag11A8, calcium hydroxide, calcium carbonate and sodium hydroxide. In order to test the efficiency of these neutralising agents a solution containing a mixture of 1% w/v arabinose, 1% w/v galactose, 1% w/v glucose , 1% w/v xylose and 10% w/v H_2SO_4 was used. 10% H_2SO_4 was employed as it was the highest concentration to be used during the hydrolysis experiments. After neutralisation the resulting liquids were desalted by the freeze-drying procedure described above, and the amount of monosaccharide recovered was

calculated from the results of HPLC analysis.

Both the ion-retardation resin and the weakly basic anion exchange resin were shown to have considerable destructive effect on monosaccharides during neutralisation. In all cases pentoses were more labile than hexoses. The anion exchange resin charged in the CO_3^{2-} form caused less decomposition than when it was charged with OH^- ions Apart from the destruction of carbohydrates, resins had another inherent disadvantage, in that large amounts of resin were needed to complete neutralisation. Thus significant volumes of hydrolysate were retained within the structure of the resin particles, and this could only be removed by repeated washing.

Of all the methods investigated, neutralisation with calcium hydroxide ($Ca(OH)_2$) had the greatest destructive effect on the carbohydrates. When $Ca(OH)_2$ was added as a slurry with water the amount of carbohydrate remaining after neutralisation was greater than when it was added as a solid. Although the mechanisms of destruction were not investigated, it was observed that the amount of heat produced during the neutralisation procedure varied with the agent used. The addition of solid $Ca(OH)_2$ which appeared to liberate most heat during neutralisation, also caused the greatest destruction of carbohydrate.

A 100% recovery of the test carbohydrates was achieved when the acid was neutralised with calcium carbonate ($CaCO_3$) or 0.5 NORMAL (N) sodium hydroxide (NaOH). Thus, it appeared that either $CaCO_3$ or 0.5 N (20% w/v) NaOH could be employed to neutralise acidic solutions of monosaccharides.

For neutralisation of pig slurry hydrolysate, NaOH offered distinct advantages over $CaCO_3$. Primarily, the whole hydrolysed pig slurry could be neutralised with NaOH immediately after the completion of hydrolysis. The product of neutralisation was a soluble salt, sodium sulphate (Na_2SO_4), and therefore the solid and liquid could be separated after neutralisation. When $CaCO_3$ reacted with H_2SO_4 insoluble calcium sulphate ($CaSO_4$) was formed. This mixture of residual slurry solids and $CaSO_4$ presented difficulties in the estimation of dry weight and cellulose content of the residual solids, and necessitated the separation of the residual slurry solids and the acidic liquid prior to neutralisation with $CaCO_3$. The products of this process were slurry solids contaminated with significant

amounts of H_2SO_4, and a neutral hydrolysate which required further separation from the insoluble $CaSO_4$. Thus, of the two techniques observed to cause no detectable loss of carbohydrate, neutralisation with NaOH appeared to be the more practical method.

In order to minimise the dilution of the hydrolysate during neutralisation with NaOH, it was proposed to use the most concentrated solution of alkali possible. It was shown in tests that 25% w/v NaOH had no destructive effect, but as the concentration was increased to 50% w/v, the yield of monosaccharides began to fall. Thus, the addition of NaOH in concentrations of 25% or below was adopted for the neutralisation of pig slurry hydrolysates prior to HPLC analysis.

From the information gained from the investigations into the methods available for the neutralisation, desalting and decolourisation of samples of acid hydrolysed pig slurry, a system of preparing samples of the liquid portion of hydrolysed slurry for HPLC analysis was developed. The hot acidic suspension was first cooled in a cold water bath. It was then neutralised to pH 7 with a NaOH solution of concentration 25% w/v or less. The pH was monitored with a pH meter. The residual solid was removed by centrifugation and duplicate 5 ml amounts of the liquid hydrolysate were dispensed into tubes and immediately freeze-dried to a pressure of $\sim$0.05 TORR. The freeze-dried samples were stoppered and stored in a vacuum desiccator over anhydrous calcium chloride ($CaCl_2$). Immediately prior to HPLC analysis, 5 ml of methanol were added to each sample, and the constituent monosaccharides were redissolved by shaking the sample for 30 min on a wrist-shaker. The sample was then decolourised by the addition of 0.1 g of activated charcoal. Finally, the insoluble salts and the charcoal were allowed to settle out and the resulting clear, colourless liquid was removed for HPLC analysis.

The complete preparatory scheme was tested using standard monosaccharide solutions and it was confirmed that none of the carbohydrate components of hydrolysed pig slurry was destroyed by the procedure.

Quantitative Analysis of the Cellulosic Constituents of Pig Slurry

The cellulosic components of pig slurry originate mainly from the cereal components of the pigs diet (Table 3). As in most plant

materials they are present in a complex mixture of other polymeric compounds such as protein and lignin. The quantitative analysis of the cellulosic components is complicated by the fact that the physical and chemical structures and the properties of α-cellulose and of the different hemicelluloses can vary considerably.

Two general methods are available for the determination of α-cellulose and hemicelluloses (Saeman, 1945; Whistler *et al*, 1948). In one reported procedure the entire sample is boiled with dilute H_2SO_4. This has the effect of hydrolysing the hemicelluloses, leaving the α-cellulose intact. The hydrolysate is then analysed for total reducing sugar, or more accurately, because of varying sensitivities to colorimetric methods, the individual monosaccharides are quantified. The residual α-cellulose is dissolved by soaking in 72% H_2SO_4, and the mixture subsequently diluted and boiled to release the glucose units from the solubilised α-cellulose. The resulting glucose is determined by normal colorimetric techniques (Saemen, 1945). A major disadvantage of this method is that the conditions necessary to hydrolyse the different cellulosic fractions vary considerably. The wide range of physical and chemical structures found in plant polysaccharides means that conditions vigorous enough to hydrolyse all the hemicelluloses will almost certainly degrade some of the α-cellulose (Gascoigne & Gascoigne, 1960). There is the added complication that the more labile monosaccharides such as xylose, which are produced by hemicellulose breakdown, can be degraded by mild acid treatment (Stamm, 1964). Thus, with this method, it is obviously possible to introduce significant errors into the quantification.

An alternative gravimetric method is more attractive for use in this work. Originally introduced to determine the cellulosic constituents of corn cobs (Whistler *et al*, 1948), it has been developed and improved by workers analysing peat (Passer *et al*, 1968). In this method the sample is subjected to the action of sodium chlorite ($NaClO_2$), which selectively removes all constituents including lignin, leaving only polysaccharides in their original state. These delignified pulps are termed holocelluloses (Passer *et al*, 1968). While some depolymerisation of the cellulosic fractions may occur, the degradative processes are minimal. The various components of the holocellulose

are differentiated by treatment with chemicals. Firstly, the hemicelluloses are dissolved in potassium hydroxide (KOH), leaving a residue of α-cellulose, which can be dried and weighed. The hemicelluloses can then be precipitated as two different fractions by chemical additions to the alkaline extract. Each fraction may contain one or more hemicellulosic polymer. A fraction termed hemicellulose-A is precipitated by bringing the pH of the alkaline solution to 5 with acetic acid. When this fraction has been removed by centrifugation, another fraction, hemicellulose-B, is precipitated by adding ethanol to the mother liquid (O'Dwyer, 1926). It has been shown that the hemicellulose precipitated by acid addition to the alkaline extract of beech wood holocellulose, is composed mainly of xylose with a small percentage of glucuronic acid. Whereas the hemicellulose precipitated in the ethanolic solution is a mixture of galacturonic acid, arabinose and galactose.

MATERIALS AND METHODS

The procedure adopted in this work was basically that used by Whistler *et al* (1948) for the isolation of the cellulosic fractions of corn cobs. However, the modifications developed by Passer *et al*, (1968) for the determination of α-cellulose in peat were introduced. Duplicate 10 g amounts of a raw or hydrolysed slurry sample which had previously been dried in an air oven at 105°C for 24 h. were used for each analysis. These samples were extracted with benzene/ethanol (32:68 w/w) azeotroph in the Soxhlet apparatus in a fume cupboard for 24 h. This treatment removed fat soluble constituents such as bitumens. The solvent was evaporated in a stream of air at room temperature for approximately 24 h. The sample was then suspended in 230 ml of distilled water contained in a wide-necked, water jacketed reaction vessel, through which water circulated to maintain a temperature of 80°C in the reaction mixture. Oxidation of the lignin was achieved by a stepwise reaction, in which the addition of 0.8 ml of glacial acetic acid was followed by careful addition of 2.5 g of $NaClO_2$ (technical grade supplied by BDH Chemicals Ltd). This procedure was repeated every 15 min, until a total of four additions had been made, and then the mixture was left stirring at 80°C for a final 15 min to ensure the completion of delignification. Throughout the oxidation procedure

the reaction vessel was contained in a fume cupboard and the reaction mixture was stirred by placing the vessel on a magnetic stirrer. A stream of nitrogen was introduced into the reaction vessel to prevent the build up of an explosive mixture of chlorine dioxide and air (Passer *et al*, 1968). When the reaction was complete, rapid cooling of the mixture was achieved by filling the water jacket with cold water. The holocellulose was isolated by centrifugation and then repeatedly washed with distilled water to remove the acid. After a final wash with acetone it was dried in an air oven at 105°C for 4 h.

The hemicellulosic constituents were removed by subjecting the holocellulose to the action of 100 ml of 24% KOH. The alkaline mixture was sealed in a flask in an atmosphere of nitrogen, which prevented oxidation of the hemicelluloses by atmospheric oxygen. The contents were continuously stirred for 24 h. The residue of α-cellulose was separated from the alkaline solution by centrifugation, and the liquid portion retained for isolation of the hemicellulosic fractions. The α-cellulose was purified by washing in 1% KOH, followed by distilled water, and finally 10% acetic acid. The residual acid was removed by repeated washings in distilled water, and the α-cellulose dried in pre-weighed moisture dishes at 105°C for 4 h until a constant weight was obtained.

The liquid alkaline extract was acidified with 50% acetic acid to a pH of 5. On standing for 4 h, a solid fraction precipitated in the liquid. This solid fraction was removed by centrifugation and is known as hemicellulose A (O'Dwyer, 1926). The slightly opalescent centrifugate was retained for further analysis. The hemicellulose A was dehydrated by dispersing in 99% ethanol and recentrifuging. This procedure was repeated once and followed by an ether wash. The hemicellulose A was dried on a pre-weighed filter paper in a vacuum dessicator over $CaCl_2$ and weighed.

The residual centrifugate from the isolation of hemicellulose A was poured into three times its volume of 95% ethanol. The ethanolic mixture was rapidly stirred, and the B-fraction hemicellulose precipitated. The slightly yellow precipitate was dehydrated by the same procedure as that described for hemicellulose A, and the dry weight was obtained.

Mild Sulphuric Acid Hydrolysis of Pig Slurry

MATERIALS AND METHODS

Preparation and Standardisation of the Sample of Pig Slurry Prior to Acid Hydrolysis

In order to compare the effect of different acid hydrolysis procedures on pig slurry, the composition of the starting material had to be consistent throughout the work. As the hydrolysis experiments would take several months to complete, it had to be accepted that the composition of the slurry would vary during the experimental period. The major factors contributing to this variation were the nutritional status, maturity and health of the pigs, and the urine content of the slurry. Standardisation of the solid/liquid ratio of the slurry prior to each experiment presented another problem. It was essential that a constant dry weight of slurry was hydrolysed in each experiment, in order to compare the efficiency of different hydrolysis procedures.

The solution to this sampling problem appeared to be the collection and storage of a bulk quantity of slurry that would be sufficient for the duration of the hydrolysis work. There appeared to be two feasible methods of stabilising and storing a large quantity of slurry. These were freezing followed by frozen storage, and oven drying followed by storage in the absence of water. Several problems were entailed in the freezing and storing of a bulk sample. A large quantity had to be collected at one time, thoroughly mixed and divided into individual quantities for each experiment, prior to freezing. Unfortunately, the volume of slurry available for collection on any one day was considerably smaller than that required to complete the hydrolysis experiments, and there was insufficient freezer space.

Oven drying of raw slurry had the advantage of making sample collection more representative and convenient. With this method several small batches of slurry were collected daily and dried in a hot air oven at 90^{o}C until a constant weight of solids was obtained. A temperature below 100^{o}C was used in order to minimise the disruption of the structure of the slurry components during the removal of the water. The bulked solids were easily mixed to achieve homogeneity. It was accepted that there were physical and chemical differences between the original raw slurry and the dry solids with water added

back, especially in the nitrogenous components. However, these experiments were designed to study the effect of H_2SO_4 hydrolysis on the fibrous cellulosic components of the slurry, which were stable to drying. Drying had the advantage that the collection of the slurry over several days produced a more representative bulk sample. Thus oven drying was the method adopted for sample preparation and standardisation. The bulk sample was stored at room temperature.

Experiments

20 g of dry slurry and 180 ml of distilled water were added to a 250 ml ground glass jointed Erlenmyer flask to provide a mixture of approximately the average solid content of raw slurry (110 g dry weight.l^{-1}). The requisite amount of H_2SO_4 was added in a volume of 20 ml. Thus, acid concentrations of 100%, 50%, 25% and 10% w/v were used to produce final concentrations of 10%, 5%, 2.5% and 1% w/v respectively in the hydrolysis mixtures.

The flasks of acidified slurry were heated in a thermostatically controlled water bath. A metal framework was constructed around the bath to hold the flasks and house a stirring mechanism for the flasks. The temperatures investigated in the hydrolysis experiments were 80°C, 90°C and 100°C. At temperatures of 80°C and 90°C evaporation from the water bath was prevented by polypropylene spheres floating on the surface. A temperature of 100°C could not be maintained in a water filled bath, and in this case polyethylene glycol (BDH Chemicals Ltd) was used. Six flasks were heated in the bath at one time. Evaporation from the flasks was prevented by Allihn condensers. The lower end of the ground glass coupling of the condenser was modified so that the shaft of a collapsible glass stirrer passed through the condenser, and was held firmly in place by a PTFE gromet in the rim of the coupling. The stirrers were driven by a chain mechanism powered by an electric motor, so that the slurry was mixed throughout the hydrolysis procedure.

For each temperature and acid concentration tested, a series of exposure times was investigated. The duration of exposure varied with each acid concentration. Duplicate flasks of slurry were analysed at each sampling time, and each test series was repeated once.

On removal from the bath the flasks were immediately plunged into

ice-cold water to prevent further hydrolysis of the sample. When the sample was cool, the acid was neutralised with NaOH. The use of NaOH has already been discussed in the section dealing with the development of a method of neutralisation. A constant volume of NaOH was added to each sample, so that the final volume of the hydrolysate was always the same. As concentrations of 25% w/v NaOH or below had been shown to have no measurable destructive effect on the sugars during neutralisation (page 49), concentrations of 25%, 12.5%, 6.25% and 2.5% w/v NaOH were used to neutralise 10%, 5%. 2.5% and 1% w/v H_2SO_4 respectively. Thus, approximately 118 ml of NaOH solution were added to each sample. In order to check that the final pH of the hydrolysate was 7, it was constantly monitored throughout neutralisation by a pH meter.

The neutralised samples were centrifuged to separate the solid and liquid portions. A trial experiment had established that washing the separated solids with 200 ml of distilled water recovered most of the free monosaccharides, and that further washing achieved negligible increase in yield. The centrifugate and washings were bulked together for analysis. The final volume of liquid obtained from 20 g of dry slurry solids varied from between 490 and 500 ml, depending on the hydrolysis conditions. More water appeared to remain bound to the solids which had been treated by milder hydrolysis procedures.

Duplicate 5 ml quantities of each sample were immediately freeze-dried for subsequent HPLC analysis. After drying, the tubes were stoppered and placed in a vacuum desiccator over anhydrous calcium chloride ($CaCl_2$).

The solid residues from hydrolysis were dried in pre-weighed moisture dishes. Hot air oven temperatures of 80°C, 90°C and 105°C were tested for drying the residual slurry solids. The standard air drying temperature of 105°C did not have any adverse effects on the sample, and required considerably less time to complete the drying procedure than did the lower temperatures. A time of 24 h was found to be sufficient to bring all samples to a constant weight. Therefore, 105°C for 24 h was adopted as a standard procedure for drying slurry residues. The dried solids were sealed in polythene bags and stored in a vacuum desiccator over anhydrous $CaCl_2$ prior to analysis for cellulose and hemicellulose content.

RESULTS AND DISCUSSION

In order to establish the proportion of cellulosic constituents in raw pig slurry, ten different samples were analysed by the $NaClO_2$ method. The results demonstrated that there is little variation in the amount of cellulosic material present in raw pig slurry. On average, approximately 43% of dry slurry solids are cellulosic in nature. The digestion method used to determine the cellulosic constituents enables three different fractions to be isolated. These are α-cellulose, hemicellulose A and hemicellulose B. It was established than on average 43% w/w of the cellulose constituents can be isolated as α-cellulose, 48% w/w as hemicellulose B and 9% w/w as hemicellulose A. However, the batch of slurry used for the hydrolysis trials was found to contain 41% w/w cellulosic constituents, of which 43% w/w was α-cellulose, 47% hemicellulose B and 10% hemicellulose A.

Figures 8, 9 and 10 show the effect of 10% H_2SO_4 at 80°C. 90°C and 100°C respectively on the cellulosic constituents of pig slurry. It can be seen from these results that the weight of all the cellulosic fractions decreases rapidly in the first 2 h and thereafter remains virtually constant throughout the rest of the hydrolysis period. Hemicellulose B, which is initially the largest fraction, disappears almost completely, and after 2 h at all temperatures, approximately 95% is hydrolysed. Approximately 60% of the fraction known as hemicellulose A disappears in 2 h. However less than 20% of the fraction isolated as α-cellulose is attacked, although at 100°C (Figure 10) there appears to be a more extensive attack of the α-cellulose with 35% loss in weight being measured.

A similar general pattern is observed when pig slurry is hydrolysed by 5% H_2SO_4 at 80°C, 90°C and 100°C respectively (Figures 11, 12 and 13). With 2.5% acid at 80°C, 90°C and 100°C (Figures 14, 15 and 16)) similar residual levels of the different cellulosic fraction are observed, but these are attained after a longer time of exposure to the acid conditions. At this acid concentration, as the temperature of hydrolysis falls, the decrease in the cellulosic constituents with time is more gradual. The same pattern appears in Figures 17 and 18, which show the effect of 1% acid at 90°C and 100°C respectively. In this case the decrease in the

The effect of hydrolysis with 10% w/v H_2SO_4 on the residual weight of the cellulosic components of pig slurry per gram of solids hydrolysed.

Figure 8: 80°C
Figure 9: 90°C
Figure 10: 100°C

● α-cellulose
○ hemicellulose A
△ hemicellulose B

Figure 8

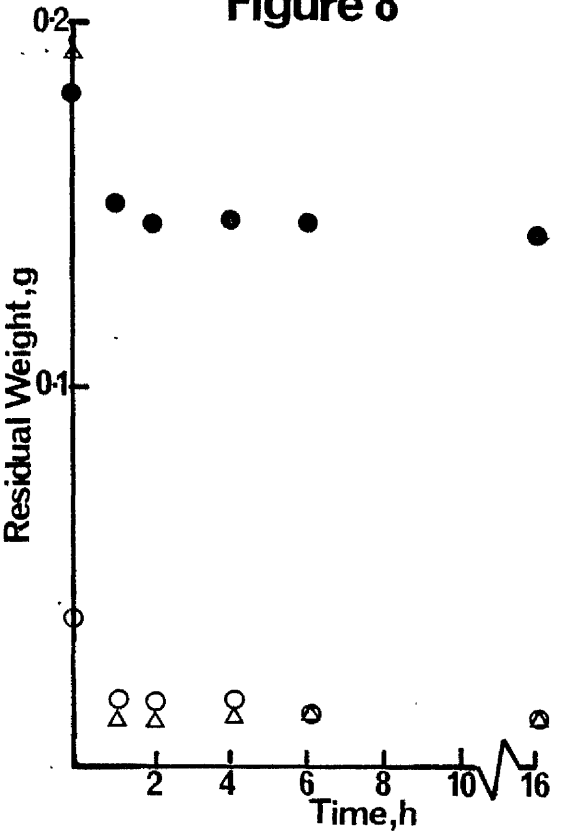


Figure 9

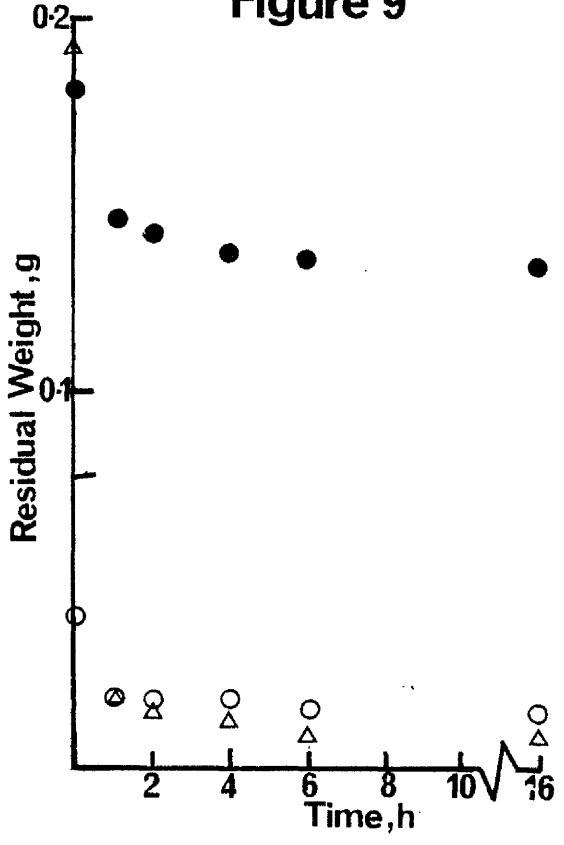
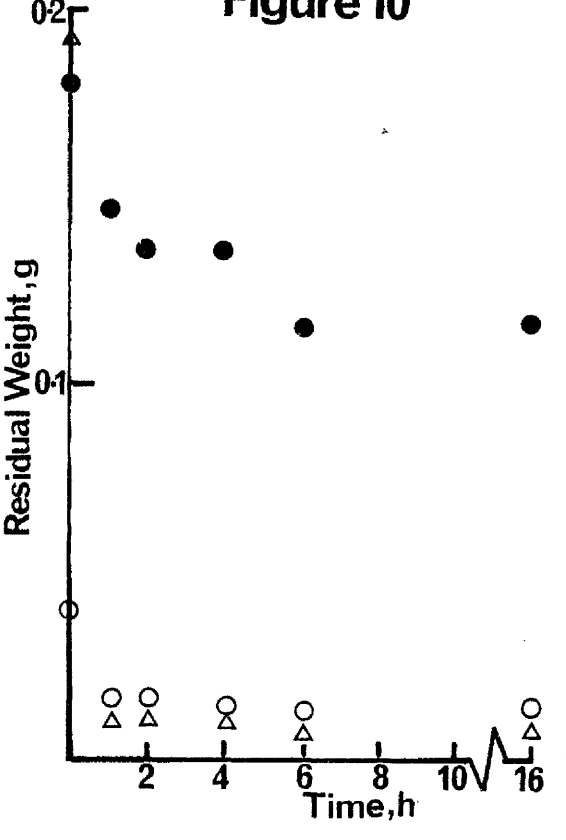


Figure 10



The effect of hydrolysis with 5% H_2SO_4 on the residual weight of the cellulosic components of pig slurry per gram of solids hydrolysed.

Figure 11: 80°C

Figure 12: 90°C

Figure 13: 100°C

- ● α-cellulose
- ○ hemicellulose A
- △ hemicellulose B

Figure 11

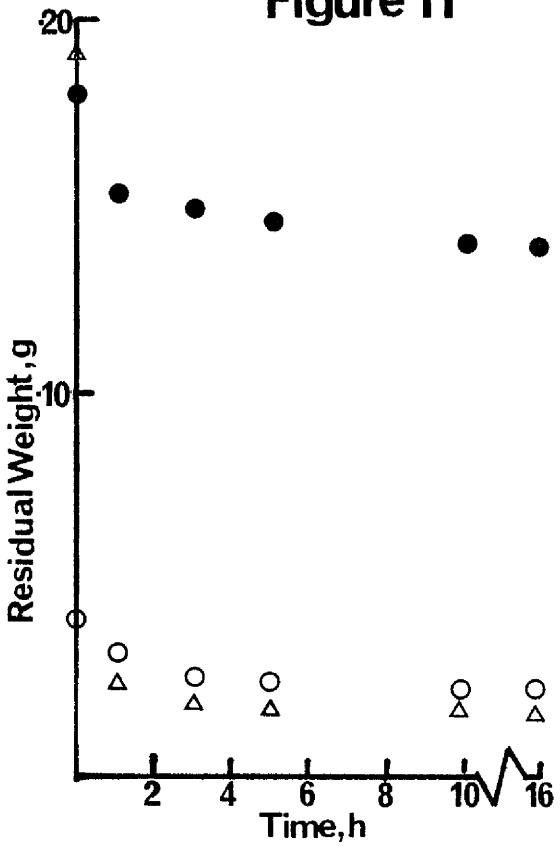


Figure 12

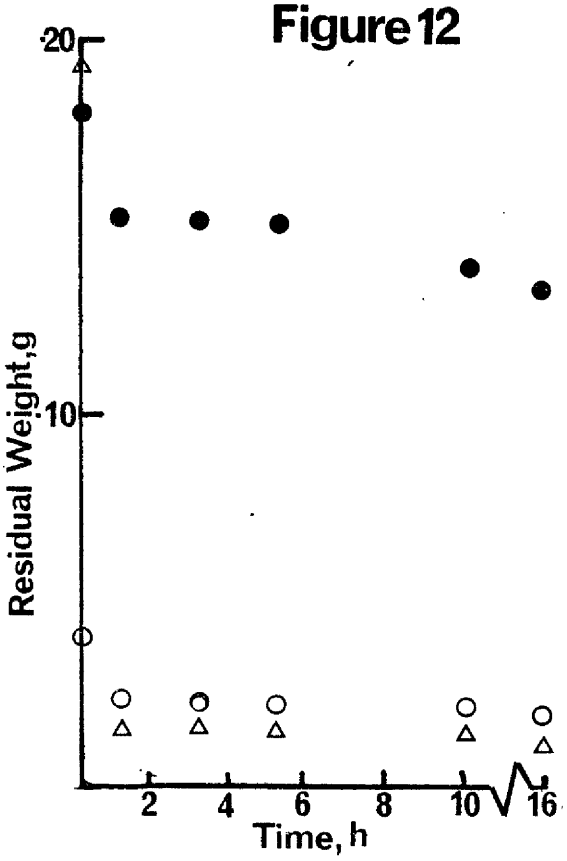
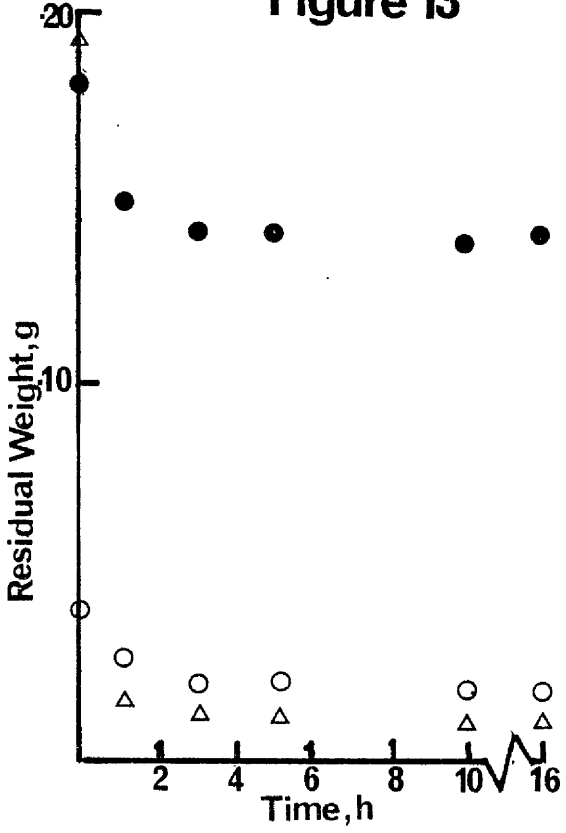


Figure 13



The effect of hydrolysis with 2.5% w/v H_2SO_4 on the residual weight of the cellulosic components of pig slurry per g of solids hydrolysed.

Figure 14: 80°C

Figure 15: 90°C

Figure 16: 100°C

● α-cellulose

○ hemicellulose A

△ hemicellulose B

Figure 14

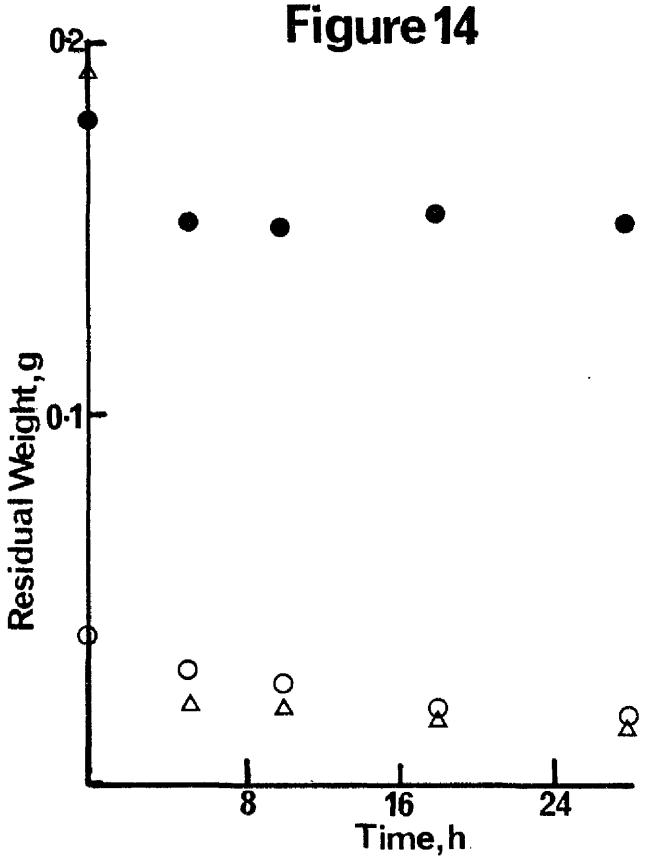


Figure 15

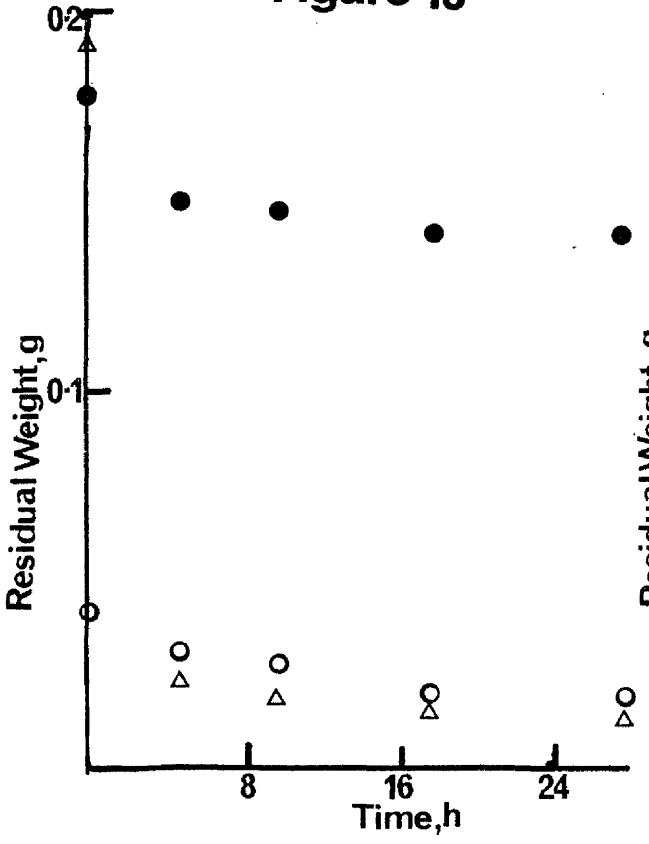
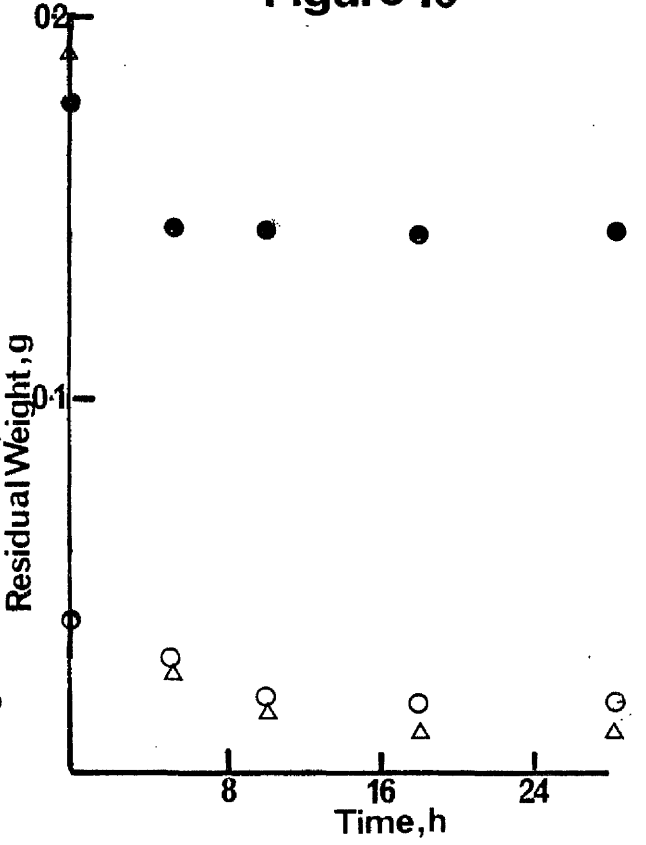


Figure 16



The effect of hydrolysis with 1% w/v H_2SO_4 on the residual weight of the cellulosic components of pig slurry per gram of solids hydrolysed.

Figure 17: 90°C

Figure 18: 100°C

● α-cellulose

○ hemicellulose A

△ hemicellulose B

Figure 17

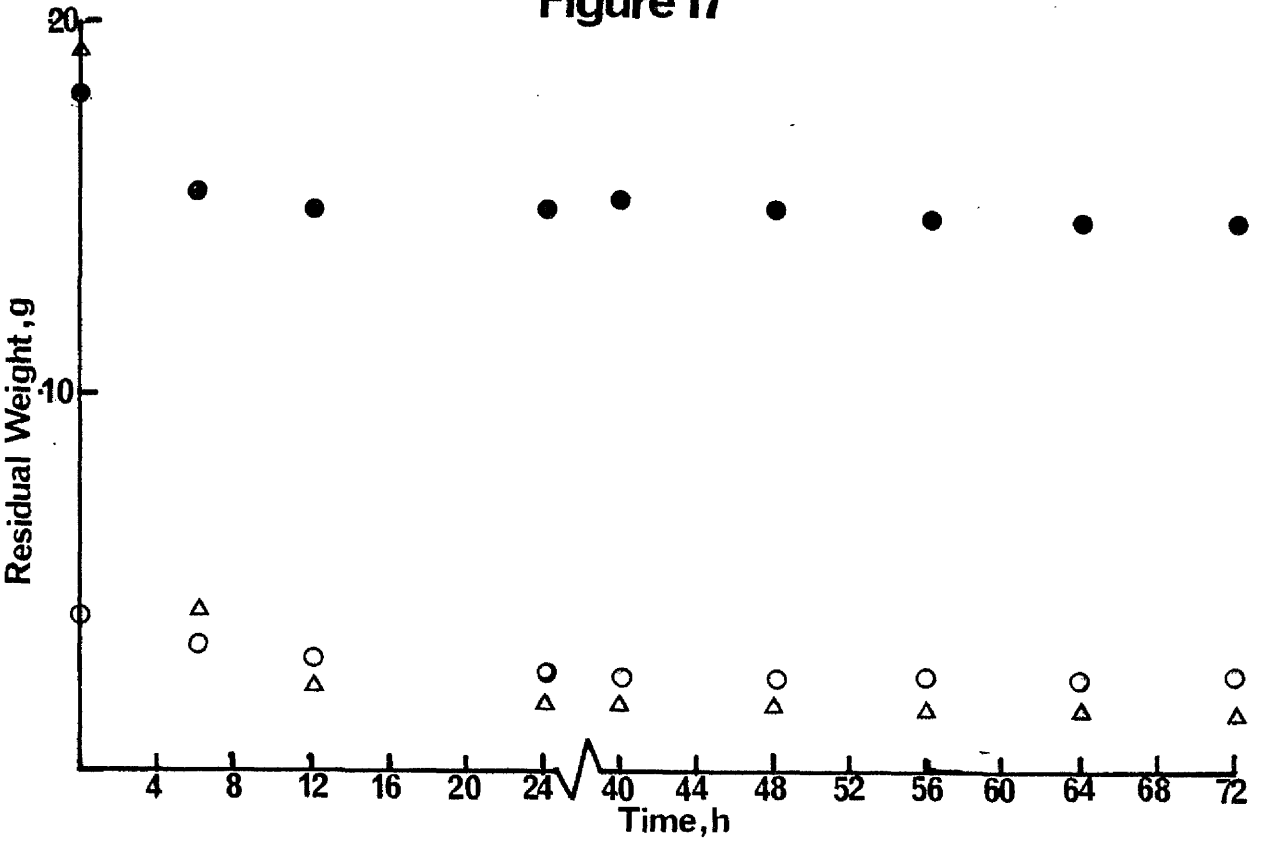
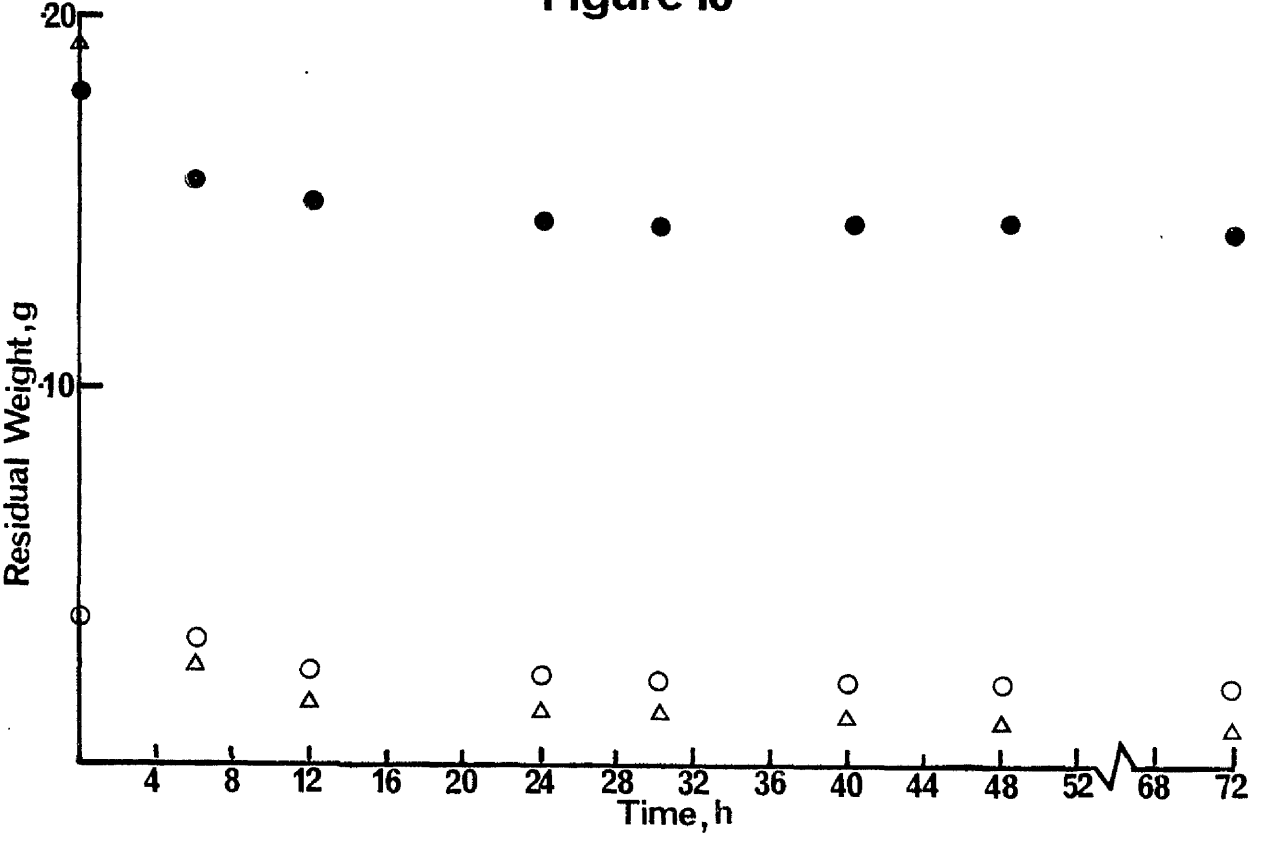


Figure 18



cellulosic constituents occurs even more slowly, the residual level becoming fairly stable after approximately 12 h. Overall, during mild H_2SO_4 hydrolysis, 50% of the insoluble components of pig slurry are degraded.

It is obvious from these results that any monosaccharides produced during the hydrolysis of pig slurry solids must be mainly the result of the breakdown of the fraction known as hemicellulose B. In fact 76% of the total cellulosic constituents broken down by the acid hydrolysis systems are hemicellulose B. The α-cellulose that disappears is 15% of the total weight loss, and hemicellulose A only 9%. It is of interest to determine the monosaccharides produced by the acid hydrolysis of these fractions. For this purpose duplicate 5 g samples of each isolated cellulosic fraction were hydrolysed with 5% H_2SO_4 at 90°C for 3 h and the resulting monosaccharides determined by HPLC. This treatment system was selected as it is one of the less severe test hydrolysis procedures, and it produces a rapid drop in the weight of the cellulosic constituents of pig slurry.

Examination of the results obtained (Table 12) show that the weight loss of the cellulosic constituents does not equal the combined weights of the monosaccharide released by hydrolysis. This is to be expected because, as Stamm (1964) has shown, monosaccharides, especially pentoses, released by acid hydrolysis of cellulosic polymers are degraded on further exposure to acid. It can also be seen from these results that almost 95% of the hemicellulose B is broken down, which is identical to the proportion of this fraction that disappears during the hydrolysis of pig slurry solids. This fraction yields xylose, arabinose and a small amount of glucose. The hydrolysis of pig slurry solids produces similar monosaccharide yields. This result is expected, as hemicellulose B was shown above to be the major cellulosic constituent of pig slurry degraded by this type of acid treatment.

It is also of interest to note that 20% of the fraction isolated as α-cellulose is broken down by acid treatment. This is the same percentage of this fraction that degrades during the hydrolysis of slurry solids. This portion, however, does not yield glucose as expected. In fact, the major monosaccharides are xylose and arabinose in proportions very similar to those found on hydrolysis of hemicellulose B. This indicates that there is some hemicellulose B which remains in

TABLE 12 The amounts of polymer degraded and the yields of monosaccharides produced by the hydrolysis of the cellulosic fractions of pig slurry with 5% H_2SO_4 at 90°C for 3 h.

CELLULOSIC FRACTION	WEIGHT LOSS $g.g^{-1}$ HYDROLYSED	YIELD OF MONOSACCHARIDE $g.g^{-1}$ HYDROLYSED		
		XYLOSE	ARABINOSE	GLUCOSE
Hemicellulose B	0.937	0.354	0.191	0.01
Hemicellulose A	0.458	0.132	-	0.071
α-cellulose	0.20	0.087	0.056	0.02

the α-cellulose fraction during the isolation procedure. Furthermore, this result shows that the α-cellulose of pig slurry is not hydrolysed by the range of acid treatments used in this study. An exception is 10% H_2SO_4 at 100°C, where more of the fraction isolated as α-cellulose disappears during hydrolysis. The overall results of the analysis of the effect of acid hydrolysis on the cellulosic components of pig slurry indicate that there is little difference in the residue produced by the various treatment systems. Thus, it was the yield of utilisable carbohydrate that was used to select the most efficient hydrolysis system.

Figures 19, 20 and 21 show the monosaccharides produced with 10% H_2SO_4 at 80°C, 90°C and 100°C respectively. In all cases it can be seen that three monosaccharides, namely xylose, arabinose and glucose, are produced in quantitative amounts. Galactose, which was qualitatively detected by TLC and paper chromatography, is not detected in quantitative amounts by HPLC. This means that galactose is present in quantities less than 0.2 $g.l^{-1}$. At all three temperatures tested, the yield of monosaccharide is fairly high after 1 h, but falls again after 2 h. The amount of each monosaccharide detected in the hydrolysate then rises again to give a maximum yield after 4 h at 80°C and 90°C and after 6 h at 100°C. Thereafter, the yield of each carbohydrate progressively decreases. In each of these hydrolysis systems xylose is always produced in larger quantities than arabinose and comparatively small amounts of glucose are released. These results agree closely with those for the degradation of the cellulosic components of pig slurry discussed previously.

In Figures 22, 23 and 24 the effect of 5% H_2SO_4 at temperatures of 80°C, 90°C and 100°C respectively are recorded. Only at 100°C does a depression in the yield/time curve occur at this acid concentration. In this case the yield of all three monosaccharides decreases between 3 and 5 h and increases again up to 10 h. The reasons for the depressions in the yield/time curves of the more severe hydrolysis processes are considered complex and are discussed later, when more explanatory data has been presented. At 80°C (Figure 22) and 90°C (Figure 23) the amount of each monosaccharide released by hydrolysis rises to a maximum yield, and then gradually falls. At 80°C (Figure 22) the yield of arabinose is higher than the yield of xylose after 1 h, although as with the other treatment systems,

The effect of hydrolysis with 10% w/v H_2SO_4 on the yields of monosaccharides released from 1 g pig slurry solids.

Figure 19: 80°C

Figure 20: 90°C

Figure 21: 100°C

▲ xylose

■ arabinose

□ glucose

Figure 19

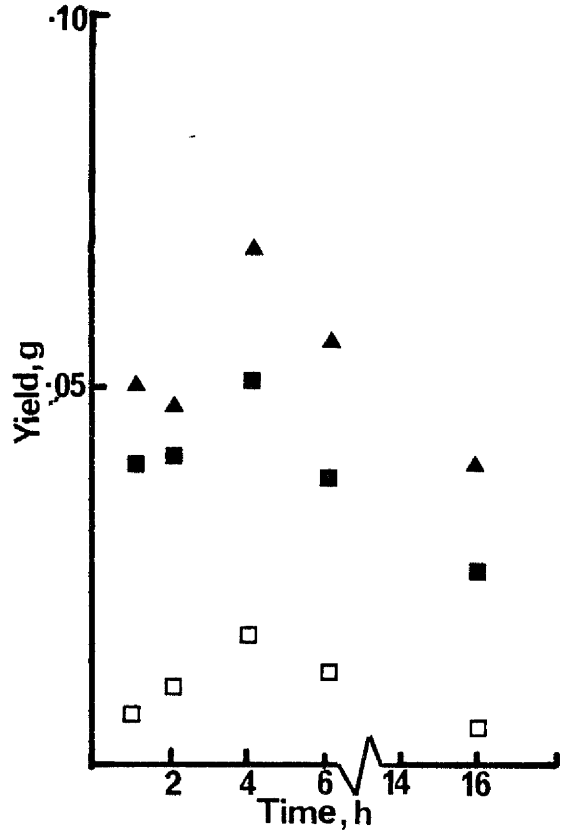


Figure 20

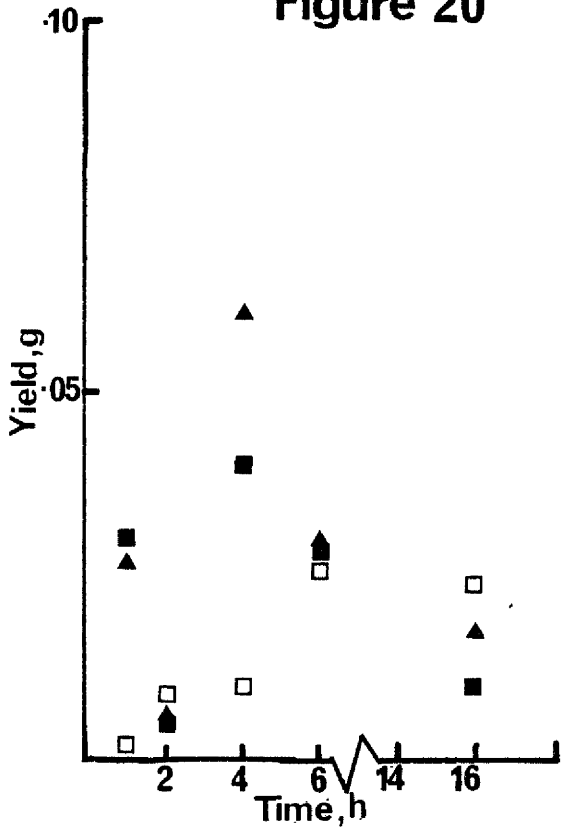
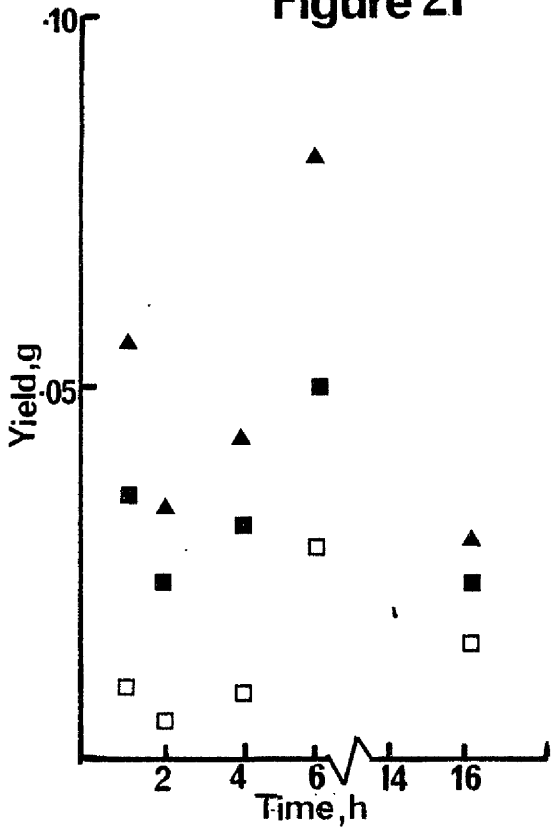


Figure 21



The effect of hydrolysis with 5% w/v H_2SO_4 on the yields of monosaccharides released from 1 g of pig slurry solids.

Figure 22: 80°C

Figure 23: 90°C

Figure 24: 100°C

▲ xylose

■ arabinose

□ glucose

Figure 22

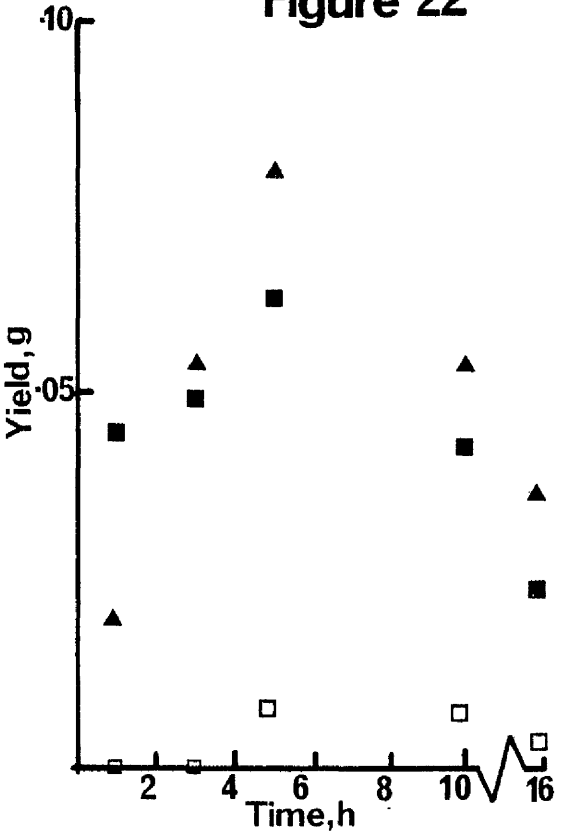


Figure 23

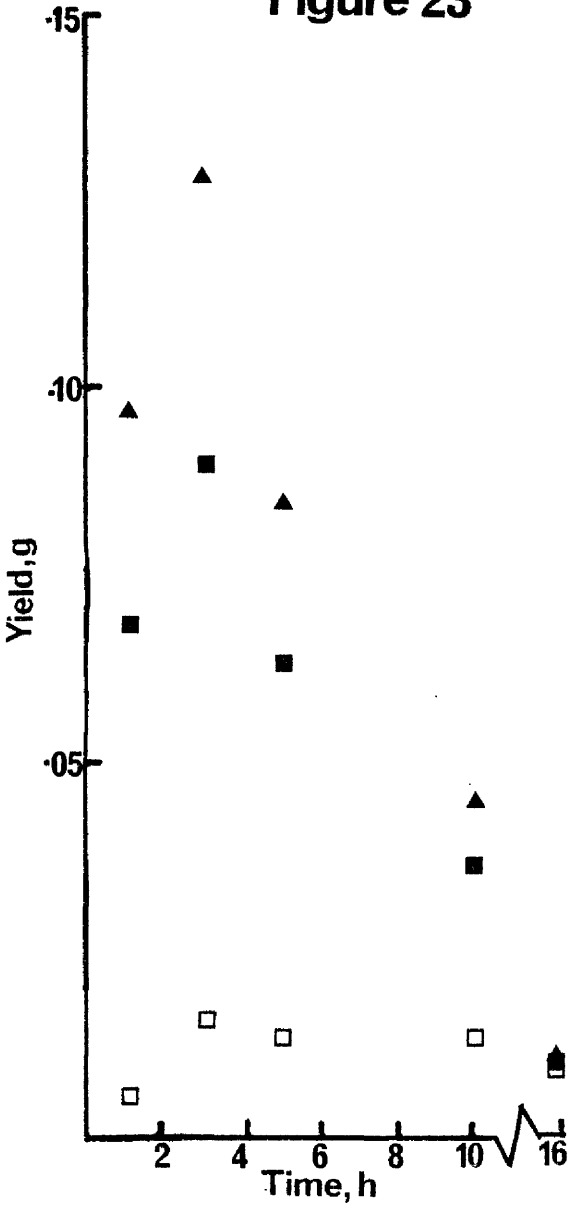
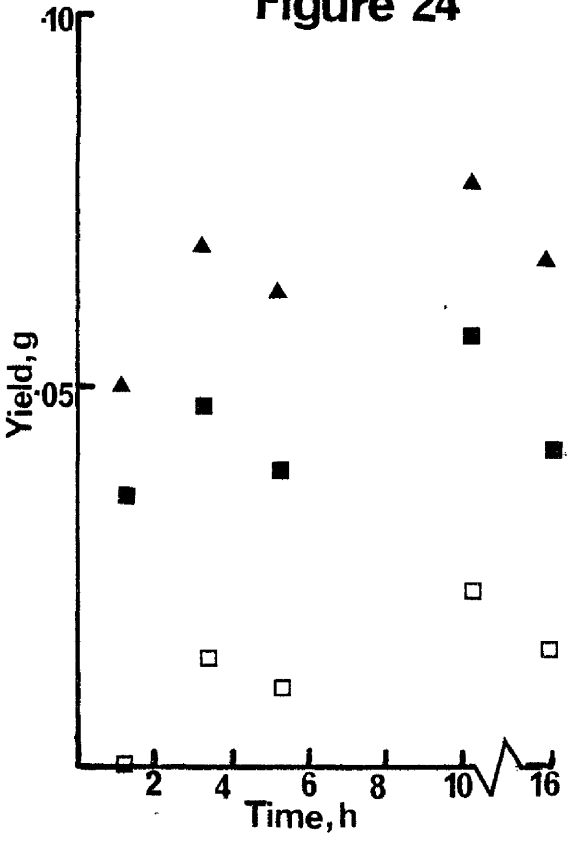


Figure 24



xylose, is ultimately detected in larger quantities. A similar pattern is observed in Figure 25, where 2.5% H_2SO_4 at 80°C initially produces a higher yield of arabinose than xylose, and this is repeated with 1% acid at 90°C and 100°C in Figures 28 and 29 respectively. The fact that this pattern is observed in the results of the mildest acid treatment systems suggests that a hemicellulosic polymer containing a larger proportion of arabinose than xylose is hydrolysed first, followed by the breakdown of a polymer rich in xylose. In the more severe treatment systems it is postulated that the hydrolysis of the more labile polymer occurs so quickly that it is not detected by the sampling regime employed.

In Figures 25, 26 and 27 the monosaccharides released by hydrolysis with 2.5% H_2SO_4 at 80°C, 90°C and 100°C respectively are shown. This acid concentration results in lower overall yields of monosaccharides than any of the treatment systems using other acid concentrations, and the rise and fall in yield with time is much more gradual. Although 1% H_2SO_4 at 90°C and 100°C (Figures 28 and 29 respectively) show the same general pattern, the yields of monosaccharides are significantly higher than those produced with any of the treatment systems using 2.5% acid. The maximum amount of monosaccharide released by hydrolysis with 1% H_2SO_4 at 90°C occurs after 56 h, whereas raising the temperature to 100°C decreases this time to 24 h.

In general, most of the hydrolysis systems tested demonstrate a trend to rise to a peak of monosaccharide production, followed by a gradual decrease in the yield of each carbohydrate with time. This indicates that there is a progressive hydrolysis of the hemicellulosic components of pig slurry accompanied by destruction of the free monomers. At the start of hemicellulose breakdown the rate of hydrolysis is faster than the rate of destruction. This leads to an overall increase in monomer yield. Finally, a point is reached at which all the readily available hemicellulose has been hydrolysed, and the destructive reactions proceed at a faster rate than the productive reactions. This results in a decrease in the amount of monosaccharide detected in the hydrolysate.

It has been shown by other workers that the hydrolysis of cellulose in concentrated H_2SO_4 at temperatures below 100°C (Stamm, 1964) and weak H_2SO_4 acid at above 100°C (Saeman, 1945) follow first order reaction kinetics. Empirical equations

The effect of hydrolysis with 2.5% H_2SO_4 on the yields of monosaccharides released from 1 g of pig slurry solids.

Figure 25: 80°C

Figure 26: 90°C

Figure 27: 100°C

▲ xylose

■ arabinose

□ glucose

Figure 25

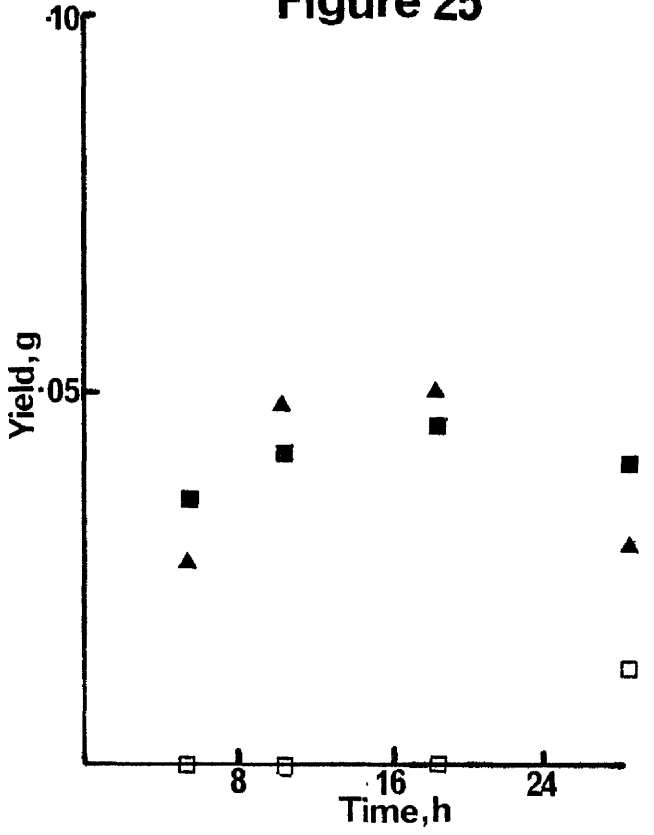


Figure 26

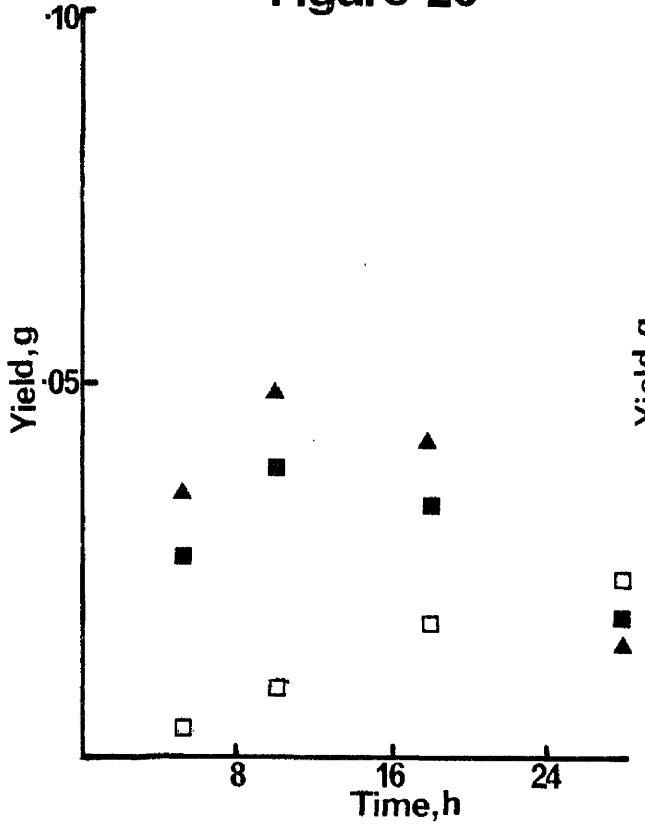
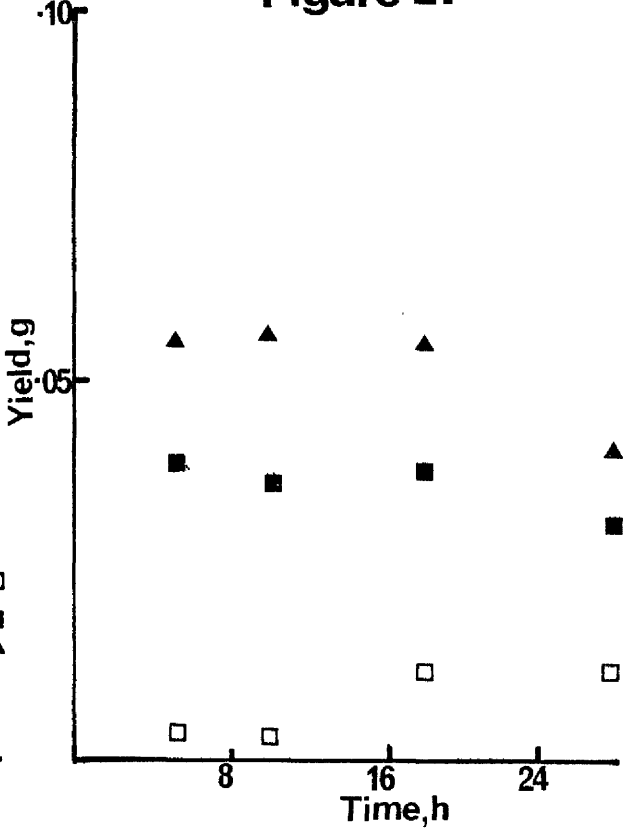


Figure 27



The effect of hydrolysis with 1% w/v H_2SO_4 on the yields of monosaccharides released from 1 g of pig slurry solids.

Figure 28: 90°C

Figure 29: 100°C

▲ xylose

■ arabinose

□ glucose

Figure 28

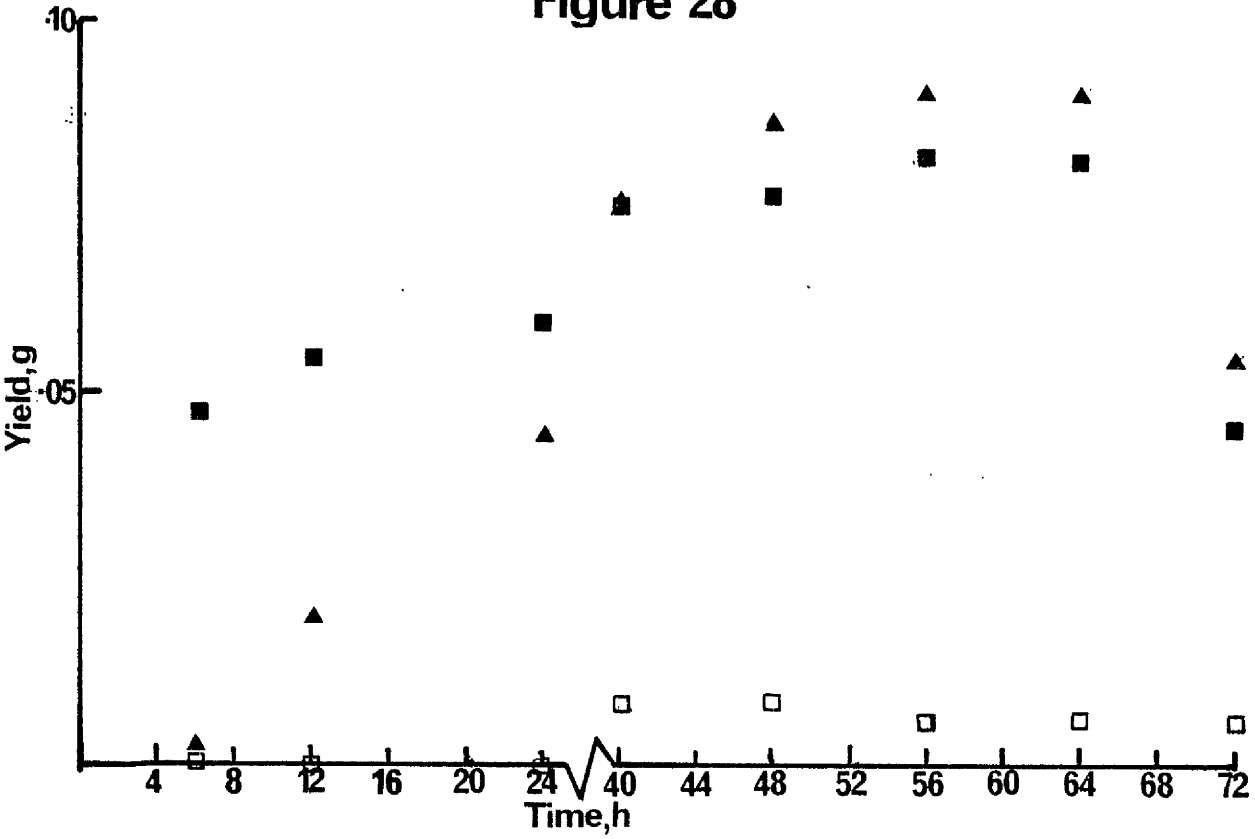
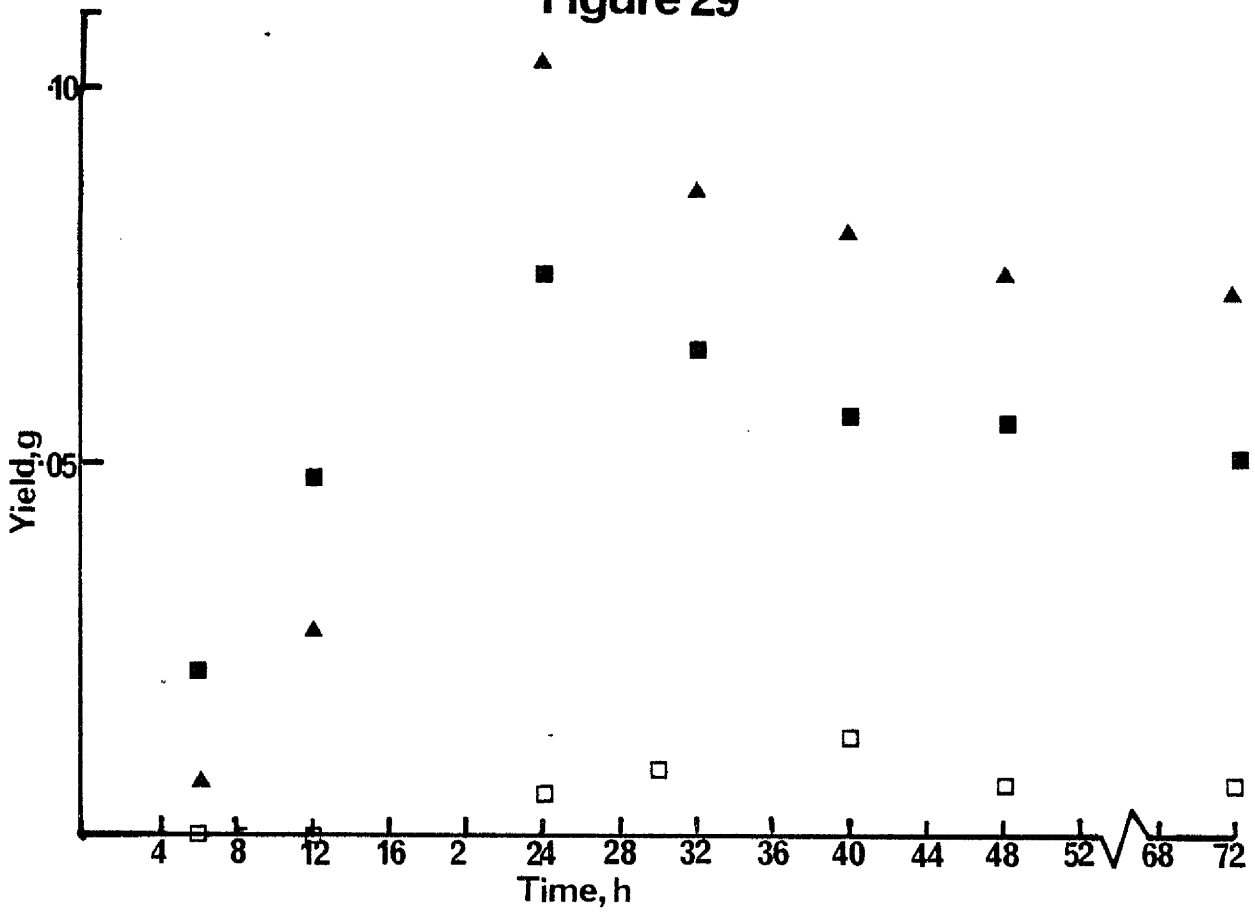


Figure 29



have been constructed for these reactions and rate constants calculated. The rate of carbohydrate decomposition has also been shown to be first order (Saeman, 1945). Thus, equations combining the rate of hydrolysis of cellulose and the rate of decomposition of glucose can be used to predict the yield of glucose theoretically attainable from any hydrolysis system. This is only possible in systems where a sample of pure cellulose is used and the acidic solution is free from contaminants (Saeman, 1945).

The reaction kinetics become much more complex when a pure hemicellulose is hydrolysed. It has been shown that there are two stages in the hydrolysis of wood hemicellulose. Both of these are first order with different rate constants (Kobayashi & Sakai, 1956). This suggests that there is a portion of the hemicellulose that is easily hydrolysed, and a second portion that is more difficult to degrade. The reaction is further complicated by differences in the rates of destruction of the monosaccharides released by acid hydrolysis of the hemicellulose. Overall, the construction of accurate reaction equations for the hydrolysis of a pure hemicellulose is very complex.

The cellulosic constituents of pig slurry are of plant origin, and constitute a complex mixture of polymers with varying acid susceptibilities. Further, in laboratory tests, the acid hydrolysis systems used in this work were shown to degrade pure solutions of xylose and arabinose. The destructive effect of 1% H_2SO_4 at 90°C (Figure 30), 5% acid at 90°C (Figure 31) and 10% acid at 100°C (Figure 32) were measured in 2% w/v solutions of the pentoses. The residual pentose was measured by HPLC analysis. Table 13 shows the time taken for half of the initial quantity of xylose and arabinose to be degraded by these acid treatment systems. It can be seen from these results that, as expected, at all acid concentrations and temperatures tested xylose is more labile than arabinose. The mildest treatment system, 1% acid at 90°C, destroys xylose but does not appear to degrade arabinose.

In pig slurry these degradative reactions are further complicated by the presence of ammonia and other amino compounds. Even in simple mixtures of pentoses and ammonia, Aso (1953) described complex carbonyl-amino condensation reactions which result in the

The destructive effect of H_2SO_4 hydrolysis on the pentoses, xylose and arabinose.

Figure 30: acid conc. 1% w/v; temp. 90°C

Figure 31: acid conc. 5% w/v; temp. 90°C

Figure 32: acid conc. 10% w/v; temp. 100°C

● xylose

○ arabinose

Figure 30

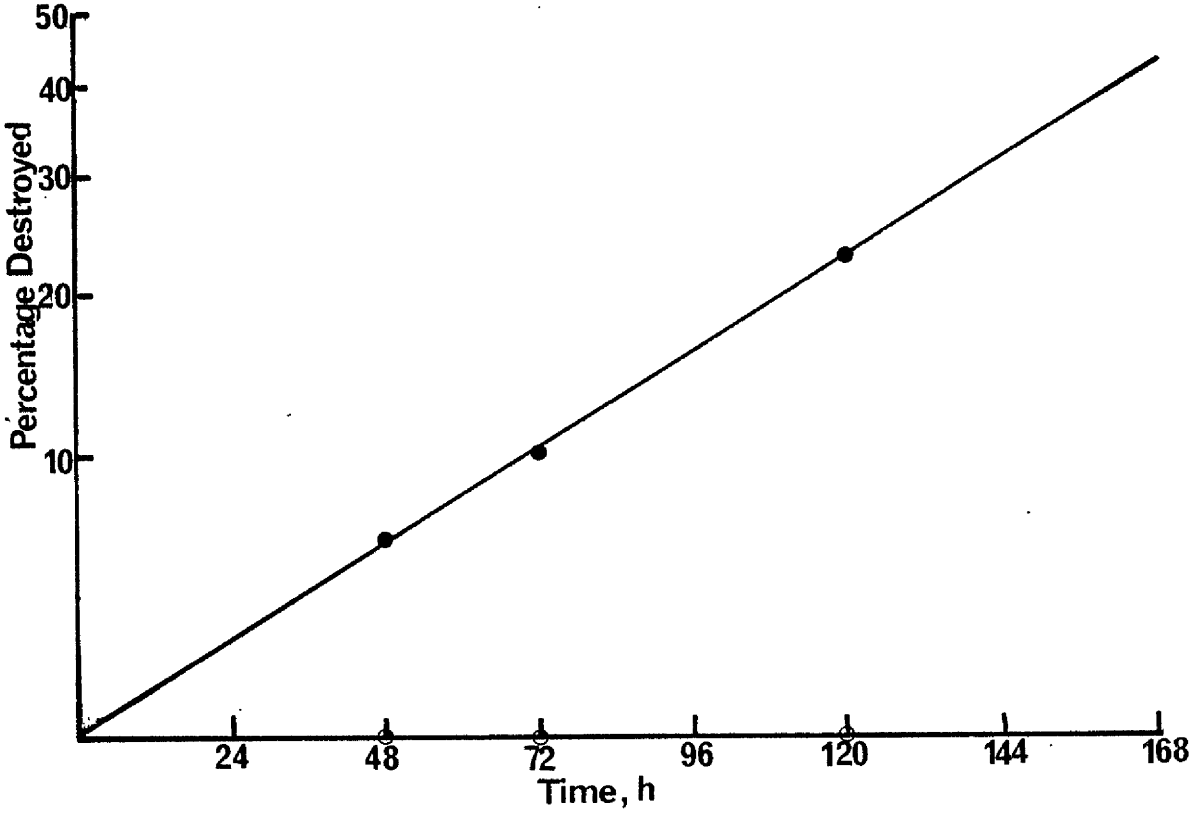


Figure 31

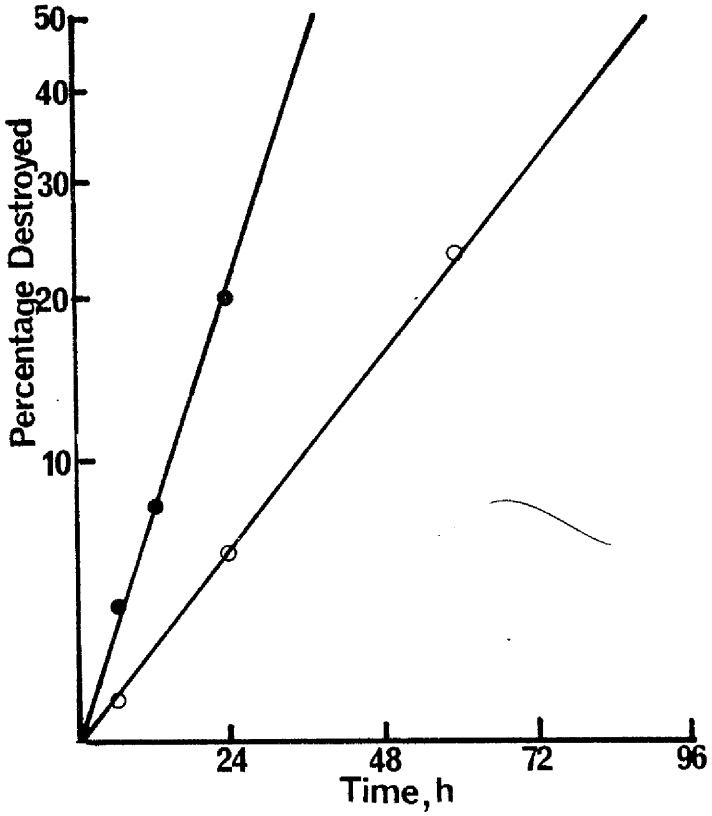


Figure 32

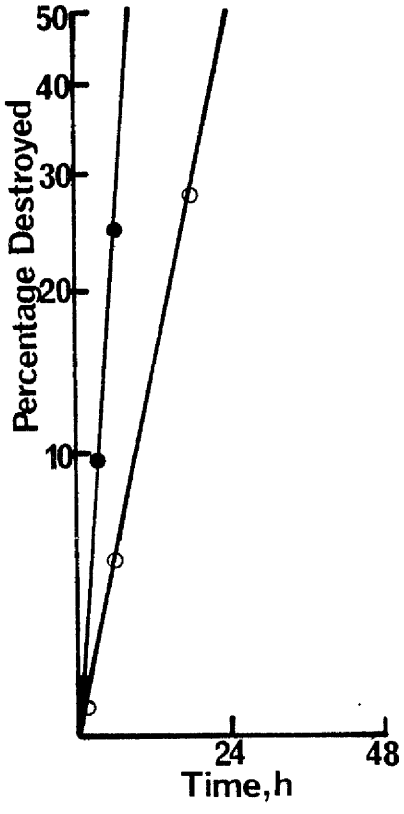


TABLE 13 Time taken for half the initial amount of monosaccharide to be destroyed by a mild H_2SO_4 hydrolysis system.

TEMP. ^{o}C	H_2SO_4 CONCENTRATION (%)	HALF-LIFE (h)	
		XYLOSE	ARABINOSE
100	10	7.8	23.4
90	1	182	∞
90	5	38	91

formation of nitrogenous aromatic compounds (Hodge, 1953). Thus, the hydrolytic and degradative processes occurring during the hydrolysis of pig slurry must be extremely complicated. It is virtually impossible to produce equations which can give any indication of the expected yields of monosaccharides for different hydrolysis systems. The observed differences in monosaccharide yields can be attributed to these variations in the ratio of rates of monomer production to rates of destruction. Furthermore, depression in the yield vs time curve, which is only observed for the most severe hydrolysis conditions, can also be accounted for by these variations in the ratio of reaction rates. In this case it is postulated that the hydrolysis of the hemicellulose B fraction follows a similar two stage pattern as that described by Kobayashi & Sakai (1956) for wood hemicellulose. The depression in yield occurs when the monosaccharides released from the easily hydrolysed portion are degraded. Subsequently, hydrolysis of the more resistant portion of this fraction overcomes this drop in yield. In the milder hydrolysis systems, the ratio of the rate of hydrolysis of the polymer to the rate of monomer degradation is such that this phenomenon is not observed.

In selecting a hydrolysis system which produces the best medium for microbial growth, a high yield of soluble carbohydrate is most important. The overall low yields of monosaccharides recorded for the hydrolysis systems employing 2.5% H_2SO_4 and 5% acid at 80°C, therefore led to their exclusion. Furthermore, it was decided to exclude all those exhibiting a depression in the yield vs time curve, because there is the unpredictability of the yield in the region of the maximum. All treatment systems using 10% H_2SO_4 and 5% H_2SO_4 at 100°C were excluded using this principle. 1% H_2SO_4 at 90°C and 100°C produce relatively high yields of soluble carbohydrate after 56 h and 24 h respectively. These milder acid treatment systems have other attractive features. Smaller quantities of acid are used in the hydrolysis process, and consequently less alkali is required for neutralisation. As a result, the final salt content of the hydrolysate is relatively low, which could be advantageous when the medium is used for the growth of microorganisms. There is also the possibility that in these mild acid conditions some microbial growth

factors such as amino acids or vitamins could survive the hydrolysis process. Unfortunately, the time taken to attain maximum yield is an inherent disadvantage. Of all the treatment systems investigated, 5% H_2SO_4 at 90°C for 3 h produces by far the highest yield of monosaccharides. Thus, it was decided to use 1% acid at 100°C for 24 h, 1% acid at 90°C for 56 h and 5% acid at 90°C for 3 h to produce test microbial growth media from pig slurry. The concentrations of the measured available monosaccharide in the liquid hydrolysates produced by these processes are given in Table 14.

Throughout the hydrolysis experiments comparable results were obtained by using a bulk sample of dried slurry solids to which the requisite amount of water was added. Normally, however, raw slurry was hydrolysed to produce a growth medium for microorganisms. The yield of monosaccharides obtained by hydrolysis of dried slurry solids was therefore compared to that obtained by hydrolysis of raw slurry solids. For this purpose a portion of a sample of raw slurry (110 $g.l^{-1}$) was air dried in the normal way. Duplicate samples of raw slurry and the reconstituted dried solids (110 $g.l^{-1}$) were hydrolysed by each of the three selected procedures. It was found that hydrolysis of raw slurry with 5% acid at 90°C for 3 h produced significantly lower yields of monosaccharides than acid treatment of the equivalent dried slurry sample. Both systems employing 1% acid gave negligible yields of soluble carbohydrate monomers from raw slurry. The reason for this phenomenon is attributed to the large concentration of ammonia in raw slurry, which neutralises a proportion of the hydrolysing acid. This effect is naturally more marked at lower acid concentrations. Raw slurry was shown to have a pH of $\sim$8.5, whereas reconstituted dried solids have a pH of $\sim$5.0. In order to establish hydrolysis procedures for raw slurry, the pH must be adjusted to 5.0 prior to the addition of the requisite amount of hydrolysing acid. When hydrolysis is repeated using reconstituted slurry solids and raw slurry adjusted to pH 5, the yields of monosaccharides obtained from the two different preparations are not significantly different. The results are shown in Table 14.

The use of raw slurry in the hydrolysis process introduced another problem. When heated, the acidified slurry was observed

TABLE 14 Comparison yield of monosaccharides obtained by H_2SO_4 hydrolysis of raw pig slurry and reconstituted dried pig slurry solids

SLURRY	TEMPERATURE	ACID CONC. (%)	TIME	MONOSACCHARIDE CONC. $g.l^{-1}$ HYDROLYSATE		
				XYLOSE	ARABINOSE	GLUCOSE
Dried	90	5	3	4.25	2.60	0.48
Raw	90	5	3	4.29	2.60	0.42
Dried	100	1	24	3.00	2.29	0.35
Raw	100	1	24	3.04	2.29	0.44
Dried	90	1	56	3.27	2.56	0.20
Raw	90	1	56	3.31	2.28	0.20

to froth violently. This was overcome in the laboratory by degassing the raw slurry under vacuum at pH 5. Analysis showed that the low pH prevents the loss of any dissolved ammonia in this process. At least a proportion of the dissolved gas was shown to be carbon dioxide, as when it was bubbled through a $Ca(OH)_2$ solution a precipitate of $CaCO_3$ was obtained.

In each of the acid treatment systems tested, there is a significant time lag between the disappearance of hemicellulose B from the solid fraction and the appearance of soluble monosaccharides in the liquid hydrolysate. This is particularly noticeable with the treatment systems using 1% H_2SO_4. It is postulated that the hemicellulose B is actually solubilised prior to its hydrolysis to monomer units. A similar process of solubilisation has been described by Kobayashi & Sakai (1958a) for α-cellulose. This phenomenon is further investigated in the following section.

CONCLUSIONS

1. Approximately 43% of the dry weight of pig slurry solids is cellulosic in nature.
2. By the $NaClO_2$ digestion method, the cellulosic constituents of pig slurry can be separated into three fractions, namely α-cellulose, hemicellulose A and hemicellulose B.
3. Approximately 43% w/w of the cellulosic constituents of pig slurry solids are isolated as α-cellulose, 48% w/w as hemicellulose B and ~9% w/w as hemicellulose A.
4. All the mild acid treatment systems tested leave solid residues with very similar cellulosic compositions.
5. Mild acid hydrolysis degrades ~60% w/w of the cellulosic constituents of pig slurry.
6. The portion of α-cellulose degraded by mild acid treatment was shown to be of the same composition as hemicellulose B. It appears to be a residue of hemicellulose B remaining in the α-cellulose after treatment with alkali.
7. Mild acid hydrolysis releases monosaccharides from the hemicellulose fractions of pig slurry.
8. Mild acid hydrolysis of hemicellulose B releases xylose, arabinose and glucose in quantitative amounts.

9. Mild acid hydrolysis of hemicellulose A releases xylose and glucose in quantitative amounts.
10. Mild acid hydrolysis of raw pig slurry releases xylose, arabinose and a small amount of glucose.
11. Xylose is produced in significantly larger quantities than arabinose.
12. After release, the monosaccharides are progressively degraded by the acid conditions.
13. Three mild acid treatment systems, namely 1% H_2SO_4 at 100°C for 24 h, 1% H_2SO_4 at 90°C for 56 h and 5% H_2SO_4 at 90°C for 3 h, appear to be most suitable for the production of growth media for microorganisms.
14. The highest yield of soluble monosaccharides from the cellulosic constituents of pig slurry is obtained from hydrolysis with 5% H_2SO_4 at 90°C for 3 h, which produces 0.53 g of monosaccharide for every 1 g of cellulosic material available in raw pig slurry.

Detection of Solubilised Polysaccharides in the Liquid Fraction of Acid Hydrolysed Pig Slurry

MATERIALS AND METHODS

Previous results (pages 56-63) indicate that during mild acid hydrolysis, the hemicellulosic constituents of pig slurry are first solubilised and then hydrolysed to monomer units. In order to establish that this is in fact the case, a system of separating low and high molecular weight carbohydrates had to be developed. For this purpose a gel filtration technique was adopted. The filtration medium was Sephadex, a modified cross-linked dextran, supplied by Pharmacia (Great Britain) Ltd, Prince Regent Road, Hounslow, Middlesex. This technique separates molecules on the basis of molecular size. Sephadex G15, fine grade, was packed into a K 15/90 column, 90 cm long and 15 cm in diameter (Pharmacia (Great Britain) Ltd). 0.02 N acetic acid was used to elute the sample from the column. An eluant flow rate of 0.0625 $ml.min^{-1}$ was maintained by gravity feed, using a constant pressure Mariotte flask (Pharmacia (Great Britain) Ltd). The eluant was collected in 2 ml quantities in a fraction collector which rotated every 32 min. The presence of carbohydrate in the eluant was detected by the phenol/H_2SO_4 method (Herbert *et al*, 1971). With reference to the colour development of standard xylose

solutions of 0.025%, 0.05% and 0.1% w/v concentration, a visual assessment of the intensity of colour in each of the eluant tubes was made.

In order to test that a separation of monosaccharide and polysaccharide was achieved by this system, a mixture of 0.5% w/v xylose and 0.5% w/v xylan in water was prepared. Application of a 1 ml sample of this mixture to the column, by means of a syringe, was shown to produce a sufficiently narrow starting zone, so that the components were eluted in discrete bands with very little tailing.

An examination of the results of the analyses of monosaccharides in the hydrolysates (pages 58-63) and cellulosic constituents in the residual slurry solids (pages 56-58) indicated that of all the hydrolysis treatments used 1% acid at 90°C produced the slowest disappearance of insoluble hemicellulose, followed by a gradual build up of soluble monosaccharide. Thus, it was considered that gel filtration of neutral desalted samples of the hydrolysates produced by 1% H_2SO_4 at 90°C, would best demonstrate the production and gradual disappearance of solubilised hemicellulose. 1 ml samples of the hydrolysates produced by heating pig slurry in 1% acid for 6 h, 12 h, 24 h and 56 h were tested. In order to test for the presence of detectable amounts of carbohydrate in the liquid portion of pig slurry prior to hydrolysis, a sample of slurry was acidified with 1% acid, and immediately neutralised, separated and desalted without any application of heat.

Membrane filtration of samples prior to application to the column was necessary in order to prevent the growth of contaminating organisms, especially fungal species, on the dextran. When the column was not in use it was eluted with a 0.002% w/v solution of hibitane (supplied by Imperial Chemical Industries Ltd, Pharmaceutical Division, Macclesfield, Cheshire). This solution prevented microbial growth.

RESULTS AND DISCUSSION

The results obtained for the elution of a mixture of xylan and xylose are recorded in Figure 33. These results indicate the xylan and xylose are eluted from the column in two discrete bands with very little tailing. Xylan, which is a large molecule, is excluded or partially excluded from the pores of swollen Sephadex, and therefore passes quickly through the column in the liquid phase. Xylose, a much

Separation of carbohydrates on a molecular size basis by the technique of gel filtration.

Figure 33: Mixture of pure xylose and xylan

Figure 34: Pig slurry hydrolysate 1 [90] 6

Figure 35: Pig slurry hydrolysate 1 [90] 12

Figure 36: Pig slurry hydrolysate 1 [90] 24

Figure 37: Pig slurry hydrolysate 1 [90] 56

Figure 38: Pig slurry hydrolysate 1 [90] 6
treated with activated charcoal

Figure 33

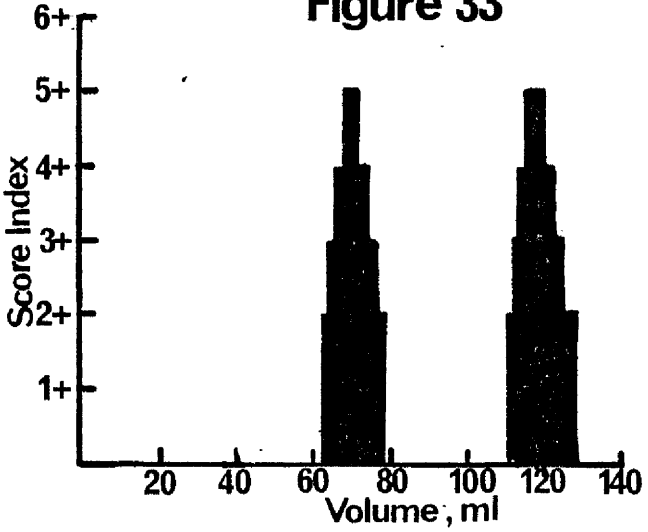


Figure 34

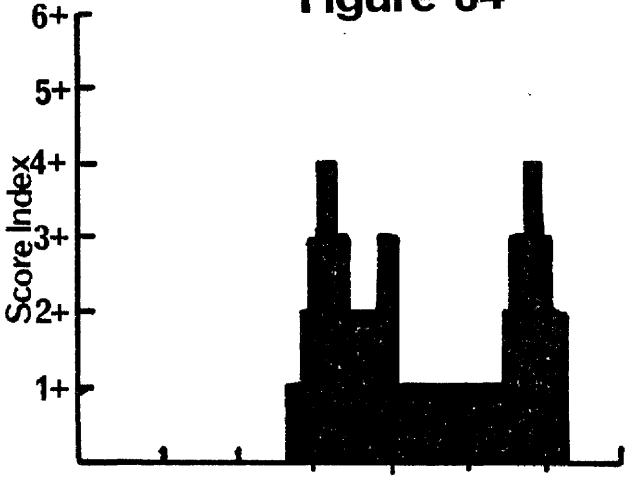


Figure 35

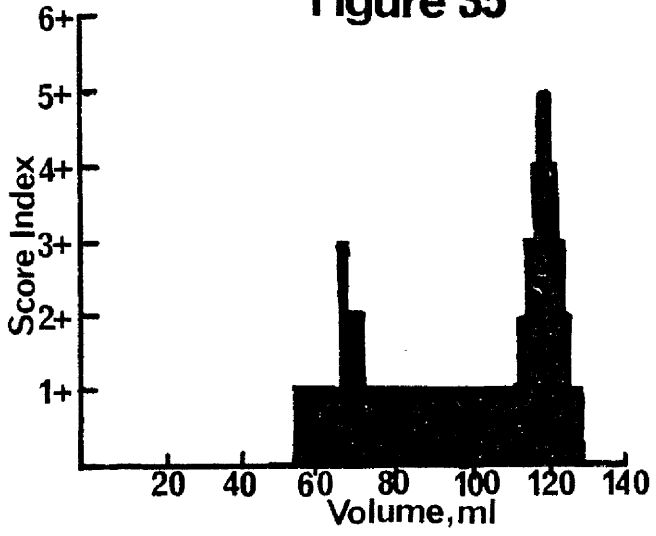


Figure 36

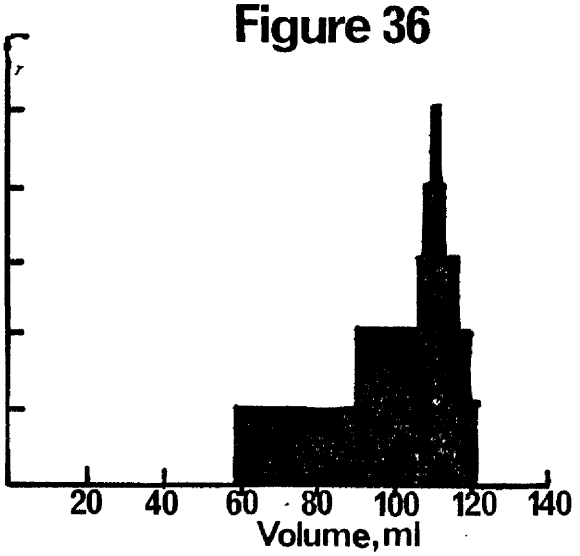


Figure 37

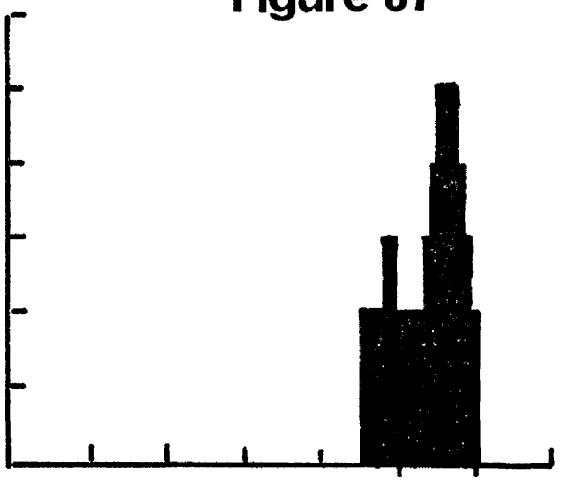
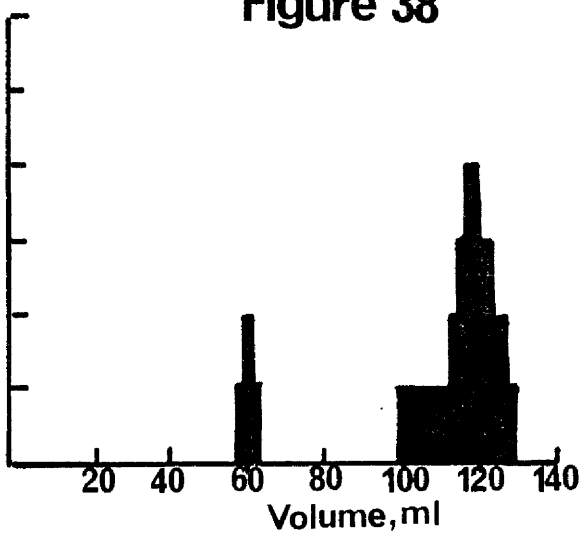


Figure 38



smaller molecule, penetrates the gel particles and is eluted from the column after a greater volume of liquid has passed.

Gel filtration of the liquid portion of slurry, which is acidified with 1% acid, but separated and analysed before it is heated, produces no detectable quantities of monomeric or polymeric carbohydrates in the column eluant. After heating for 6 h, however, Figure 34 shows that hydrolysis in the presence of 1% acid produces two distinct peaks of carbohydrate corresponding roughly to the elution volumes of xylan and xylose. There also appears to be small amounts of carbohydrates of intermediate molecular weight present in the sample. This result indicates that soluble hemicellulosic polymers are produced in significant quantities on contact with 1% H_2SO_4 at 90°C, and that these polymers are progressively broken down by the weak hydrolysis conditions finally yielding monomers. This is similar to the process for cellulose in concentrated H_2SO_4 reported by Kobayashi & Sakai (1958a). In this case a portion of the cellulose dissolved in concentrated H_2SO_4, and the solublised glucose polymers were subsequently hydrolysed to monomer units in dilute H_2SO_4.

In Figure 35 it can be seen that after 12 h very little polymer remains in solution. The reduction of soluble polymer is even more marked after 24 h exposure to acid (Figure 36). After 56 h, all the polymeric carbohydrates and most of the carbohydrates of intermediate molecular weight disappear (Figure 37). This result shows that the low molecular weight carbohydrates produced by hydrolysis with 1% acid at 90°C for 56 h are eluted over a wider range of volumes than is pure xylose (Figure 33). This can probably be explained by the presence of a mixture of the monosaccharides arabinose, xylose and glucose in the hydrolysate. These carbohydrates vary slightly in molecular weight, size and shape, which causes them to pass through the column at slightly different speeds. There is also the possibility that trace amounts of dimers and trimers are still present in the hydrolysate.

At this stage it was thought to be of some interest to show the effect of activated charcoal treatment on the solubilised polymer in the hydrolysate. For this purpose the sample obtained by hydrolysing slurry with 1% acid at 90°C for 6 h was treated with charcoal

by the standard method of adding 2 g charcoal to 100 ml of hydrolysate (see page 45). This decolourised sample was applied to the gel filtration system and the results are shown in Figure 38. This figure indicates that, as expected, charcoal treatment removes most of the soluble polymer and intermediate molecular weight carbohydrates, leaving the monomeric carbohydrates in solution. It was shown that the addition of a small excess of charcoal removed all residual polymer.

CONCLUSIONS

On mild H_2SO_4 hydrolysis, some of the hemicellulosic constituents of pig slurry are solubilised, and subsequently broken down to low molecular weight carbohydrates.

2. STUDIES ON THE GROWTH OF SELECTED YEAST SPECIES ON PIG SLURRY HYDROLYSATE

INTRODUCTION

Several strains of yeast have been considered for food and feed yeast production (Birch *et al*, 1976; Davis, 1974). Important characteristics include growth rate, yield from a given substrate, pH and temperature tolerance, aeration requirements and genetic stability. *Saccharomyces cerevisiae* has a long history of use in the production of bakers' yeast and for brewing and alcholic beverage production (Snyder, 1970). It is therefore not surprising that spent brewers' and distillers' yeasts were sold for food or feed use at an early date. However, for primary food and feed yeast production *Saccharomyces cerevisiae* has the disadvantages of being unable to utilise lactose, pentoses or hydrocarbons, and of the need for supplementation with organic nitrogen sources and B-vitamins (Lodder, 1971a). Thus, the production of food and feed yeast is based mainly on *Candida utilis* (Sobkowicz, 1976), but many other species are in fact suitable for SCP production.

The readily available carbon sources in pig slurry hydrolysate are mainly in the form of pentose sugars. *Candida utilis* is unable to utilise arabinose and some strains only use xylose weakly as a carbon source (van Uden & Buckley, 1971). This yeast species is therefore unsuitable for growth on pig slurry hydrolysate. The major source of nitrogen in the hydrolysate is present in the form of ammonia. Although it is known that other forms of non-protein nitrogen and amino-nitrogen are present in raw slurry (Evans *et al*, 1978), the effect of acid treatment on these compounds is not known. It is also probable that raw slurry contains several microbial growth factors such as amino acids and vitamins, however, many of these compounds are acid labile, and therefore it cannot be assumed that hydrolysed slurry will contain growth factors. Thus, in order to ensure a good yeast yield on pig slurry hydrolysate, without nutrient or growth factor additions, it is necessary to select a yeast species that grows well on pentose sugars, uses ammonia as a nitrogen source, and does not have a requirement for growth factors. However, the addition of growth factor to the hydrolysate medium may ultimately be necessary to obtain maximum yields of biomass.

Screening of Yeast Species for Growth on Pig Slurry Hydrolysate

MATERIALS AND METHODS

Yeast Strains

After studying the descriptions of over 350 yeast species (Lodder, 1971a, van der Walt *et al*, 1973; van der Walt & Nakase, 1973), 11 species were selected as having suitable characteristics for growth on pig slurry hydrolysate. These yeasts, listed in Table 15, were obtained as pure cultures from the Centraalbureau Voor Schimmelcultures (CBS), Oosterstraat 1, Baarn, Holland.

TABLE 15 Yeast species selected as suitable for growth on pig slurry hydrolysate

Yeast species	Culture number	References
Candida blankii	CBS 1898	Lodder, 1971a
Candida ciferrii	CBS 4856	"
Candida homilentoma	CBS 6312	van der Walt & Nakase, 1973
Candida mogii	CBS 2032	Lodder, 1971a
Candida neodendra	CBS 6032	van der Walt *et al*, 1973
Cryptococcus terreus	CBS 1895	Lodder, 1971a
Lipomyces starkeyii	CBS 1007	"
Endomycopsis burtonii	CBS 6141	"
Rhodosporidium toruloides	CBS 14	"
Rhodotorula glutinis	CBS 20	"
Torulopsis fujisanensis	CBS 4551	"

Preliminary work had indicated (Bruce, 1973) that, of the yeast species held in the departmental culture collection, one isolate, 53A, was particularly suited to growth in pig slurry hydrolysate. It was therefore proposed to include this yeast species with those listed in Table 15 in the selection procedures to be undertaken.

Maintenance of Cultures

Sheda & Yarrow (1966) have shown that the carbohydrate assimilation abilities of yeast strains may change when they are kept on malt agar, whereas lyophilised cultures revived and maintained on a glucose medium have the same characteristics as the original culture. Thus, all stock cultures of yeast species were lyophilised and stored in the freeze-dried state (Lapage & Redway, 1974) and

revived on a glucose medium prepared according to the formula of Ogur & St John (1956): peptone 0.35%; yeast extract 0.3%; KH_2PO_4 0.2%; $MgSO_4.7H_2O$ 0.1%; $(NH_4)_2SO_4$ 0.1%; glucose 2%. The working cultures were maintained on slopes of this medium solidified with 2% agar.

Preparation of Inoculum

A cell suspension was prepared by adding a loopful of a 48 h culture grown on glucose medium, to sterile distilled water in a 16 mm tube. This suspension was aseptically diluted with sterile water until the black lines, approximately ¾ mm wide, drawn on white cardboard, became visible through the tube as dark bands (van der Walt, 1971). In order to make this method of standardisation more reproducible, a nephelometer reading of this suspension was taken. This was found to give a value of 30. Thus, throughout the growth trials the yeast suspensions for inoculation were always diluted to give a nephelometer reading of 30.

Culture Media

In order to check that the yeast species selected utilise both arabinose and xylose, with ammonia as the sole nitrogen source and in the absence of growth factors, a basal salts medium was used. This medium was prepared using the mineral salts base of van der Walt (1971), viz:- 0.01% KH_2PO_4; 0.05% $MgSO_4.7H_2O$; 0.01% $CaCl_2.2H_2O$; 0.1% NaCl to which 0.5% $(NH_4)SO_4$ and 2% xylose or arabinose was added. The pH of this medium was adjusted to 4.5, as this was the pH at which it was hoped to grow the yeast in order to minimise the growth of contaminants.

As indicated on pages 56-64 , the media produced by three different hydrolysis systems were considered worthy of further investigation as substrates for yeast production. These were the hydrolysates produced by treating pig slurry with 1% H_2SO_4 at 90°C for 56 h (1 [90] 56), 1% H_2SO_4 at 100°C for 24 h (1 [100] 24) and 5% H_2SO_4 acid at 90°C for 3 h (5 [90] 3). These hydrolysates were all produced from the same batch of raw slurry by the method described on pages 53-55. For trials of yeast growth the pH of the hydrolysate was raised to 4.5 by the addition of NaOH. After the completion of the hydrolysis procedure, the acidified suspension was sterile. It was therefore essential that

the adjustment of pH, separation of the solid and liquid fractions and the washing of the solids was completed aseptically, in order to maintain the sterility of the final hydrolysate growth medium. As a final precautionary measure, the hydrolysate was membrane filtered to ensure the exclusion of any contaminating organisms.

Cultural Conditions

The yeasts were cultured in 20 ml volumes of media contained in 50 ml Erlenmyer flasks which were covered with metal caps. They were continually shaken throughout the incubation period in an orbital incubator (Gallenkamp & Co Ltd, Registered Office, PO Box 290, Technic House, Christopher Street, London), operating at 125 rev/min. The temperature of the incubator was controlled at 25^{o}C, a temperature at which all the selected yeast species were known to grow reasonably well (Lodder, 1971a; van der Walt *et al*, van der Walt & Nakase, 1973).

Assessment of Biomass Yield

In selecting a yeast species suitable for use as a source of SCP it was important to maximise the total biomass yield. Thus, a method of measuring biomass yield was adopted to gauge the suitability of a particular species, for growth on pig slurry hydrolysate. The determination of dry weight is a simple and accurate method of measuring the total yield of yeast from a liquid medium. Thus, the method of determining the dry weight described below was developed. For this purpose, throughout the growth cycle of a yeast species in each hydrolysate, two flasks were removed every 24 h for dry weight analysis. The contents of each flask were separated by centrifugation, the yeast was then washed with distilled water, recentrifuged and then transferred to dried and pre-weighed foil dishes. The contents of these dishes were dried in a hot air oven at 105^{o}C for 2 h. Prior to re-weighing, the dishes were cooled in a vacuum desiccator over $CaCl_2$ and the results for the two samples averaged.

RESULTS AND DISCUSSION

All the yeast species tested were found to grow well in basal salts medium, with either xylose or arabinose as carbon sources. Yields of between 0.26 and 0.43 $g.g^{-1}$ available carbohydrate were recorded for all test species.

In trials in which the hydrolysate media were used as substrates, none of the yeastsgrew in any of the hydrolysates tested, even after 6 days incubation.

As several factors could be responsible for the failure of yeast growth, it was decided to investigate each in turn, starting with the most obvious cultural parameters. Although yeasts had grown in pentose/basal salts medium at pH 4.5, it was thought that if the hydrolysate was a less favourable medium, this pH might be too low for yeast growth. However, the failure of test yeasts to grow in hydrolysate medium at pH 5.0 indicated that this was not the case. The growth of test yeasts on pentose/basal salts showed that they have the ability to synthesise all their cellular components from simple inorganic substances. The presence of an inhibitory agent in the hydrolysate, which could block one or more of these anabolic pathways, would account for the lack of yeast growth, and this was investigated by adding a solution of growth factors (van der Walt, 1971) to the hydrolysate. Once again, no yeast growth was observed. The standard inoculation procedure was then investigated, as it was considered that the number of viable cells introduced into the hydrolysate medium might be too low to survive a lengthy lag period and establish growth. However, it was found that doubling the size of inoculum had no effect.

The above results suggested that the inhibition of yeast growth in the hydrolysate is most probably due to the presence of a toxic agent. Before carrying out more elaborate investigations into the nature of the inhibition, it was established that this was not a problem confined to one batch of slurry. For this purpose, hydrolysates were prepared from pig slurry samples obtained from the College Farm and a variety of commercial farms. No yeast growth was observed in any of these preparations, which indicated that the inhibitory agent was common to hydrolysates produced from the slurry of all fattening pigs fed similar diets.

An anomolous feature of this problem was that in preliminary trials (Bruce, 1973) in which the feasibility of this approach to slurry utilisation had been investigated, the yeast isolate 53A was reported to have grown on a medium prepared by hydrolysing pig slurry with 10% w/v H_2SO_4 at 100°C for 2 h. This hydrolysate had been neutralised with $Ca(OH)_2$, which produced an

insoluble precipitate of $CaSO_4$, and this is the major difference between the preliminary trials and the procedure adopted for the work described here, in which neutralisation with NaOH resulted in the formation of a soluble salt. In the preliminary trials, both the $CaSO_4$ precipitate and the solid residue of hydrolysis had been washed several times with large quantities of water to ensure the maximum recovery of soluble carbohydrates, however, when NaOH was the neutralising agent, good carbohydrate recovery was achieved with only one washing of the solid residue (page 55). Thus, it seems possible that in the preliminary trials the toxic effect had been overcome by dilution, or that the $CaSO_4$ precipitate had completely or partially absorbed some toxic substance from the hydrolysate.

In order to investigate the effect of dilution of the hydrolysate on the inhibition of yeast growth, a hydrolysate produced with 5% w/v acid at 90°C for 3 h was diluted with the xylose basal salt medium to give the following proportions of hydrolysate to xylose basal salts: 0:10; 1:9; 2:4 ; 3:7; 2:3; 1:1; 3:2; 7:3; 4:1; 9:1; 10:0 v/v. At this point it was decided that if inhibition was the cause of the failure of yeast growth it appeared to be universal, and therefore the number of yeast species used in further investigations was limited to two, viz. *Endomycopsis burtonii* and *Rhodosporidium toruloides*. Both of these yeasts were found to grow when the proportion of hydrolysate in the medium was 4:6 v/v or less. Repeating the experiment with distilled water instead of xylose basal salts medium produced similar results. It therefore appears that the acid hydrolysates of pig slurry contain an inhibitory agent, the effect of which can be overcome by dilution. Several origins of the toxin can be postulated, and a number of these were considered worthy of further investigation.

It is known that fattening pigs are fed large amounts of copper and zinc in their diet (Table 3). Much of this passes through the digestive system and accumulates in the faeces (Evans *et al*, 1978). As it is known that large amounts of heavy metals inhibit the growth of microorganisms, three different hydrolysates were analysed for heavy metal content by Atomic Absorption Spectroscopy[1] (Table 16).

1. *The analysis for heavy metals by Atomic Absortpion Spectroscopy was undertaken by Dr J. Dixon of the Chemistry Department, The West of Scotland Agricultural College.*

TABLE 16 The heavy metal content of the H_2SO_4 hydrolysates of pig slurry.

CONDITIONS OF PRODUCTION OF THE HYDROLYSATE			HEAVY METAL CONTENT ($mg.l^{-1}$)			
Acid Concentration	Temperature	Time	Zn	Cu	Fe	Mn
1	90	56	19	0.19	4.5	15.1
5	90	3	28	3.9	12.1	16.5
1	100	24	28	0.16	4.2	9.5

These results indicate that the levels of the heavy metals Cu and Fe in the hydrolysates are insignificant. Zn is present in the largest quantities at a maximum detected level of 28 $mg.l^{-1}$. It was demonstrated, by adding Zn and Cu to the xylose/basal salts medium in quantities of up to 500 $mg.l^{-1}$, that both of the test yeasts grow in Zn and Cu concentrations of almost 20 times those encountered in the hydrolysates. The fact that the heavy metals in the hydrolysates do not appear to be present at inhibitory levels, was confirmed by two supplementary experiments. In the first, the chelating agent ethylene diamine acetic acid (EDTA) was used to lower the heavy metal content of the hydrolysates. The test yeasts still failed to grow in these hydrolysates. In the second experiment, hydrolysates were prepared from the slurry of breeding sows which had no Zn or Cu supplements in their diet. Again, both yeasts failed to grow in the resulting hydrolysates. Thus, it appears that the heavy metal concentrations of pig slurry hydrolysates are not the reason for the failure of the yeasts to grow.

Two compounds known to be present in the hydrolysates in large amounts are ammonia and sodium sulphate. Although ammonia and sodium sulphate may not be inhibitory in themselves, it was thought that the concentrations in which they are present in the hydrolysates may have a toxic effect on yeast growth. In the hydrolysate produced with 5% acid, the level of sodium sulphate in the resulting medium is approximately 3% w/v. In the xylose/basal salts medium, however, additions of sodium sulphate to give concentrations of up to 5% w/v were not inhibitory to yeast growth.

It is known that the amount of urine in the slurry affects the ammonia level of the resulting hydrolysate (Evans *et al*, 1978). For this reason, several different hydrolysates were analysed for ammonium nitrogen by the standard distillation technique (American Public Health Association,1971). In all the samples tested, the highest level of ammonia encountered was 1200 $mg.l^{-1}$. Additions of ammonium sulphate to xylose/basal salts medium to give final concentrations of ammonium nitrogen of up to 5000 $mg.l^{-1}$ allowed the yeast to grow. It is apparent that from these results neither the concentration of sodium sulphate nor the level of ammonia in the hydrolysates is normally toxic to yeasts.

Microscopic examination of the non-viable yeast cells in the hydrolysate media revealed that small salt crystals congregated around the cells. This precipitate formed in the membrane filtered hydrolysate medium when it was left to stand for a few hours. At first it was thought that these crystals might have a toxic effect on the yeast cells but even after they were removed by a secondary filtration procedure freshly inoculated yeast failed to grow.

There remained one other obvious source of toxicity in pig slurry hydrolysate. This is the formation of carbohydrate breakdown products in the presence of acid, ammonia and other nitrogenous compounds. It had already been shown (pages 60-61) that, even in the absence of ammonia, xylose and arabinose were broken down by the hydrolysis conditions used for the preparation of hydrolysate for yeast growth trials. The presence of ammonia and other nitrogenous materials is likely to complicate the degradative reactions. These products will probably have a non-ionic organic nature, which means that they may be adsorbed by activated charcoal, if they are of sufficiently large size. Indeed it was shown that hydrolysates which were treated with activated charcoal,by the standard method used to prepare samples for HPLC analysis (page 45), supported good yeast growth. This indicated that the toxic agent was removed from solution by the activated charcoal, and was some type of organic compound. In order to test the ionic nature of the inhibitor, 10 ml of the hydrolysate were freeze-dried. The addition of 10 ml of methanol was known to dissolve organic substances such as carbohydrates, but to leave all ionic salts completely undissolved (page 47). The two fractions were separated, and the methanol removed by evaporation

under reduced pressure. A test was devised in which each of these fractions was used as a component of a yeast growth medium. The hydrolysate salts were dissolved in a 2% xylose solution, and the methanol soluble fraction which should contain the carbohydrates of the hydrolysate was added to the yeast basal salts medium. Both these media were inoculated with yeast. It was found that the hydrolysate salts and xylose medium supported good growth, but no growth was observed when basal salts were added to the methanol soluble substances. However, this fraction supported good growth after charcoal treatment. This indicated that the inhibitor was methanol soluble and therefore non-ionic in nature.

Yeast growth trials could therefore be continued using either diluted or charcoal treated hydrolysates, and further attempts to identify the unknown toxic agent(s) abandoned. However, as it is proposed to utilise the yeast biomass as an animal feed, the possible accumulation of unidentified potentially toxic substances in the yeast cells is considered to be toxicologically unacceptable. Charcoal treatment is a more attractive alternative, but involves the inclusion of another step in the production process, which may be uneconomic. Even although yeast grows in the treated medium, it cannot be guaranteed that the unidentified compound has been completely removed by the charcoal.

For these reasons it is considered desirable to make further attempts to identify the nature and origin of the toxic compound(s). It is hoped that this information will allow the compound(s) to be prevented from forming initially, be safely and completely removed, or be diluted to a level at which growth is possible, in the knowledge that the product is unlikely to be toxic to animals.

Qualitative Detection of the Toxic Components of Hydrolysed Pig Slurry

Sugars are known to decompose readily in acid or alkaline media, in the presence of various amines, or simply by heating in the dry state. Pentoses have been shown to be more labile than hexoses (Stamm, 1964).

The mechanisms involved in the breakdown of carbohydrates are complex and include transformations of the carbohydrates, fragmentation, recombination of fragments and ring closures. Table 17 lists

several compounds that have been isolated and identified as breakdown products of pentoses in acidic conditions (Anderegg & Neukom, 1974; Popoff & Theander, 1972).

TABLE 17 Breakdown products of pentoses in acidic conditions

COMPOUND	REFERENCE
2-furfuraldehyde	Popoff & Theander, 1972
3,8-dihydroxy-2-methylchromone (alginetin)	" "
5,6,7,8-tetrahydro-3,5-dihydroxy-2-methyl-8- oxobenzopyrone (a precursor of alginetin)	" "
2,3-dihydroxyacetophenone	" "
4-hydroxy-5-methyl-2(2H)-furanone	Anderegg & Neukom, 1974

Several so far unidentified compounds have also been found (Popoff & Theander, 1972). 2-furfuraldehyde is regarded as the main precursor of the non-aromatic chromones and catechols, which are intermediates in the formation of dark brown phenolic or enolic condensation and polymerisation products.

In acidified pig slurry the browning reactions are complicated still further by the presence of ammonia and various amino compounds. In this case complex carbonyl-amino condensation reactions can take place. Aso (1953) has isolated 3-hydroxy-pyridine and 2,3-dihydroxy-pyridine when xylose is heated in the presence of ammonia. Ultimately, a complex range of brown nitrogenous polymers and co-polymers called melanoidins are produced (Hodge, 1963).

MATERIALS AND METHODS

It was considered necessary to analyse for three different types of compounds which could occur as a result of carbohydrate breakdown. The first compound that is likely to be formed by the initial degradation of pentoses is the aldehyde, 2-furfuraldehyde. Then in the presence of ammonia, carbonyl-amino reactions may produce amino compounds. Finally, the culmination of the breakdown reactions produce phenolic/enolic condensation products. For detection of these three different types of compounds, TLC was regarded as the most useful qualitative method, and for this purpose the general TLC technique described on pages 30-32 was used.

TLC for Aldehydes (Stahl & Jork, 1969)

Sample

The sample used for this method of TLC was basically the 5[90]3 slurry hydrolysate, which had been ultrafiltered to remove all solutes above a molecular weight of 500. This ultrafiltrate was then desalted as for HPLC, but in this case the solutes were redissolved in methanol so that they were 5 times more concentrated than in the original hydrolysate. A portion of this sample was charcoal treated.

Reference Compound

5 µl of 2-OH furfuraldehyde prepared in methanol at a concentration of 2.5 µg/ml was used as a reference standard.

TLC Procedure

Thin-layer plates were prepared with silica gel G at a thickness of 250 µ, and run in a chloroform solvent. The aldehydes were visualised as yellow spots with 2,4-dinitrophenylhydrazine in 2N hydrochloric acid.

TLC for Amino Compounds (Lewis & Smith, 1969)

Sample

5 µl of the sample used in the TLC for aldehydes was employed.

Reference Compounds

Two amino-sugars, glucosamine hydrochloride and galactosamine hydrochloride, and two amino acids, hydroxyproline and glycine, were used as standards to test that the system separates amino compounds.

TLC Procedure

Thin-layer plates were prepared with "Avicel" cellulose at a thickness of 250 µ, developed in n-butanol:pyridine:glacial acetic acid:water (60:45:4:30 by volume) and the separated amino compounds visualised with 0.2% w/v ninhydrin in ethanol.

TLC for Phenolic and Enolic Compounds (Anderegg & Neukom, 1974)

Sample

The phenolic and enolic constituents of pig slurry hydrolysate were extracted and purified prior to application to the TLC plate. In this procedure the aqueous hydrolysate was extracted with an equal volume of ethyl acetate. This extraction was repeated five times. The combined ethyl acetate extract was evaporated to dryness on a rotary evaporator under reduced pressure. The solutes were then

resuspended in methanol so that the concentration of the extract was 10 times that of the original hydrolysate. The product was a straw coloured liquid. A portion of this sample was treated with activated charcoal.

Reference Compounds

A study of the relevant literature (Anderegg & Neukom, 1974; Aso, 1953; Hodge, 1963; Popoff & Theander, 1972) indicates that several compounds are likely to be formed by the breakdown of pentoses in acid conditions. These compounds are listed in Table 17 and of these, only 3-OH pyridine is commercially available. It appears that most of the laboratories working in the field of carbohydrate breakdown have the facilities to isolate, identify and purify their own reference compounds (Popoff & Theander, 1972). Another compound, 2,3-dihydroxy-2-cyclopenten-1-one (reductic acid) is also commercially available. Although this compound has not been detected as a breakdown product of pentoses, it is known to be formed when uronic acids are subjected to acid conditions at temperatures approaching 100°C (Popoff & Theander, 1972). Uronic acids were qualitatively detected in pig slurry hydrolysate (pages 32-36), and thus it is possible that reductic acid will be present in the hydrolysate. Finally, as some of the phenolic breakdown products of carbohydrates are related in structure to pyridine, it was also decided to use this compound as a reference to test the separation system.

The three reference substances were prepared in methanol at a concentration of 2.5 $g.l^{-1}$. 5 μl of each solution was applied to the TLC plate.

TLC Procedure

Thin layer plates were prepared with silica gel GF used at a thickness of 250 μ. This adsorbent contains a fluorescent inorganic pigment. On this material, substances which adsorb in the ultra-violet (UV) region appear as dark, quenching spots on a fluorescent background. A developing solvent system of benzene:ethyl acetate (3:1 by volume) was used.

UV light at a wavelength of 254 nanometres (nm) was used to detect substances which adsorb in the UV region and the phenolic nature of these compounds confirmed by spraying with a 5% w/v solution of ferric chloride in 0.5 N hydrochloric acid ($FeCl_3$ spray).

Phenols are blue-purple in colour on visualisation with this spray.

RESULTS AND DISCUSSION

TLC of the hydrolysate for the presence of 2-furfuraldehyde produced an almost undetectable yellow spot at the hRf of the reference standard, and no other aldehydes were visualised. The reference 2-furfuraldehyde had an hRf of 56 and was an intensely yellow spot. This indicated that there was little free furfuraldehyde present in pig slurry hydrolysate.

The results of TLC for amino compounds are shown in Chromatograph 4, and the corresponding hRf values are given in Table 18. This chromatograph indicates 11 ninhydrin positive components in pig slurry hydrolysate. A twelth component at hRf 97 shows up as a yellow spot prior to spraying, but does not react with ninhydrin.

The reference amino acids and amino sugars are well separated in this system. The sample has one component that could correspond to either glucosamine or galactosamine, and another component has the same hRf as the amino acid, glycine. The other ninhydrin positive spots are likely to be other amino acids or amino sugars not included as references. None of the ninhydrin positive components were removed when the sample was treated with activated charcoal. The only spot that disappeared was the non-reactive yellow spot at hRf 97. This indicates that the inhibitor present in pig slurry hydrolysate is not an amino compound. Further, when a similar plate was visualised for phenolic/enolic substances instead of amino compounds, only the component with hRf 97 was found to be sensitive. This suggests that there is a yeast growth inhibitor present in pig slurry hydrolysate that is phenolic or enolic in nature.

Chromatograph 5 was produced when pig slurry hydrolysate was analysed for the presence of phenols and enols. It can be seen that the hydrolysate produces 4 components which quench UV light and also react with $FeCl_3$ spray. A fifth spot with an hRf of 16 shows up on the plate as brown prior to spraying with $FeCl_3$, and does not quench UV light (Table 19). With $FeCl_3$ treatment this component develops the blue colour which indicates the presence of a phenolic or enolic compound. If the plate is left unsprayed, the spots which quench UV light turn brown. Popoff & Theander (1972) found that on standing chromones and catechols turned brown on unvisualised silica

CHROMATOGRAPH 4 Thin-layer chromatography of amino compounds on cellulose layers run in n-butanol:pyridine:glacial acetic acid:water solvent and visualised with ninhydrin spray.

Gam	Galactosamine hydrochloride
Glm	Glucosamine hydrochloride
G	Glycine
H	Hydroxyproline
Sa	Pig slurry hydrolysate

▩ purple

≡ yellow

Chromatograph 4

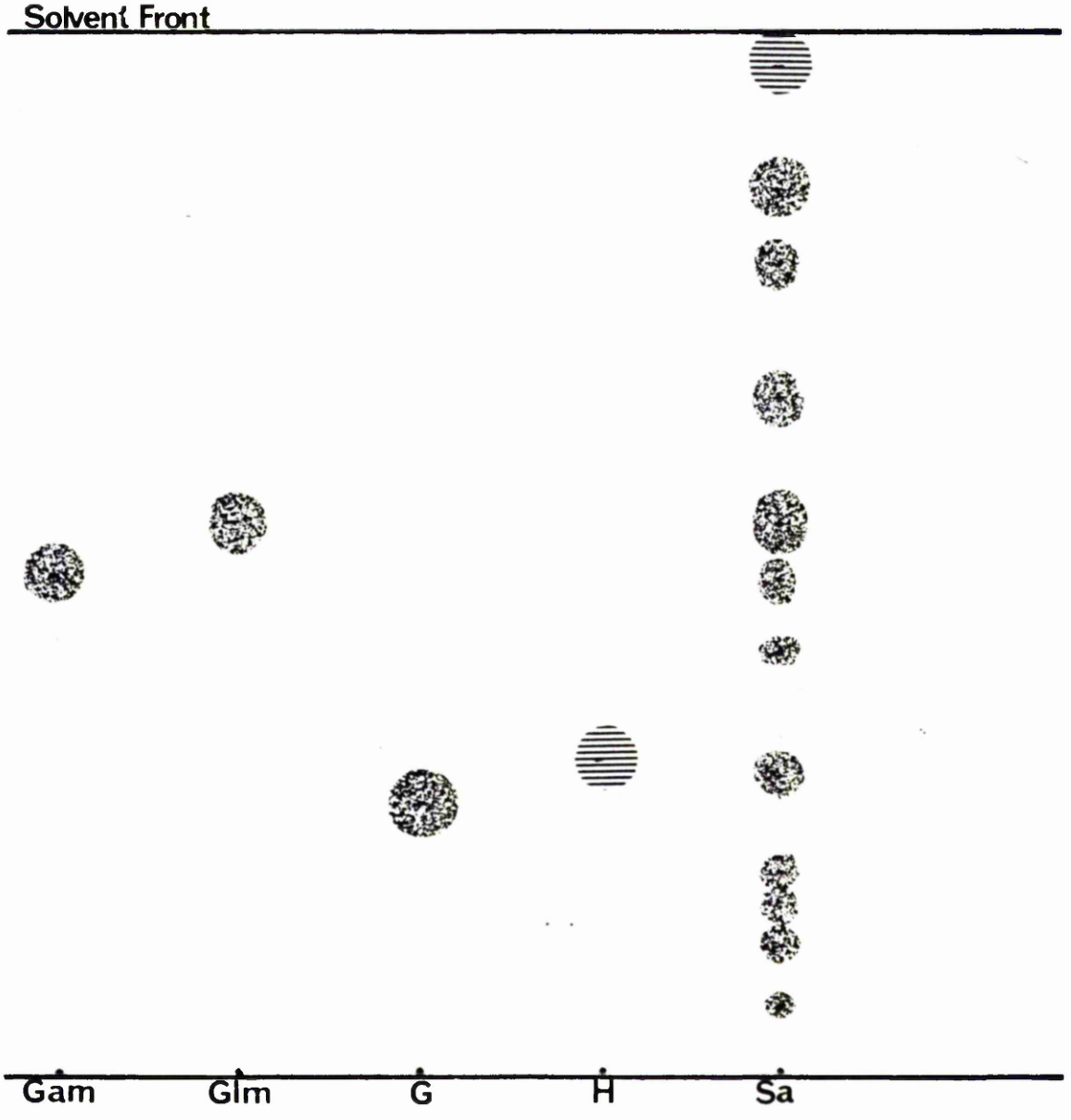


TABLE 18 hRf values and colour development of amino compounds developed on cellulose layers run in n-butanol:pyridine:glacial acetic acid:water solvent and visualised with ninhydrin spray.

SUBSTANCE	hRf	COLOUR
Galactosamine hydrochloride	30	Purple
Glucosamine hydrochloride	27	Purple
Glycine	54	Purple
Hydroxyproline	48	Yellow
Acid Hydrolysed Pig Slurry:		
Component 1	7	Purple
2	13	Purple
3	17	Purple
4	20	Purple
5	29	Purple
6	41	Purple
7	48	Purple
8	53	Purple
9	65	Purple
10	78	Purple
11	85	Purple
12	97	Yellow (prior to spraying, does not react with ninhydrin)

CHROMATOGRAPH 5 Thin-layer chromatography of phenolic/enolic compounds on silica Gel GF plates run in benzene:ethyl acetate solvent and visualised under UV light and by spraying ferric chloride.

P	Pyridine
OH-P	3-OH pyridine
R	Reductic acid
Sa	Pig slurry hydrolysate
▒	blue

Chromatograph 5

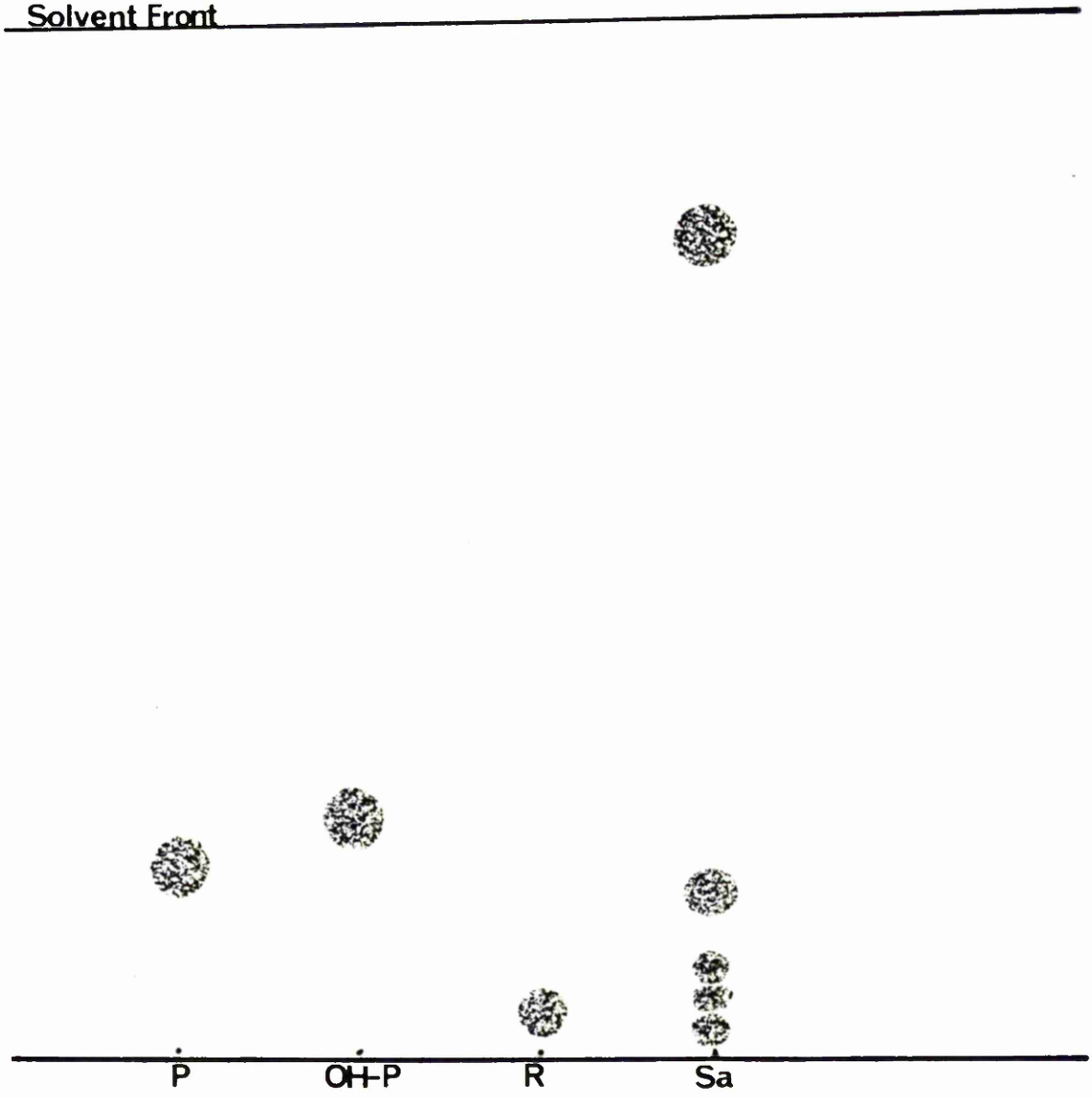


TABLE 19 hRf values and colour development of phenolic/enolic compounds developed on silica gel GF layers in benzene:ethyl acetate solvent and visualised under UV light and with $FeCl_3$ spray.

SUBSTANCE	hRf	UV LIGHT AT 254 nm	COLOUR WITH $FeCl_3$ SPRAY
Pyridine	18	UV quenching	Blue
3-OH	23	UV quenching	Blue
Reductic acid	5	UV quenching	Blue
Acid Hydrolysed Pig Slurry:			
Component 1	3	UV quenching	Blue
2	5	UV quenching	Blue
3	8	UV quenching	Blue
4	16	Non UV quenching	Blue
5	78	UV quenching	Blue

gel plates. They postulated that this reaction was due to the coupling of these compounds with traces of iron in the silica gel.

Comparing the hRf values of the reference compounds to those of the sample components indicates that pig slurry hydrolysate may contain some reductic acid, which probably results from the breakdown of the uronic acids.

On treatment of the phenolic/enolic extract of pig slurry hydrolysate with charcoal, the spots at hRf 16 and hRf 78 disappear. Charcoal treatment is known to remove the inhibitory effect on yeast growth, and it is therefore possible that either or both of these phenolic components are toxic to yeast.

An experiment was now undertaken to show that phenolic/enolic compounds can be produced by the breakdown of the pentoses, arabinose and xylose, as these are the major cabohydrates produced by mild acid hydrolysis of pig slurry. For this purpose, a solution containing a mixture of 0.5% w/v xylose and 0.5% w/v arabinose was hydrolysed in 5% w/v H_2SO_4 at $90^{o}C$ for 3 h. The phenolic/enolic compounds were extracted for the resulting liquid and chromatographed against the reference standards and the extract of pig slurry hydrolysate. The results are shown in Chromatograph 6 and the hRf values are presented in Table 20. These results indicate that the three phenolic components with the hRf's of 3, 5, and 78 found in pig slurry hydrolysate can be produced by the breakdown of pure pentoses in mild acid conditions. The components with hRf values of 8 and 16, which are found in slurry hydrolysate, do not appear to be produced by the breakdown of pure pentoses. This experiment was repeated with the addition of 0.5% w/v ammonium sulphate ($(NH_4)_2SO_4$), as this was thought to more closely represent the situation encountered during the hydrolysis of pig slurry. In fact it was found that the presence of $(NH_4)_2SO_4$ did not alter the pattern of phenolic compounds obtained by acid hydrolysis of pentoses. Both systems produced three components with hRf values of 3, 5 and 78.

It was thought that the phenolic component of pig slurry hydrolysate which has an hRf of 5 was possibly reductic acid produced by the breakdown of uronic acids. Aso (1953) has isolated this compound from acidified solutions of glucuronic acid and galacturonic acid, but so far it has not been identified as a breakdown product of pentoses (Anderegg & Neukom, 1974; Aso, 1953; Popoff & Theander, 1972). Confirmation of the identity of this and the other components

CHROMATOGRAPH 6 Thin-layer chromatography of phenolic/enolic compounds on silica gel GF plates run in benzene:ethyl acetate solvent and visualised under UV light and by spraying with ferric chloride.

P	Pyridine
OH-P	3-OH pyridine
R	Reductive acid
Sa	Pig slurry hydrolysate
AP	Acid treatment of pure pentoses
[illegible]	blue

Chromatograph 6

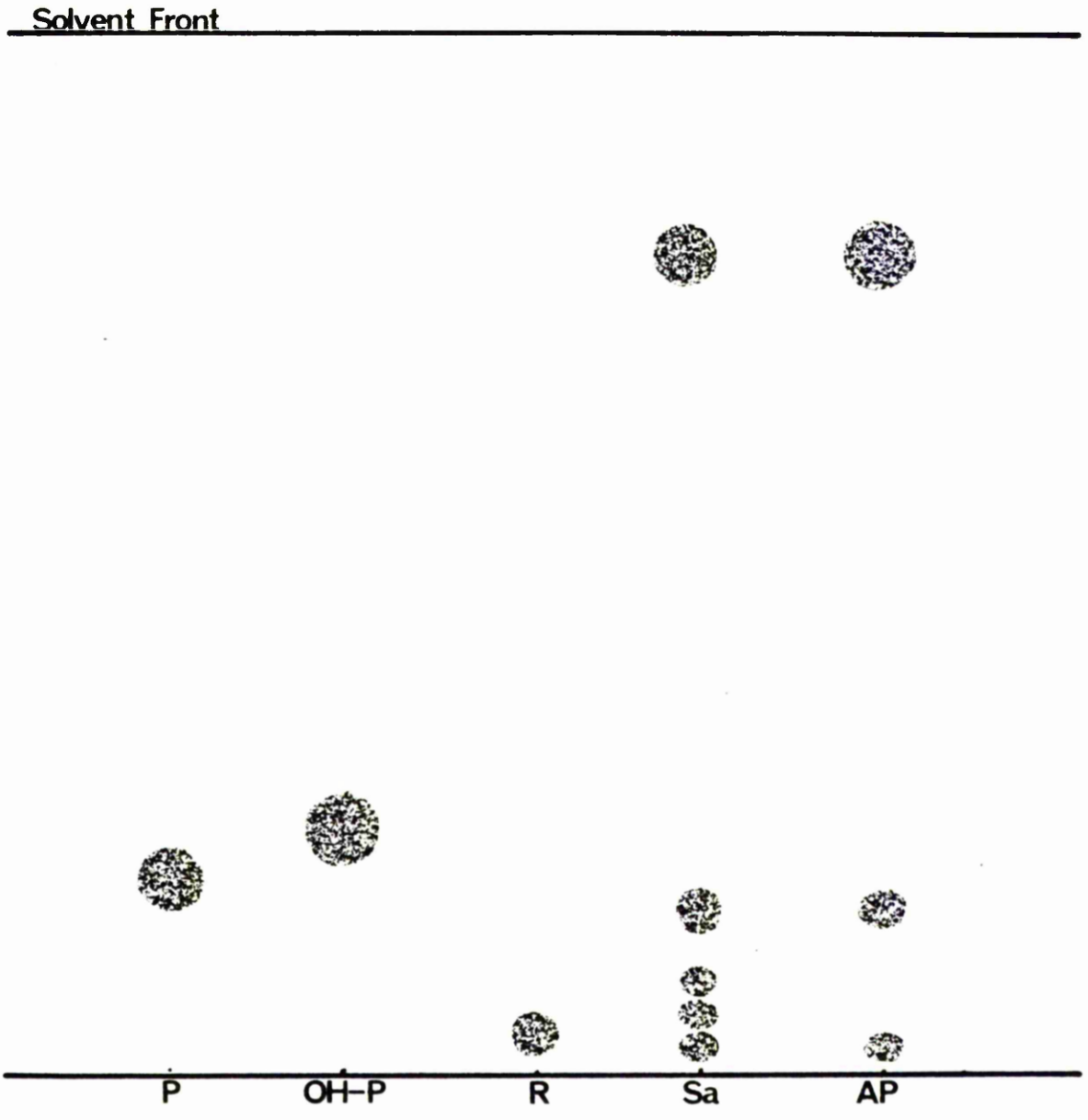


TABLE 20 hRf values and colour development of phenolic/enolic compounds developed on silica gel GF layers in benzene:ethyl acetate solvent and visualised under UV light and with $FeCl_3$ spray.

SUBSTANCE	hRf	UV LIGHT AT 254 nm	COLOUR WITH $FeCl_3$ SPRAY
Pyridine	18	UV quenching	Blue
3-OH pyridine	23	UV quenching	Blue
Reductic acid	5	UV quenching	Blue
Acid Hydrolysed Pig Slurry:			
Component 1	3	UV quenching	Blue
2	5	UV quenching	Blue
3	8	UV quenching	Blue
4	16	Non UV quenching	Blue
5	78	UV quenching	Blue
Acid Treated Pentoses:			
Component 1	3	UV quenching	Blue
2	5	UV quenching	Blue
3	78	UV quenching	Blue

would require a much more extensive chemical analysis, which is outwith the scope of this work.

Overall, these experiments indicated that mild acid treatment of pig slurry inevitably produces a certain amount of the phenolic/enolic compound(s) that inhibit(s) yeast growth. Consequently, growth in hydrolysed slurry can only be carried out if the compound(s) is removed or diluted to a level at which it is not inhibitory. As this type of phenolic breakdown product of carbohydrates is commonly found in baked and cooked foods (Hodge, 1963), it may have little toxicological significance in animal feed and therefore dilution of the hydrolysate may be acceptable. In the long term, however, charcoal treatment may be the more attractive alternative, as stringent control of the substrate may be required by law to guarantee the toxicological quality of the SCP product.

If diluted pig slurry hydrolysate is to be used for yeast growth, the ultimate fate of the growth inhibitor(s) is of interest. For this reason, a sample of hydrolysate was diluted 4:6 v/v with water and inoculated with *Rhodosporidium toruloides*. Samples were taken every 12 h throughout the growth cycle and the separated hydrolysate was extracted for phenolic constituents by the standard method. TLC of the phenolic extracts indicated that one of the phenolic constituents disappeared during the growth cycle of the yeast. A visual assessment of the intensity of the spots indicated that the five phenolic components, including the inhibitory compound, have neither been metabolised nor accumulated by the yeast during growth. This suggests that these phenolic breakdown products may be completely removed by washing the yeast during the separation process.

CONCLUSIONS

1. 2-Furfuraldehyde is not present in the hydrolysate in significant amounts.
2. The hydrolysate contains several amino compounds.
3. Treatment of the hydrolysate with activated charcoal does not remove any amino compounds.
4. The hydrolysate contains 5 phenolic/enolic components in detectable quantities.

5. Without the use of more sophisticated analytical techniques, it is not possible to identify these compounds.
6. Two of the phenolic components of pig slurry hydrolysate are removed by treatment with activated charcoal, namely those with an hRf of 16 and 78, which indicates that they may be inhibitory to yeast growth.
7. Three of the phenolic/enolic components of pig slurry hydrolysate can be produced when xylose, arabinose and $(NH_4)_2SO_4$ are subjected to mild H_2SO_4 hydrolysis conditions, namely those of hRfs 3, 5 and 78
8. The phenolic components of pig slurry hydrolysate do not appear to be metabolised or accumulated during the growth of a yeast species.
9. It is possible to use the hydrolysate in the diluted or charcoal treated form for the growth of yeast species.

Investigation of the Toxicity of the Amino Components and the Phenolic/Enolic Components of Pig Slurry Hydrolysate on Yeast Growth

The analytical techniques described in the previous Section only separate microgram (µg) amounts of test compounds. A preparative technique was therefore used to separate milligram (mg) amounts of components of pig slurry hydrolysate. These compounds were then extracted from the plate and added to xylose/basal salts media to test their effect on yeast growth. The preparative technique was used to separate and isolate both amino compounds and phenolic/enolic compounds from hydrolysate.

MATERIALS AND METHODS

Preparative TLC

Application of the Sample

In the preparative technique the sample was applied in a band using the cumulative spot technique (Stahl, 1969). It was found that 1 ml of sample could be applied to the plate in 5 µl fractions by this method.

TLC Procedure

Cellulose or silica gel GF were prepared by the method described by Stahl (1969).

The solvents used to develop the plates were the same as those used in the corresponding analytical TLC method.

Procedures which do not harm the separated substances were used to identify the zones. In the case of phenolic/enolic compounds, visualisation was achieved non-destructively by exposing the plate to UV light at a wavelength of 254 nm. With amino compounds the plate was covered except for 2 strips at either edge. These strips were visualised with ninhydrin. Although the best colour development was not achieved, heating the plate to 50°C was found to be sufficient to visualise the bands.

Collection and Extraction of Separated Components

Separated components were most easily collected by scraping the zones off with a spatula. In order to extract the compounds from the adsorbent, 5 ml of ethyl acetate for phenolic compounds or 5 ml of methanol for amino compounds were added to the scrapings, and the suspension vigorously shaken on a wrist shaker for 1 h. Each extract was separated from the adsorbent by centrifugation, the extraction procedure was repeated and the combined extracts evaporated to dryness by rotary evaporation under reduced pressure. The dry solute was then tested for its inhibitory effect on yeast growth. A control sample was prepared by scraping off a layer of adsorbent on which there was no separated component, and extracting the adsorbent by the standard method. This was done to ensure that no inhibitory substance was extracted from the adsorbent layer itself.

Demonstration of the Effect that Substances Separated by Preparatory TLC Have on Yeast Growth

The solutes separated and extracted by the preparatory TLC procedure were resuspended in 1 ml of xylose/basal salts medium (page 70). As the 1 ml sample applied to the TLC plate was 5 or 10 times more concentrated than the original hydrolysate, even allowing for losses incurred during the preparatory procedure, the concentration of the extracted compounds in the growth medium should be at least as great as that encountered in the hydrolysate. The growth medium was inoculated with *Rhodosporidium toruloides* by the standard method and incubated at 25°C. Presence or absence of growth was assessed visually and microscopically.

RESULTS AND DISCUSSION

When the sample was subjected to preparative TLC for amino compounds and the 12 components extracted and tested for their ability to support yeast growth, it was found that the control tube and tubes with extracts from layers 1-11 showed considerable turbidity after 2 days incubation, while the tube which contained the extract of the ninhydrin sensitive component with an hRf of 97 showed no visible growth even after 6 days. Microscopic examination of the contents of this tube indicated that no healthy reproducing yeast cells were present. This confirmed the results obtained previously (page 80), where the only spot removed by charcoal treatment was that with hRf 97, indicating that this fraction was inhibitory to yeast growth. The phenolic/enolic nature of this extracted fraction was confirmed by subjecting it to TLC for phenolic/enolic compounds (pages 78-80). In this procedure components with hRfs 3, 8, 16 and 78 were visualised. It had previously been shown that pig slurry hydrolysate contained 5 phenolic/enolic components with hRfs 3, 5, 8, 16 and 78 (Table 20). Thus the component with hRf 5 was absent from the extracted fraction, which could be explained either by the fact that it was not recovered in detectable quantities during the preparatory procedure, or that it separated at a different hRf from the other phenolic/enolic components during the TLC for amino compounds.

By using the preparative TLC to extract the 5 phenolic/enolic components of pig slurry hydrolysate, their toxicity to yeast growth was assessed. Of all the components tested, only the compound with an hRf of 78 prevented yeast growth. This agreed with the results of charcoal treatment (page 81), which demonstrated that this component was adsorbed by activated charcoal. The other component with hRf 16 which was also adsorbed by activated charcoal, did not have any inhibitory effect on yeast growth. Thus, it appears that there is at least one phenolic/enolic compound present in pig slurry hydrolysate that inhibits yeast growth.

CONCLUSIONS

1. None of the amino compounds present in pig slurry hydrolysate appears to inhibit yeast growth.
2. Using a preparatory TLC technique, only 1 of the 5 phenolic components of pig slurry hydrolysate is shown to inhibit yeast growth.

3. The inhibitory phenolic/enolic compound has an hRf of 78.

Selection of a Yeast Species for Growth on Pig Slurry Hydrolysate

MATERIALS AND METHODS

In the preceeding sections it was established that pig slurry hydrolysate medium contains a phenolic/enolic substance that inhibits the growth of yeasts. However, it was shown that this inhibitory effect can be overcome by dilution or removed by adsorption of the toxic agent with activated charcoal. During the investigations into the nature of the inhibition, only two of the yeast species listed in Table 15, viz *Endomycopsis burtonii* and *Rhodosporidium toruloides* were shown to grow on pig slurry hydrolysate 5 [90] 3 which had been diluted 4:6 v/v. It was therefore necessary to establish which of the test yeasts listed in Table 15, including yeast species 53A, would grow best on diluted hydrolysate medium. For this purpose, the hydrolysates 5 [90] 3, 1 [100] 24 and 1 [90] 56 were diluted with water to give final concentrations of 2:3, 1:1 and 3:2 v/v. Six flasks, each of which contained 20 ml of a diluted hydrolysate preparation, were inoculated with a test yeast species. After 2, 3 and 4 days duplicate flasks were removed and the amount of growth assessed. The cultivation conditions in the orbital incubator, preparation of inoculum, and method of assessing biomass yield were as described previously (pages 70-71).

RESULTS AND DISCUSSION

The results of all yeast growth experiments are reported as g biomass per g measured available monosaccharide. Although it is appreciated that the yeast may also utilise other minor carbon sources such as uronic acids or amino sugars, it is felt that this method of expressing yield enables a reasonable comparison of the efficiency of biomass production in each test media to be made. The monosaccharide content of each of the hydrolysate media was therefore measured by HPLC and the results are given in Table 21. The biomass yields obtained when the test yeast species were grown on dilutions of the hydrolysates 5 [90] 3, 1 [100] 24 and 1 [90] 56 are given in Tables 22, 23 and 24 respectively.

It can be seen from these results that all 12 test yeast species grow reasonably well in the 2:3 v/v dilution of the 1 [100] 24, 1 [90] 56 and 5 [90] 3 hydrolysates. With the hydrolysate 1 [90] 56, raising the

TABLE 21 The monosaccharide content of the bulk hydrolysates used in yeast growth trials.

ACID TREATMENT SYSTEM			YIELD OF CARBOHYDRATE $g.l^{-1}$		
Acid Concentration (% w/v)	Temperature (^{o}C)	Time (h)	Xylose	Arabinose	Glucose
5	90	3	4.9	3.7	0.4
1	100	24	3.6	2.4	0.2
1	90	56	3.3	2.1	0.9

TABLE 22 The yield of biomass in $g.g^{-1}$ measured available monosaccharide obtained when selected yeast species are grown at 25°C on dilutions of pig slurry hydrolysate 5[90]3

Yeast species	Dilution of hydrolysate with water (v/v)								
	2:3			1:1			3:2		
	Yield after 2 days	Yield after 3 days	Yield after 4 days	Yield after 2 days	Yield after 3 days	Yield after 4 days	Yield after 2 days	Yield after 3 days	Yield after 4 days
Candida blankii	0.062	0.144	0.153	-	-	-	-	-	-
Candida ciferrii	0.191	0.215	0.201	-	-	-	-	-	-
Candida homolentoma	0.193	0.182	0.156	-	-	-	-	-	-
Candida mogii	0.050	0.174	0.177	-	-	-	-	-	-
Candida neodendra	0.221	0.202	0.159	-	-	-	-	-	-
Cryptococcus terreus	0.133	0.166	0.187	-	-	-	-	-	-
Lipomyces starkeyii	0.075	0.119	0.124	-	-	-	-	-	-
Endomycopsis burtonii	0.201	0.208	0.213	-	-	-	-	-	-
Rhodosporidium toruloides	0.092	0.157	0.165	-	-	-	-	-	-
Rhodotorula glutinis	0.111	0.174	0.166	-	-	-	-	-	-
Torulopsis fujisanensis	0.143	0.187	0.092	-	-	-	-	-	-
Yeast species 53 A	0.230	0.216	0.204	0.205	0.245	0.206	0.022	0.041	0.085

TABLE 23 The yield of biomass in $g.g^{-1}$ measured available monosaccharide obtained when selected yeast species are grown at 25°C on dilutions of pig slurry hydrolysate 1 [100] 24

Yeast species	Dilution of hydrolysate with water (v/v) 2:3 Yield after 2 days	2:3 Yield after 3 days	2:3 Yield after 4 days	1:1 Yield after 2 days	1:1 Yield after 3 days	1:1 Yield after 4 days	3:2 Yield after 2 days	3:2 Yield after 3 days	3:2 Yield after 4 days
Candida blankii	0.081	0.165	0.132	-	-	-	-	-	-
Candida ciferrii	0.208	0.212	0.201	-	-	-	-	-	-
Candida homolentoma	0.214	0.201	0.185	-	-	-	-	-	-
Candida mogii	0.013	0.092	0.132	-	-	-	-	-	-
Candida neodendra	0.214	0.192	0.161	-	-	-	-	-	-
Cryptococcus terreus	0.121	0.178	0.192	-	-	-	-	-	-
Lipomyces starkeyii	0.125	0.143	0.126	-	-	-	-	-	-
Endomycopsis burtonii	0.146	0.204	0.207	-	-	-	-	-	-
Rhodosporidium toruloides	0.083	0.142	0.157	-	-	-	-	-	-
Rhodotorula glutinis	0.063	0.143	0.085	-	-	-	-	-	-
Torulopsis fujisanensis	0.098	0.165	0.992	-	-	-	-	-	-
Yeast species 53 A	0.226	0.219	0.204	0.165	0.235	0.219	0.080	0.126	0.128

TABLE 24 The yield of biomass in $g.g^{-1}$ measured available monosaccharide obtained when selected yeast species are grown at 25°C on dilutions of pig slurry hydrolysate 1[90]56

Yeast species	Dilution of hydrolysate with water (v/v) 2:3 Yield after 2 days	2:3 Yield after 3 days	2:3 Yield after 4 days	1:1 Yield after 2 days	1:1 Yield after 3 days	1:1 Yield after 4 days	3:2 Yield after 2 days	3:2 Yield after 3 days	3:2 Yield after 4 days
Candida blankii	0.123	0.135	0.126	0.114	0.146	0.132	0.126	0.168	0.170
Candida ciferrii	0.193	0.201	6.187	0.167	0.201	0.192	0.162	0.213	0.192
Candida homolentoma	0.187	0.185	0.162	0.167	0.192	0.184	0.168	0.213	0.208
Candida mogii	0.021	0.087	0.126	0.016	0.046	0.180	0.068	0.123	0.204
Candida neodendra	0.191	0.178	0.171	0.183	0.192	0.171	0.204	0.198	0.167
Cryptococcus terreus	0.108	0.145	0.167	0.126	0.167	0.192	0.139	0.167	0.216
Lipomyces starkeyii	0.098	0.136	0.101	0.121	0.152	0.148	0.135	0.162	0.163
Endomycopsis burtonii	0.151	0.204	0.200	0.190	0.224	0.216	0.178	0.204	0.200
Rhodosporidium toruloides	0.141	0.160	0.161	0.131	0.192	0.174	0.136	0.203	0.152
Rhodotorula glutinis	0.123	0.162	0.153	0.137	0.187	0.154	0.154	0.192	0.123
Torulopsis fujisanensis	0.141	0.156	0.137	0.167	0.183	0.152	0.198	0.197	0.143
Yeast species 53 A	0.201	0.223	0.221	0.213	0.234	0.228	0.203	0.240	0.222

concentration to 1:1 or 3:2 v/v does not prevent yeast growth. In these concentrations of the 1 [100] 24 and 5 [90] 3 hydrolysate, however, only the yeast species 53A was observed to be able to grow. It appears that only yeast species 53A is able to cope with the level of toxic component present in these more concentrated hydrolysate preparations. It is also apparent that this yeast consistently produces the highest biomass yields from all diluted hydrolysates, and it was therefore decided to use yeast species 53A in all further growth trials. Furthermore, the ability of all yeast species to grow on all three dilutions of the hydrolysate 1 [90] 56 indicates that this milder acid preparation contains significantly less of the toxic component than either of the hydrolysates 1 [100] 24 or 5 [90] 3.

Growth of Selected Yeast Species on Diluted and Charcoal Treated Pig Slurry Hydrolysate

MATERIALS AND METHODS

Preparation of Growth Media

Since it was envisaged that small scale growth trials in the orbital incubator would ultimately be scaled up in a fermenter, it was necessary that all results should be comparable. For this purpose bulk quantities of the three selected hydrolysates, viz 5 [90] 3, 1 [90] 56 and 1 [100] 24 were prepared from one large batch of slurry, which had been collected over a period of 3 to 4 days. In order to minimise microbial growth and prevent any loss of ammonia during storage, the pH of the slurry was lowered to 5 immediately after collection, and the bulk sample was stored in a refrigerator at $5^{o}C$.

Prior to hydrolysis, the slurry was thoroughly mixed and a sample dried at $105^{o}C$ for 24 h to assess its dry weight. On the basis of the results obtained, the solids content was then adjusted to a level of 110 $g.l^{-1}$ of liquid by the addition of water.

The basic method of hydrolysis was similar to that described on page 54 , although some minor modifications were necessary due to the increase in scale. A 20 l round bottomed flask heated in an electrothermal heating mantle (Electrothermal Engineering, 270 Neville Road, London) was used to hydrolyse 10 l quantities of slurry

at one time. The reaction mixture was continuously stirred with a polypropylene paddle throughout the procedure, and the temperature was controlled by a thermocouple contained in an oil-filled tube, which was immersed in the acidified slurry.

On completion of hydrolysis, the pH of the reaction mixture was brought to 4.5, by the addition of NaOH (page 55). The liquid and solid fractions were separated by centrifugation in sterile 1 litre centrifuge buckets, and the solids washed with sterile distilled water. The hydrolysate and washings were then bulked together, membrane filtered and stored at 5°C in sterile glass containers until the growth medium was needed for a particular growth trial. A portion of each hydrolysate was also treated with activated charcoal by the method described previously (page 45).

Conditions of Cultivation

In order to obtain the maximum biomass yield when yeast species 53A was grown on pig slurry hydrolysate, an experiment was undertaken to establish the optimum level of dilution for the three different media. For this purpose the hydrolysates 1 [100] 24 and 5 [90] 3 were diluted with sterile water to give 3:7, 2:3, 1:1 and 3:2 v/v concentrations of the original hydrolysate. As the hydrolysate 1 [90] 56 appeared to have a slightly lower concentration of the inhibitor, 2:3, 1:1, 3:2, 7:3 and 4:1 v/v dilutions of this preparation were used. The standard growth procedure in theorbital incubator was used (pages 70-71). When the optimum dilution level had been established, growth trials in the fermenter were used to compare the yields of biomass obtained from different hydrolysates. The media used in these trials were charcoal treated hydrolysates and hydrolysates that were diluted to the level that produced the maximum yield of yeast biomass. A continuously stirred 3 l BIOTEC fermenter (LKB House, 232 Addington Road South, Croydon, Surrey, CR2 8YD) fitted with equipment to control aeration, stirring rate and temperature, was used in thiswork. The temperature was controlled at 25°C and an aeration rate of 500 cc.min^{-1} was found to be sufficient to maintain a dissolved oxygen (DO) level of above 90% throughout the growth of the yeast.

100 ml samples were taken from the fermenter every 24 h. This was done by increasing the air pressure in the fermenter until a

sample was forced through a sample loop attached to the top of the fermenter. When it was not in use, the loop was closed with a clip and the external end was immersed in alcohol. Throughout the growth trials the contents of the fermenter were regularly checked for the presence of contaminating microorganisms by plating out on nutrient and malt agar plates.

The biomass yield was estimated by the dry weight method described previously (page 71). Ultimately, growth trials with the preparations which produced the best yeast yields in the 3 l fermenter were repeated in a 15 l BIOTEC fermenter. The procedures used were the same as those reported for the trials in the smaller fermenter. The residual hydrolysate was retained for analysis of pH, ammonia level, total nitrogen level, residual monosaccharides and in some cases Biological Oxygen Demand (BOD). The results of this analysis are reported later in the text (pages 116-117).

RESULTS AND DISCUSSION

Figures 39 and 40 show the biomass yields obtained when 3:7, 2:3, 1:1 and 3:2 v/v dilutions of 1 [100] 24 and 5 [90] 3 respectively are used for the growth of 53A. It can be seen from these results that the 3:2 v/v dilutions of both hydrolysates partially inhibit yeast growth. Of the three other dilutions tested, the 1:1 v/v dilutions appear to produce the maximum recorded yield after 3 days. In the case of hydrolysate 5 [90] 3, this is 0.244 g biomass.g^{-1} measured available monosaccharide and for hydrolysate 1 [100] 24 it is 0.246 g biomass.g^{-1} measured available monosaccharide. As the concentration of these two hydrolysates drops below this optimum level, it can be seen from the results that the maximum attainable yield also falls. This suggests that some essential nutrient or growth factor becomes limiting at the lower hydrolysate concentrations. When hydrolysate 1 [90] 56 is used in similar trials a maximum recorded yield of 0.248 g.g^{-1} measured available monosaccharide is attained after 4 days in a 7:3 v/v dilution (Figure 41). This suggests that this milder acid treatment system produces slightly less of the inhibitory compound. At concentrations lower than 7:3 v/v a smaller overall yield is obtained, whereas when the concentration is increased from 7:3 v/v to 4:1 v/v a marked inhibitory effect is observed. On the basis of

FIGURE 39 The yield of biomass in $g.g^{-1}$ measured available carbohydrate obtained when yeast 53A is grown in an orbital incubator on dilutions of the liquid hydrolysate medium 1 [100] 24.

□ 3:7 v/v dilution

■ 2:3 v/v dilution

● 1:1 v/v dilution

▲ 3:2 v/v dilution

Figure 39

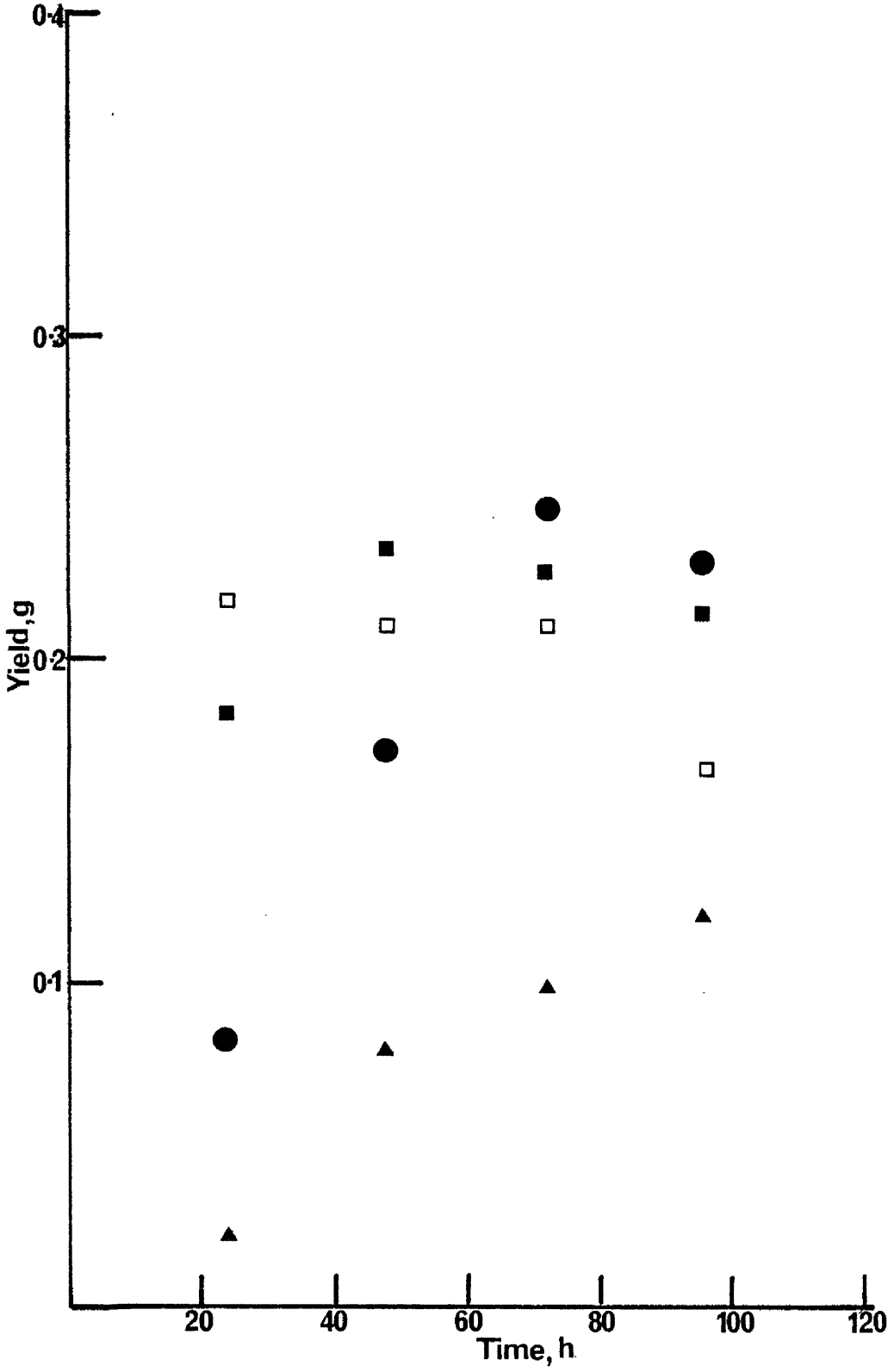


FIGURE 40 The yield of biomass in $g.g^{-1}$ measured available carbohydrate obtained when yeast 53A is grown in an orbital incubator on dilutions of the liquid hydrolysate medium 5[90] 3.

□ 3:7 v/v dilution

■ 2:3 v/v dilution

● 1:1 v/v dilution

▲ 3:2 v/v dilution

Figure 40

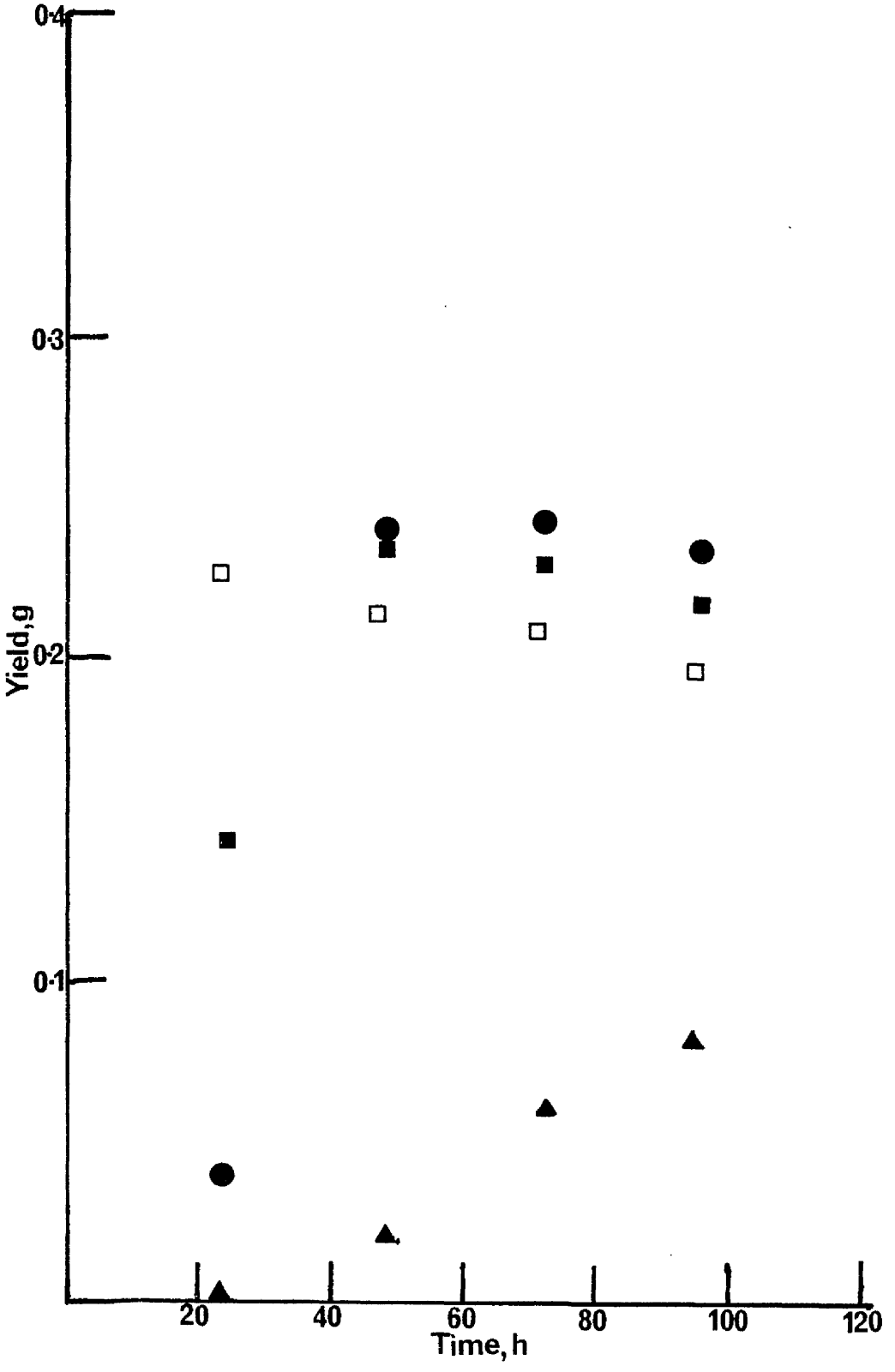


FIGURE 41 The yield of biomass in $g.g^{-1}$ measured available carbohydrate obtained when yeast 53A is grown in an orbital incubator on dilutions of the liquid hydrolysate medium 1 [90] 56.

■ 2:3 v/v dilution

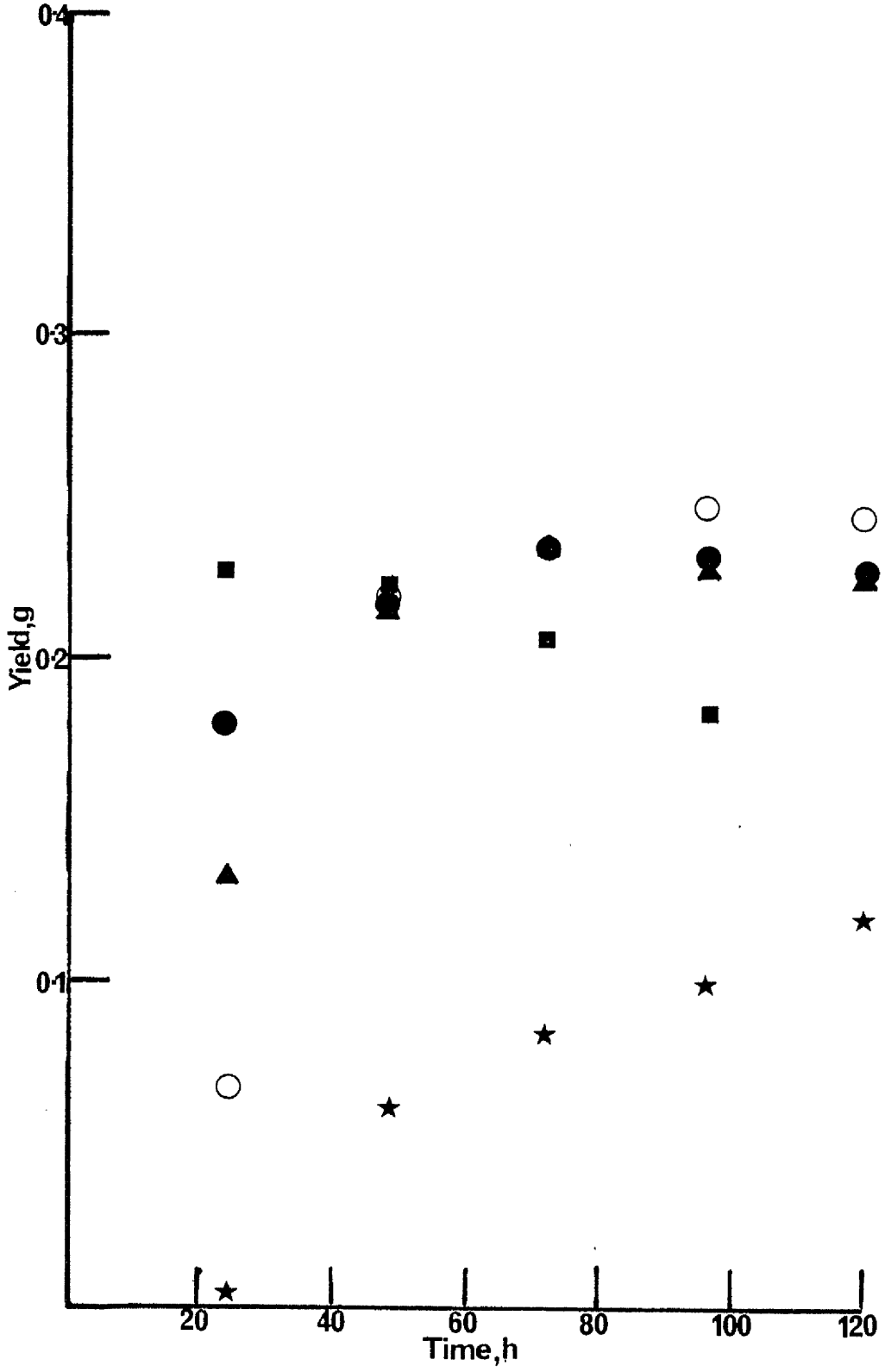
● 1:1 v/v dilution

▲ 3:2 v/v dilution

○ 7:3 v/v dilution

★ 4:1 v/v dilution

Figure 41



these results, it was decided that a 7:3 v/v dilution of 1 [90] 56 hydrolysate and 1:1 v/v dilutions of both the 1 [100] 24 and the 5 [90] 3 hydrolysates should be used for further growth trials in the fermenter.

A 3 l fermenter was used to compare the yields obtained from each of these diluted preparations, and the original undiluted hydrolysates that had been treated with activated charcoal. Each fermenter run was completed in duplicate and the results averaged. In Figure 42 a 1:1 v/v dilution of 1 [100] 24 produces a maximum biomass yield of 0.338 $g.g^{-1}$ measured available monosaccharide after 2 days, whereas the charcoal treated medium yields a maximum of 0.260 $g.g^{-1}$ measured available monosaccharide. This indicates that treatment with activated charcoal completely or partically removes a growth factor or nutrient. With a 1:1 v/v dilution of 5 [90] 3 hydrolysate 0.332 $g.g^{-1}$ measured available monosaccharide is obtained and in the corresponding charcoal treated medium 0.282 $g.g^{-1}$ measured available monosaccharide is the yield (Figure 43). In this case, the maximum yields are both attained after 2 days incubation. In Figure 44 it can be seen that with the hydrolysate 1 [90] 56 it takes longer for the maximum yield to be attained in the dilute hydrolysate than in the charcoal treated medium. After 3 days the recorded yield in the 7:3 v/v dilution of the hydrolysate 1 [90] 56 is 0.350 $g.g^{-1}$ measured available monosaccharide, and in the charcoal treated hydrolysate a maximum yield of 0.244 $g.g^{-1}$ measured available monosaccharide is obtained after 2 days.

These results indicate that treatment of the hydrolysates with activated charcoal lowers the yield that can be obtained from pig slurry hydrolysate. They also indicate that the efficiency with which the yeast utilises the available carbohydrate in the diluted hydrolysates is similar in each case. Thus, to obtain the best yield of yeast from the media produced from the hydrolysis of pig slurry, the initial carbohydrate content is the main parameter that controls the overall yield of biomass obtained. The results presented in Table 21 demonstrate that, of the three chosen acid treatment systems, the hydrolysate produced with 5% acid at 90°C for 3 h contains appreciably more monosaccharide than either of those produced with 1% acid. With this hydrolysate the yield of yeast per 1 litre of undiluted hydrolysate is 3.0 g, compared to 2.1 g with 1 [100] 24 and 2.2 g with 1 [90] 56.

FIGURE 42 The yield of biomass in $g.g^{-1}$ measured available carbohydrate obtained when yeast 53A is grown in a 3 litre fermenter on liquid hydrolysate medium 1 [100] 24.

● hydrolysate diluted 1:1 v/v

□ hydrolysate treated with activated charcoal.

Figure 42

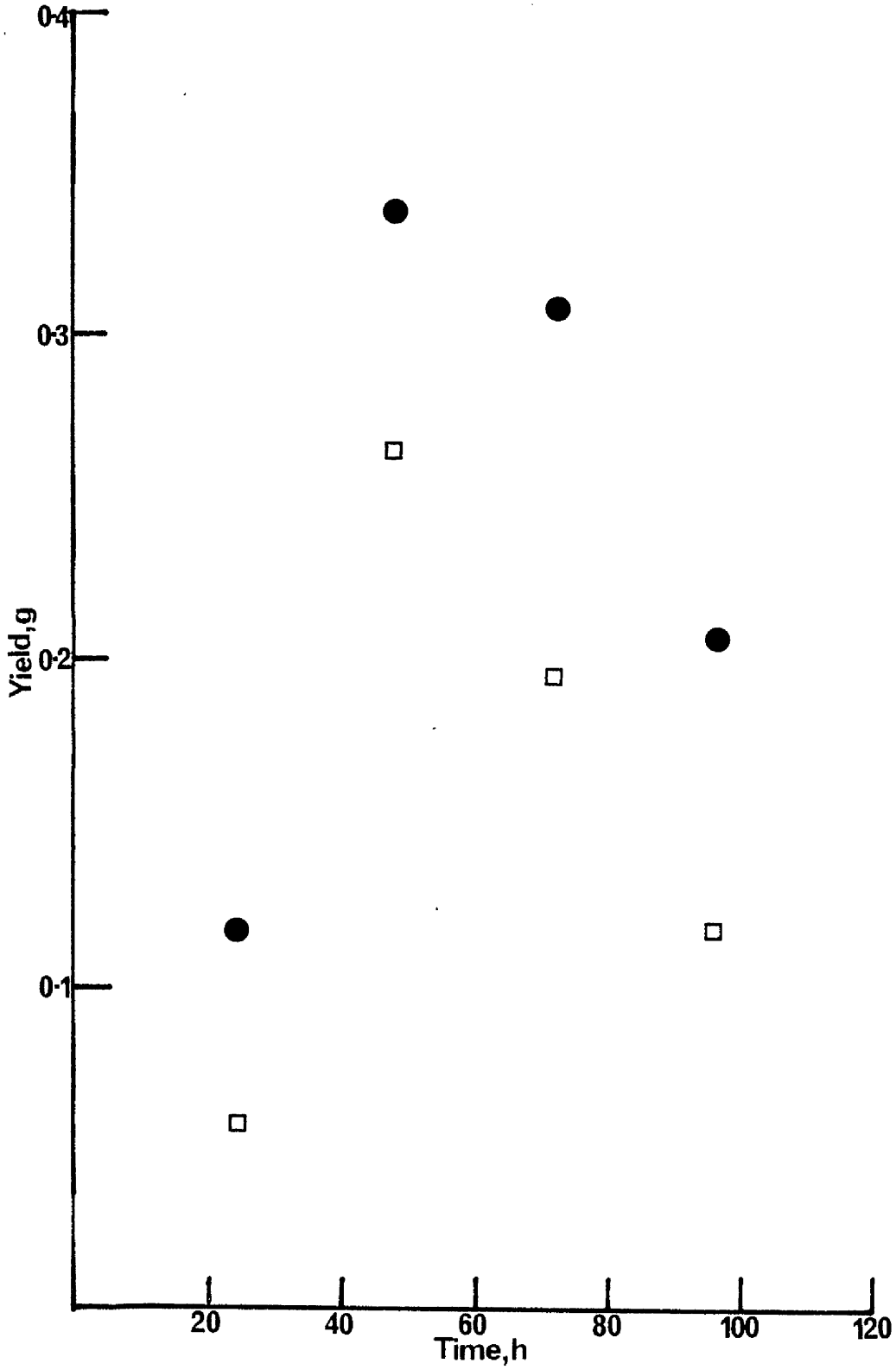


FIGURE 43 The yield of biomass in $g.g^{-1}$ measured available carbohydrate obtained when yeast 53A is grown in a 3 litre fermenter on liquid hydrolysate medium 5 [90] 3

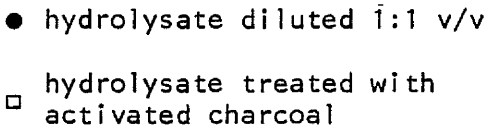


Figure 43

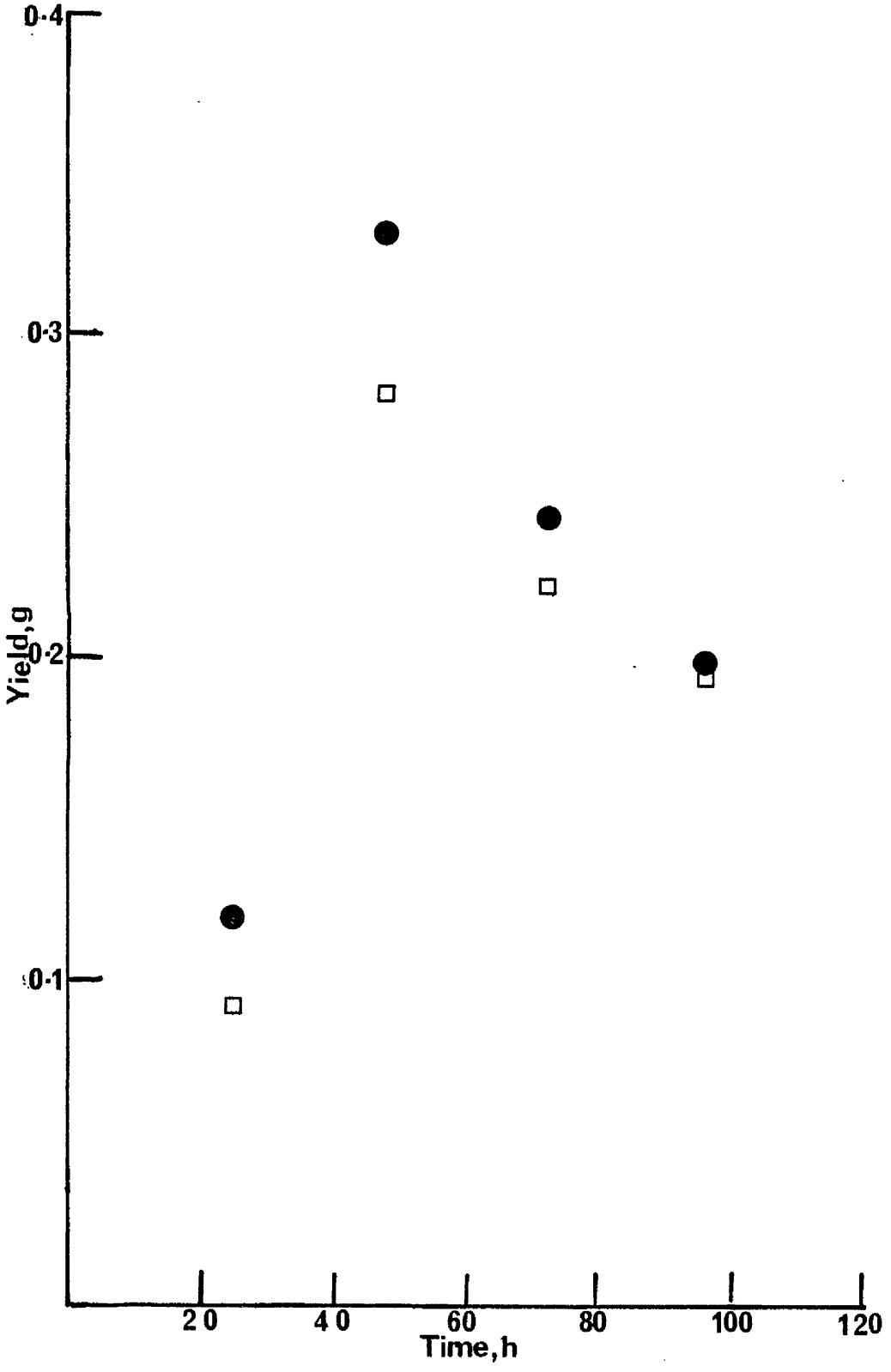


FIGURE 44 The yield of biomass in $g.g^{-1}$ measured available carbohydrate obtained when yeast 53A is grown in a 3 litre fermenter on liquid hydrolysate medium 1 [90] 56.

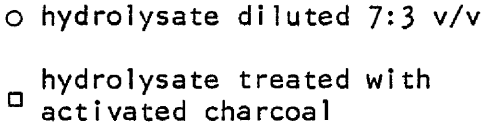
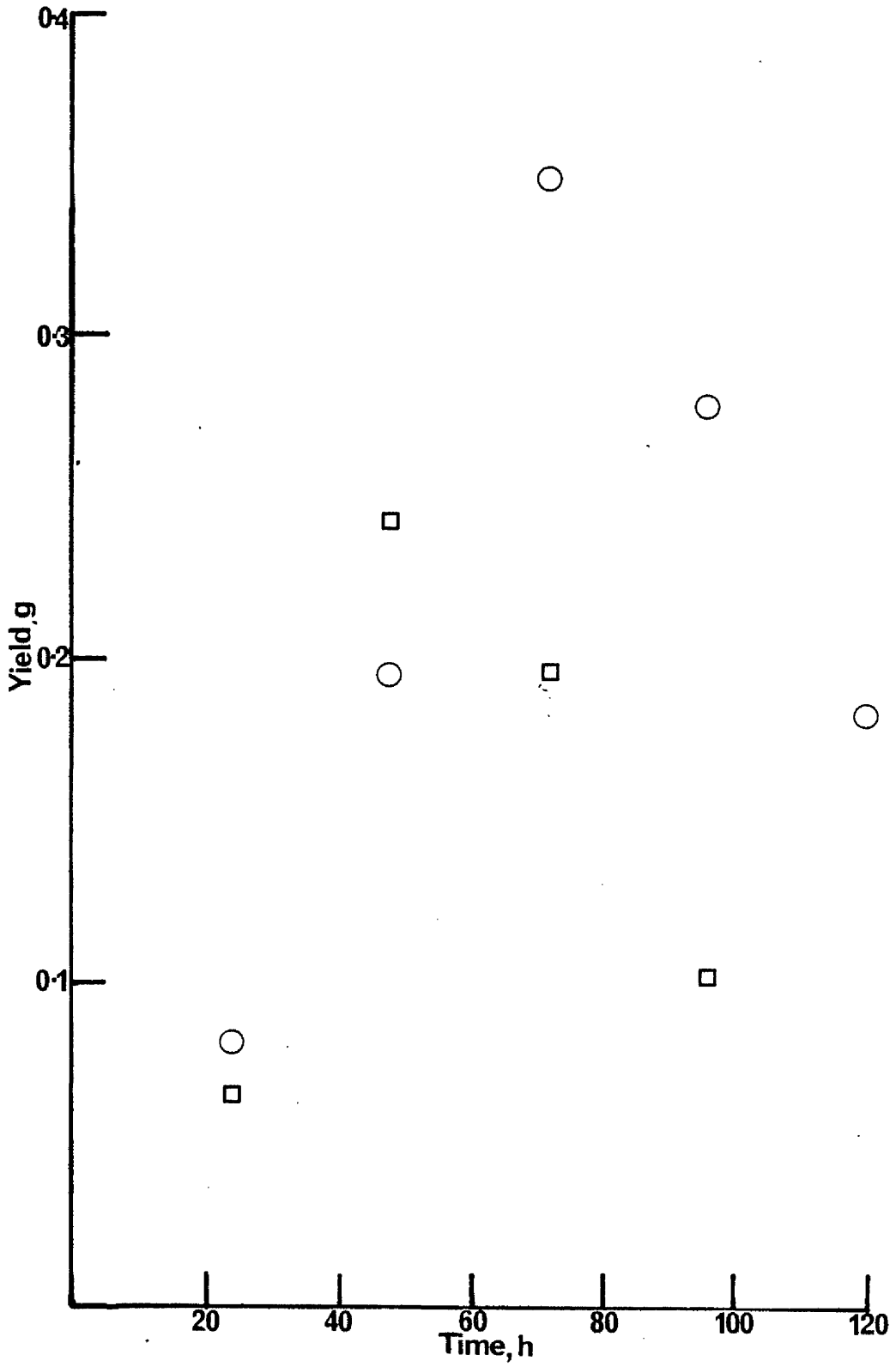


Figure 44



It was therefore the hydrolysate 5 [90] 3 that was used when the growth experiments were scaled up to 10 l in a 15 l fermenter. The growth trials in this fermenter were conducted in the same way as on the 3 l scale. Both diluted and charcoal treated hydrolysates were tested. Apart from the standard preparations, the growth of yeast on a 1:1 v/v dilution of the charcoal treated hydrolysate was also used in an attempt to show the effect that dilution of the nutrient concentration has on the yeast yield. The time intervals between sampling were shorter in this trial. The results are given in Figure 45. It can be seen from these that the maximum biomass yield in $g.g^{-1}$ available carbohydrate from the charcoal treated hydrolysate is not significantly affected by dilution of the substrate. They also confirm that a 1:1 v/v dilution of the non-treated hydrolysate produces a significantly higher biomass yield than charcoal treated hydrolysate. This medium produces 0.358 $g.g^{-1}$ measured available monosaccharide in 42 h. The charcoal treated hydrolysate gives a 0.338 $g.g^{-1}$ measured available monosaccharide in 30 h and the 1:1 v/v dilution a yield of 0.342 $g.g^{-1}$ measured available monosaccharide in 30 h. Thus, from the results of these growth trials, it seems to be possible to achieve a maximum yield of 3.2 g of yeast per litre of original hydrolysate, when a 1:1 v/v dilution of the 5 [90] 3 hydrolysate is used as the substrate in 10 l amounts in a 15 l fermenter. This means that for every litre of pig slurry with a solids content of 110 $g.g^{-1}$ of liquid, approximately 9 g of yeast can be produced.

CONCLUSTIONS

1. Of all the yeasts tested in pig slurry hydrolysate medium, the unidentified isolate 53A is able to grow in the most concentrated hydrolysate medium.
2. The best overall yields of the yeast 53A are obtained when the selected hydrolysates are diluted 1:1 v/v with water for the 1 [100] 24 and 5 [90] 3 preparations, and 7:3 v/v for the 1 [90] 56 preparation.
3. Charcoal treatment of each of the three test hydrolysates produces a medium that supports a lower yield of yeast/g measured available carbohydrate than the corresponding hydrolysate that has been diluted to its optimum concentration.

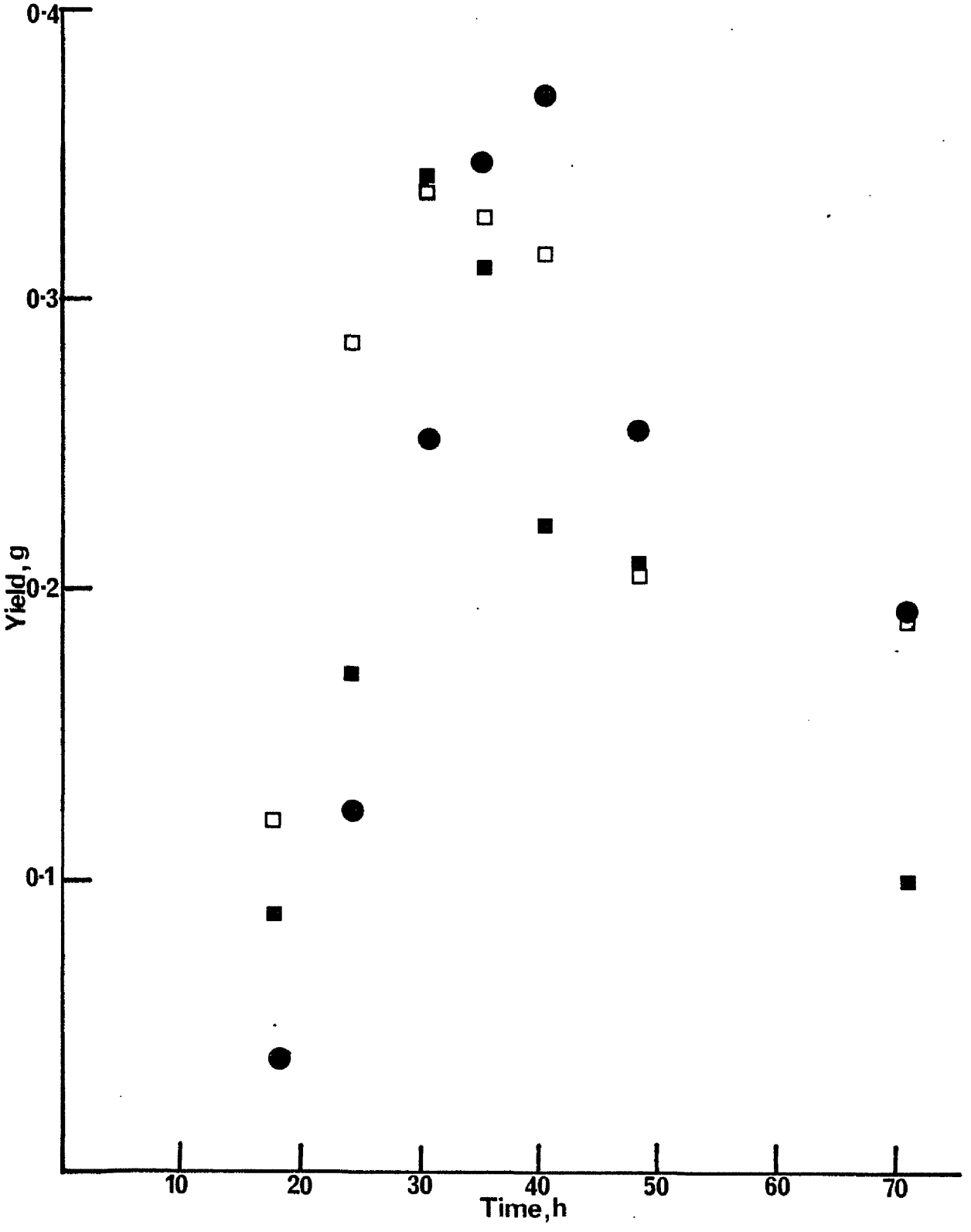
FIGURE 45 The yield of biomass in $g.g^{-1}$ measured available carbohydrate obtained when yeast 53A is grown in a 15 litre fermenter on liquid hydrolysate medium 5 [90] 3.

● hydrolysate diluted 1:1 v/v

□ hydrolysate charcoal treated

■ hydrolysate charcoal treated and diluted 1:1 v/v

Figure 45



4. When diluted to the concentration that gives maximum yield, all three test hydrolysates produce almost the same amount of yeast biomass/g measured available carbohydrate.
5. Overall, the hydrolysate 5 [90] 3 produces more yeast biomass than either the 1 [90] 56 hydrolysate or the 1 [100] 24 hydrolysate, because the initial concentration of measured available carbohydrate is significantly higher in this medium.
6. A yield of 9 g of yeast can be obtained by growing the unknown yeast species 53A on a medium produced by hydrolysing 1 litre of pig slurry with 5% w/v H_2SO_4 at 90°C for 3 h, when the initial solids content of the slurry is 110 $g.l^{-1}$.

Classification of the Yeast Species 53A

MATERIALS AND METHODS

The routine media and methods described by van der Walt (1971) were employed for the classification of the yeast 53A. Lodder's (1971b) key was used to identify the genus, and the key to the species of the genus *Candida* (van Uden & Buckley, 1971) was used to complete the classification.

RESULTS AND DISCUSSION

In the system of classification used, morphological and reproductive characteristics are employed to decide the genus of the yeast, and physiological criteria are used to differentiate the species. The morphological and reproductive characteristics of the yeast species 53A are listed in Table 25. These characteristics place the unknown yeast in the genus *Candida* using Lodder's key (1971b). In Table 26, the physiological characteristics of the yeast are given. These enable the species of the yeast to be decided by using the key of van Uden & Buckley (1971). Using these results, the yeast 53A was identified as *Candida tropicalis*. This identification was confirmed by the Centraal Bureau Voor Schimmelcultures (Baarn, Holland).

The identification of yeast species 53A as *Candida tropicalis* led to consideration of the implications of its toxicology and pathogenicity on its use as a source of SCP. Volfova & Kyslikova (1979) have recently reported the growth of a strain of *Candida*

TABLE 25 The morphological and reproductive characteristics demonstrated by the unknown yeast 53A.

Cells are short and ovoid in shape
Pseudomycelium formed
No ascospores produced
No ballistospores produced
No telliospores produced
No arthrospores produced
No pigment produced in steak cultures

TABLE 26 Physiological characteristics of the unknown yeast 53A

TEST	RESULT
Glucose fermentation	+
Galactose fermentation	+
Sucrose fermentation	+
Maltose fermentation	+
Melibiose fermentation	-
Raffinose fermentation	-
Nitrate assimilation	-
Glucose assimilation	+
Maltose assimilation	+
Sucrose assimilation	+
Lactose assimilation	-
Inositol assimilation	-
Cellobiose assimilation	+
Raffinose assimilation	-
L-rhamnose assimilation	-
Rititol	+
Erythritol	-
D-galactose	+
Inulin	-
Salicin	-
Melibiose	-
Trehalose	+
L-arabinose	+
D-xylose	+
Growth at 39°C	+

tropicalis on straw hydrolysates. This product has a high protein content and in toxicity trials with rats it has so far proved harmless. However, the recent development of processes using *Candida* species as sources of SCP has focused attention on their potential pathogenicity (Ahearn *et al*, 1979; Gargani, 1979; Holzschu *et al*, 1979). Some strains of *Candida tropicalis* have been shown to be pathogenic to animals and man (Ahearn *et al*, 1979). Thus, it has to be accepted that this may limit its usefulness as a source of SCP. However, the growth of *Candida tropicalis* on pig slurry hydrolysate has established the principle that a yeast species can be cultivated, and although it may require further dilution or charcoal treatment of the hydrolysate, it is possible that one of the eleven yeasts listed in Table 15 could be substituted in the process. Another alternative organism is *Candida maltosa*, which has similar carbohydrate fermentation and assimulation patterns to *Candida tropicalis* and has not as yet been associated with human or animal pathogenicity (Ahearn *et al*, 1979). Although van Uden & Buckley (1971) grouped *Candida maltosa* with *Candida sake*, Meyer (1979) and Komagata (1979) have recently shown that on the basis of DNA reassociations, *Candida tropicalis, Candida maltosa* and *Candida sake* are distinct species. It has also been stated that these species can be physiologically differentiated on the basis of growth on soluble starch and the maximum temperature permitting growth.

The identification of the unknown yeast species as a strain of *Candida tropicalis* necessitated a reappraisal of the cultivation conditions employed in the foregoing growth trials. This yeast species is known to tolerate fairly high incubation temperatures, and to be stimulated by the presence of the vitamin biotin. Some strains are also stated to be dependent on the presence of thiamine in the growth medium (van Uden & Buckley, 1971). The conditions used in the growth trials (pages 87-89) were selected to suit the growth of a vitamin independent, mesophilic yeast species.

The stimulatory effect of the addition of biotin and thiamine was therefore tested in the laboratory using a vitamin-free medium (van der Walt, 1971) to which these vitamins were added independently, and in combination (Figure 46). It was found from this study that biotin is essential for the growth of this strain of *Candida tropicalis,* but that the addition of thiamine does not have a

FIGURE 46 The yield of biomass in $g.g^{-1}$ measured available carbohydrate obtained when *Candida tropicalis* is grown in an orbital incubator on mineral salts medium with and without the addition of biotin and thiamine.

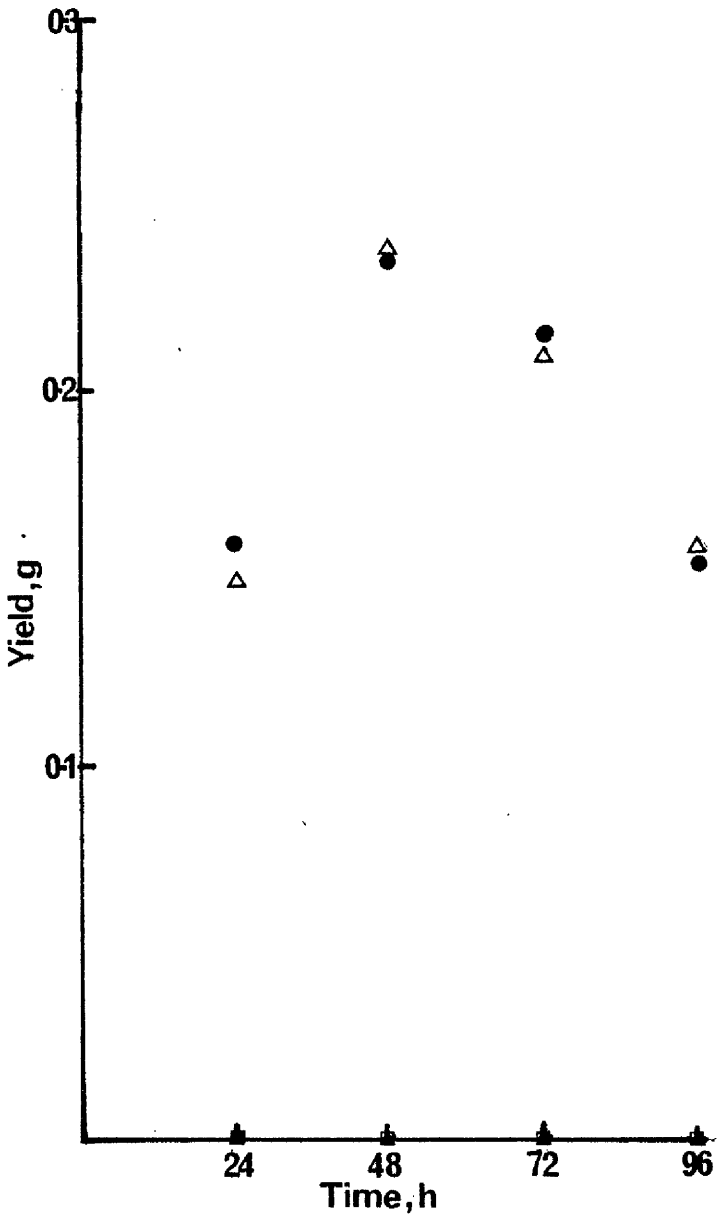
▲ no vitamins added

● biotin added

□ thiamine added

△ biotin and thiamine added

Figure 46



marked stimulatory effect. It was therefore possible that the addition of biotin to pig slurry hydrolysate would increase the biomass yield. This was tested in the laboratory, and it was found that the presence of added biotin has no significant effect on the yield of *Candida tropicalis* (Figure 47). This results suggests that the pig slurry hydrolysate medium contains biotin or some substance that can act as a substitute for this vitamin.

The effect of increasing the incubation temperature was tested in the laboratory by growing *Candida tropicalis* on pig slurry hydrolysate at 25°C, 30°C and 35°C (Figure 48). These experiments show that raising the temperature from 25°C to 30°C does not significantly increase the yield of yeast biomass obtained from the hydrolysate medium, and increasing the temperature to 35°C has a deleterious effect on the growth of this strain of *Candida tropicalis*. Thus, it appears that the biomass yields previously reported (pages 89-91) would not be significantly altered by the addition of biotin or by raising the temperature of incubation.

CONCLUSIONS

1. The unknown yeast species 53A is identified as *Candida tropicalis*.
2. The growth of this strain of *Candida tropicalis* is stimulated by the addition of biotin, but thiamine does not improve growth.
3. The pig slurry hydrolysate 5[90]3 appears to contain biotin or a substance that acts as a substitute for biotin.
4. The yield of *Candida tropicalis* obtained from the 5[90]3 hydrolysate of pig slurry is not significantly affected by raising the incubation temperature from 25°C to 30°C, whereas incubation at 35°C causes a decrease in the amount of biomass recovered.

FIGURE 47 The yield of biomass in $g.g^{-1}$ measured available carbohydrate obtained when *Candida tropicalis* is grown in an orbital incubator on a 1:1 v/v dilution of the liquid hydrolysate medium 5 [90] 3 with and without the addition of biotin.

● with biotin

○ without biotin

Figure 47

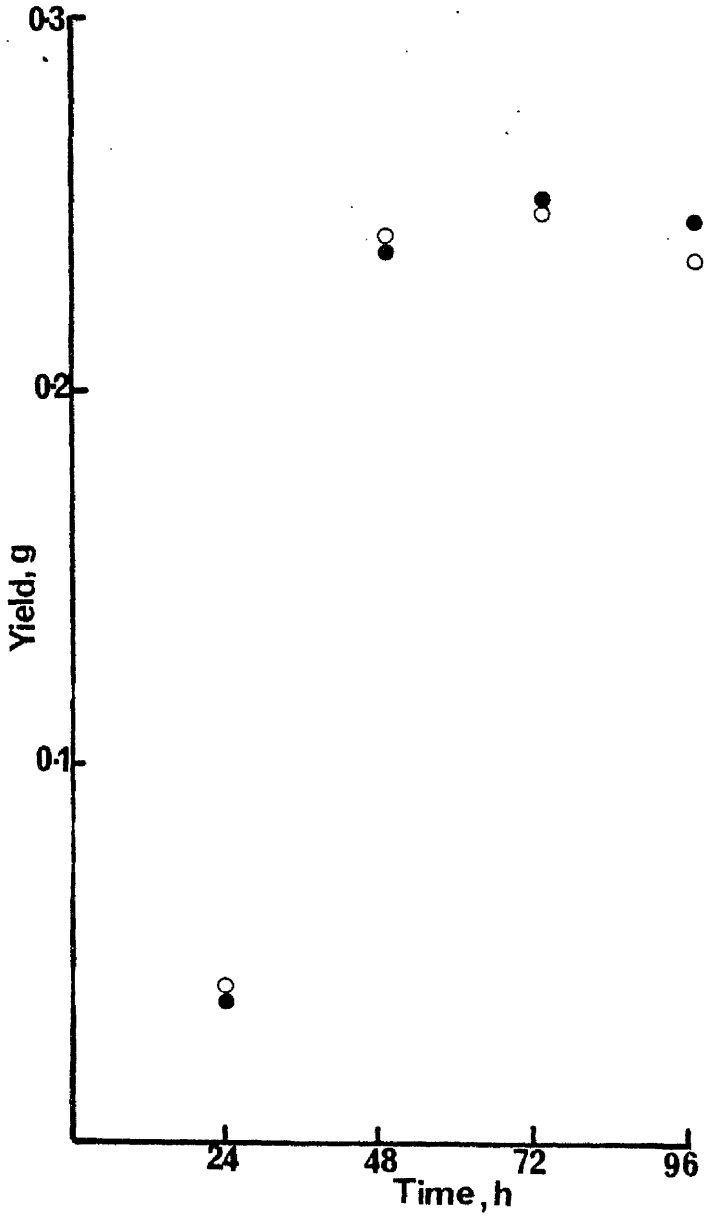


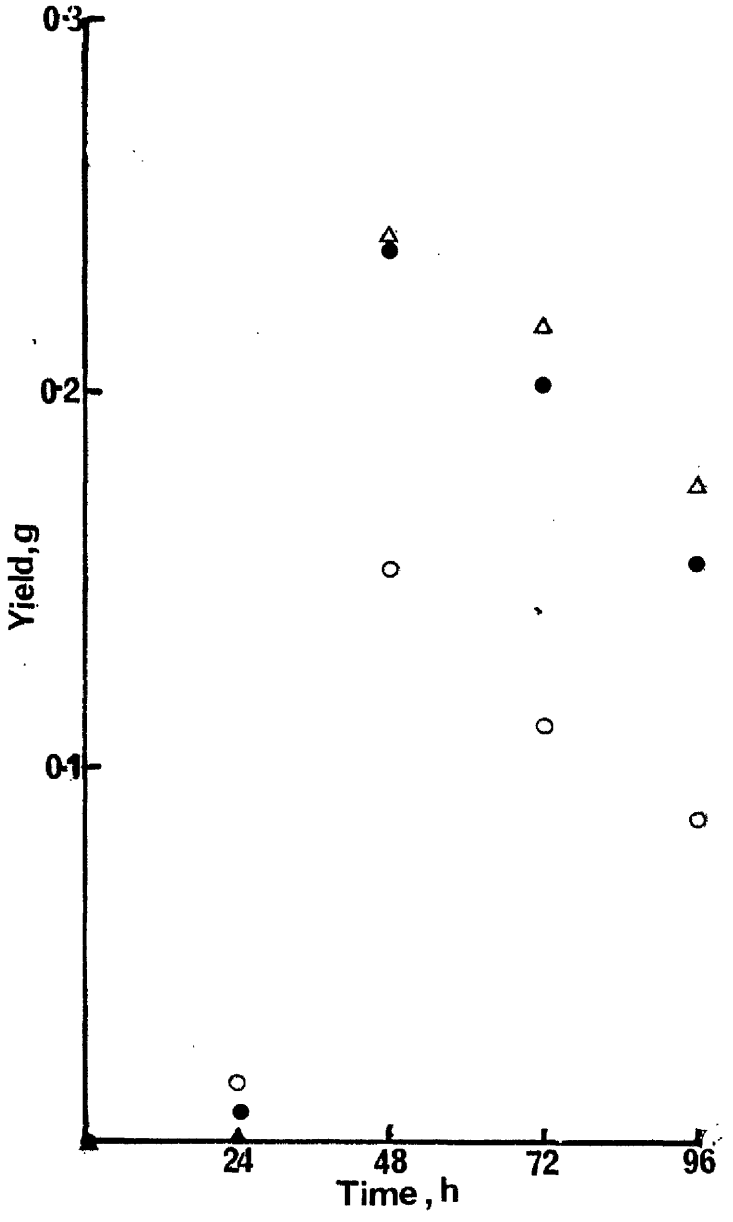
FIGURE 48 The effect of incubation temperature on the yield of biomass in $g.g^{-1}$ measured available carbohydrate obtained when *Candida tropicalis* is grown in an orbital incubator on a 1:1 v/v dilution of the liquid hydrolysate medium 5[90]3.

△ 25°C

● 30°C

○ 35°C

Figure 48



3. STUDIES ON THE GROWTH OF THE CELLULOLYTIC FUNGAL SPECIES *SPOROTRICHUM PULVERULENTUM* ON HYDROLYSED PIG SLURRY

INTRODUCTION

The growth of cellulolytic fungi on cellulosic waste materials has been extensively investigated by Worgan (1976), and this work has resulted in the successful cultivation of *Trichoderma viride* on various plant materials that have been pre-treated to open up the structure of the cellulose. However, this organism has the disadvantage that it cannot utilise cellulose that is bound in a ligno-cellulose complex. In recent studies in Sweden aimed at producing microbial protein from ligno-cellulosic material (von Hofsten & von Hofsten, 1974; von Hofsten, 1976), several thermo-tolerant basidiomycetes of the white rot type have been used. It has been established that of the organisms screened, *Sporotrichum pulverulentum* degrades cellulose most efficiently.

The extra-cellular enzyme systems used by *Sporotrichum pulverulentum* have been characterised (Eriksson & Rzedowski, 1969a and 1969b; Streamer *et al*, 1975). Eriksson & Larsson (1975) have shown that these cellulase enzymes are bound to the cellulose fibres rather than the fungal mycelium. The production of extra-cellular phenol oxidases for the degradation of lignin has also been investigated (Ander & Eriksson, 1976).

Eriksson & Larsson (1975) studied the growth and protein production of *Sporotrichum pulverulentum* in submerged culture. They claimed that in experiments with cellulose of different degrees of crystallinity and polymerisation, the protein content of the biomass decreased as the complexity of the substrate increased. On barley flour the nitrogen content of the *Sporotrichum pulverulentum* biomass produced in stirred fermenters is usually 6-7% of the dry cell material (von Hofsten & Ryden, 1975). These values cannot be directly converted to protein concentrations by multiplying by the conventional factor of 6.25, because the cell walls of the fungus contain substantial amounts of non-protein nitrogen (NPN). The mycelium also contains nucleic acids, but the concentration is lower than that reported for more rapidly growing moulds and yeasts. The nucleic acid content has been shown to be between 2.5 and 3.5% of the dry weight.

The amino acid composition of *Sporotrichum pulverulentum* biomass has been shown to be favourable for use as an animal feed, and the concentration of lysine, the first limiting amino acid in cereals, is high. Small scale animal experiments have indicated that *Sporotrichum pulverulentum* is not toxic (von Hofsten & Ryden, 1975). The ability of *Sporotrichum pulverulentum* to efficiently utilise cellulosic and ligno-cellulosic materials indicated that it might be used to advantage to utilise the cellulose which remained intact in the solid residue after the mild H_2SO_4 hydrolysis of pig slurry. After yeast growth the liquid hydrolysate contained a considerable quantity of ammonium-nitrogen (see pages 116-117), and it was proposed to add this to the solid residue from hydrolysis to provide a source of nitrogen for fungal growth (Figure 49). It was also thought that the use of the residual hydrolysate medium might have the added advantage that yeast growth may have released substances which could promote growth of the fungus.

Prior to investigating the growth of *Sporotrichum pulverulentum* on hydrolysed slurry solids, preliminary growth trials were conducted to establish that the fungus could grow with hexose sugar, pentose sugar and cellulose as carbon sources. The optimum temperature and pH for the growth of *Sporotrichum pulverulentum* on cellulose were established, and the reported requirement of this organism for thiamine was also tested.

Preliminary Investigations to Establish the Basic Growth Requirements of *Sporotrichum pulverulentum*

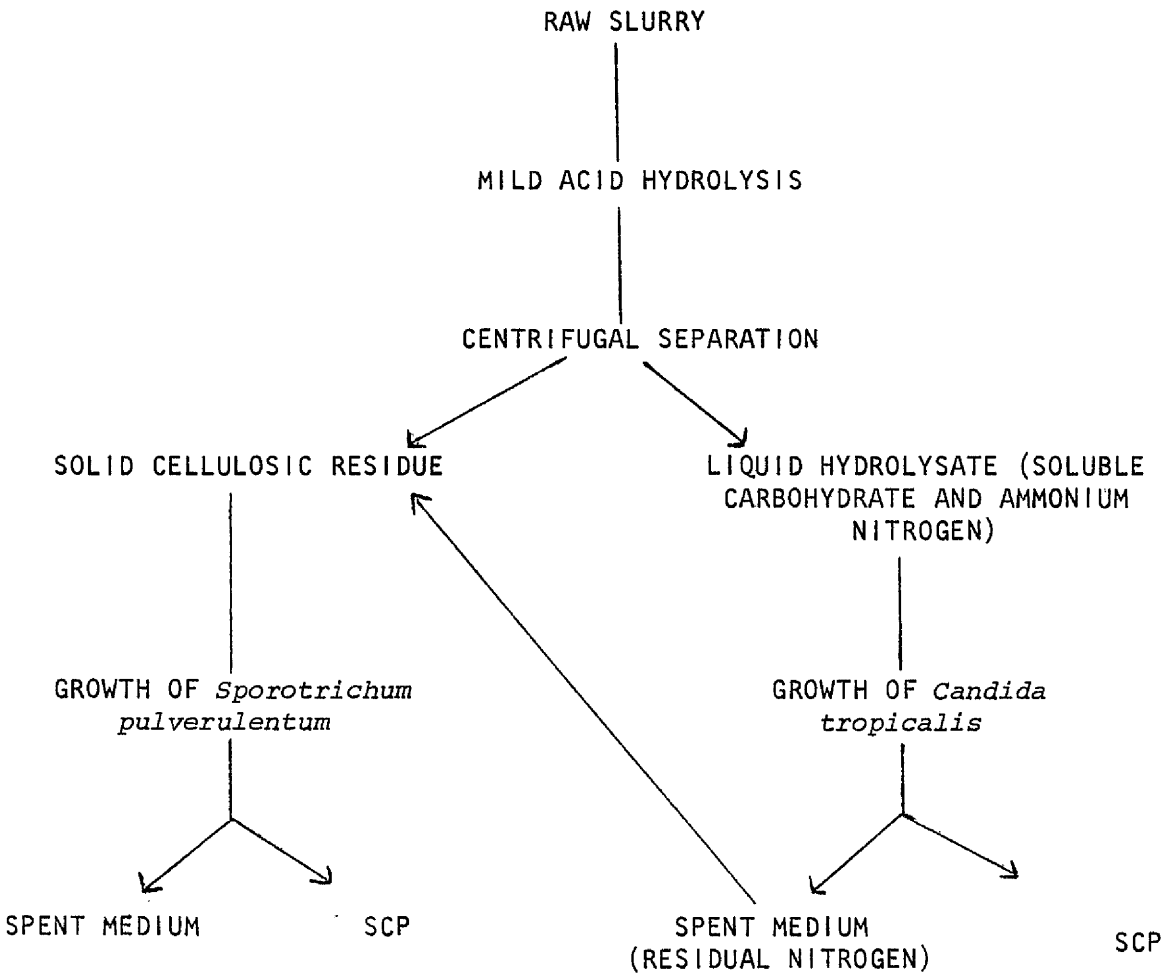
MATERIALS AND METHODS

Fungal Strain

The organism used in this study was isolated in 1964 by Thomas Nilsson, College of Forestry, Stockholm, and was obtained from the Centraalbureau Voor Schimmelcultures, Baarn, Holland, where it has the number CBS 671.71.

Although this organism was previously classified as a *Chrysosporium* species, it has been shown to be identical to a strain of *Sporotrichum pulverulentum* (von Hofsten & von Hofsten, 1974). Recent studies showed that it is the imperfect state of the basidiomycete described as *Phanerochaete chrysosporium*, family Corticiaceae

FIGURE 49 Outline of the process developed for the utilisation of the separated liquid and solid residues of acid hydrolysed slurry.



(Burdsall & Eslyn, 1974).

Maintenance of Cultures

All stock cultures of *Sporotrichum pulverulentum* were freeze-dried and stored in the freeze-dried state (Lapage & Redway, 1974). Working cultures were maintained on malt agar (von Hofsten & Ryden, 1975).

Preparation of Inoculum

Sporotrichum pulverulentum forms a large number of vegetative spores (conidia) when it is grown for 4 to 5 days on solid media such as malt agar. These spores are hydrophobic but can be suspended homogeneously in water containing 0.01% Tween 80 (von Hofsten & Ryden, 1975).

It is important to control the growth morphology of the fungus as far as possible if the mycelial biomass is to be used as a source of protein. This fungus grows either in filamentous or pellet form, depending on the nature of the inoculum, composition of the medium or other physical conditions (von Hofsten & Ryden, 1975).

von Hofsten & von Hofsten (1974) have shown that large pellets of *Sporotrichum pulverulentum* contain a high proportion of partially autolysed cells with a protein content of about 20%. von Hofsten & Ryden (1975) have found that as the size of the inoculum increases, the size of the pellets decreases and homogeneous suspensions of small irregular mycelial granules with a higher protein content are formed when the inoculum is large. For this reason a standard method of preparing a large inoculum was adopted. *Sporotrichum pulverulentum* was grown for 5 days at 30°C on malt agar slopes in universal bottles. The spores from each slope were then suspended in 2 ml of a sterile solution of 0.01% v/v Tween 80 in distilled water, and 0.2 ml of this inoculum added to every 10 ml of growth medium.

Growth Requirement Trials

von Hofsten (1976) has reported that *Sporotrichum pulverulentum* requires the presence of the vitamin thiamine for optimum growth. This phenomenon was checked in the laboratory using the basic mineral salts medium described by Eriksson & Larsson (1975) with and without the addition of thiamine and using glucose as a carbon

source. This medium was prepared as follows: 0.06% KH_2PO_4 w/v; 0.05% $MgSO_4 7H_2O$; 0.04% K_2HPO_4; 0.0074% $CaCl_2.2H_2O$; 0.0012% ferric citrate; 0.0066% $ZnSO_4.7H_2O$; 0.005% $MnSO_4.4H_2O$; 0.001% $CoCl_2.6H_2O$; 0.0001% thiamine; 0.05% $(NH_4)_2SO_4$; 1% of the appropriate carbon source. The pH was adjusted to 5 unless otherwise stated.

In order to test the ability of *Sporotrichum pulverulentum* to utilise pentose sugar, the above experiment was repeated with xylose as a carbon source.

Growth trials were then carried out to establish the optimum temperature and pH levels for the growth of *Sporotrichum pulverulentum* on cellulose. Temperatures of 25, 30, 35 and 40°C and pH levels of 4.5, 5 and 5.5 were tested. "Avicel" microcrystalline cellulose (Merck, Darmstadt, Germany) was used as carbon source in the basic mineral salts medium.

All these preliminary trials were conducted in 50 ml Erlenmeyer flasks in an orbital incubator operating at 125 rev/min. Each flask contained 20 ml of the appropriate growth medium, and in each experiment duplicate flasks were analysed at each sampling time. The temperature was controlled at 30°C unless otherwise stated, as this is the normal temperature for the growth of *Sporotrichum pulverulentum* (Eriksson & Larsson, 1975).

Determination of Biomass Yield

When *Sporotrichum pulverulentum* was cultivated on media containing soluble carbon sources, it was possible to measure the biomass yield by the dry weight method employed in the yeast growth trials (page 71). However, when cellulose was the carbon source, dry weight analysis did not reflect the yield of fungal biomass, as the microbial cell material was inextricably mixed with the residual substrate. Thus, an alternative method of assessing biomass yield had to be adopted, and measurement of protein content was selected as being most suitable, as it has been commonly used to determine the yield of *Sporotrichum pulverulentum* on cellulosic substrates, when growth conditions are strictly controlled (Eriksson & Larsson, 1975; von Hofsten & Ryden, 1975). Eriksson & Larsson (1975) have shown, however, that the protein content of the fungus can alter with changes in the cultivation conditions.

The protein content of the fungus was determined by the Biuret method (Herbert *et al*, 1971), and a standard curve relating fungal biomass to absorbance of protein in the Biuret reaction was constructed (Figure 50). The fungus used to prepare this standard curve was grown on glucose mineral salt medium with added thiamine. It can be seen from this result that, up to a weight of 10 g of fungus, the response of the Biuret reaction is linear. In order to obtain some indication of the protein content of the fungus, a standard curve for a pure protein was constructed using bovine serum albumen (Figure 50). From these results it was calculated that the fungus contains 35 g protein.g^{-1} of fungal biomass. This value agrees closely to the protein content reported by von Hofsten (1976) for *Sporotrichum pulverulentum* grown on barley flour.

RESULTS AND DISCUSSION

Figure 51 clearly shows that *Sporotrichum pulverulentum* can grow on mineral salts medium using either xylose or glucose as a carbon source, and that the yield of biomass is enhanced by the presence of the vitamin thiamine. It is therefore apparent that the fungus has a requirement for this vitamin for optimum growth and it was added at the 0.001% w/v level to the media used in subsequent growth trials.

The normal pH reported for the growth of *Sporotrichum pulverulentum* is 5.5, but it has been shown to grow well at pH levels between 5 and 6 and to grow at pH 4 (Eriksson & Larsson, 1975; von Hofsten & Ryden, 1975). A low starting pH has the advantage of minimising the risk of contamination of the fungal culture, and therefore the ability of *Sporotrichum pulverulentum* to utilise cellulose at different pH levels was tested in the laboratory. Figure 52 shows the yield of fungal biomass produced when this fungus is grown on cellulose mineral salts medium at pH 4.5, 5.0 and 5.5 and at an incubation temperature of 30°C. It can be seen that the highest yield of fungus is obtained when the starting pH is 5. At pH 4.5 the fungus appears to grow slightly better than at pH 5.5.

A similar experiment was conducted in which the incubation temperature was varied. Temperatures of 25°C, 30°C, 35°C and 40°C

FIGURE 50 Standard curve relating weight of sample in mg to absorbance in nm obtained in the Biuret reaction.

o pure bovine serum albumen

■ *Sporotrichum pulverulentum*

Figure 50

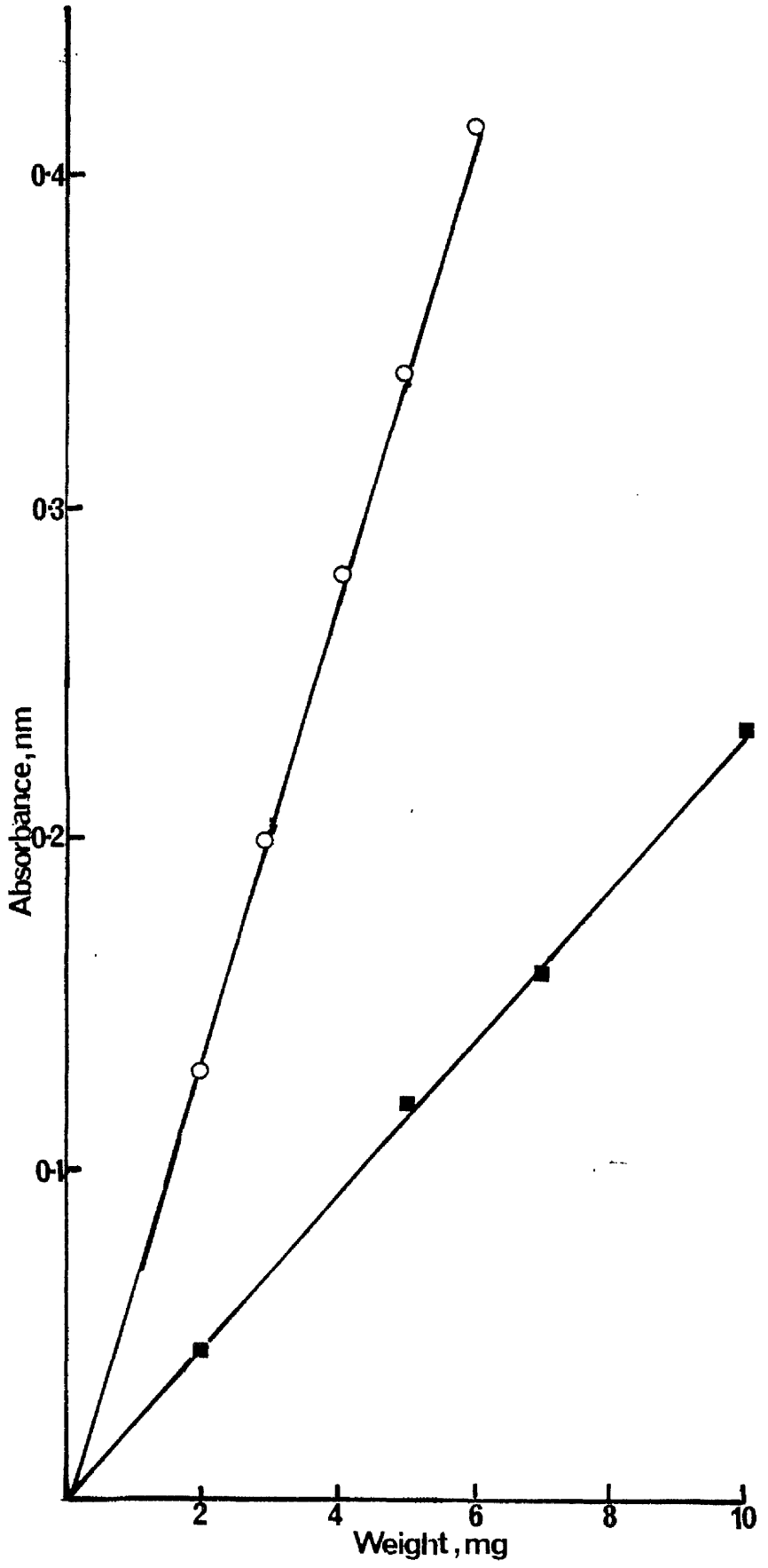


FIGURE 51 The yield of biomass in $g.g^{-1}$ measured available carbohydrate obtained when *Sporotrichum pulverulentum* is grown in an orbital incubator on mineral salts medium with xylose or glucose as a carbon source and with and without the addition of thiamine.

● with thiamine:carbon source glucose
▲ with thiamine:carbon source xylose
○ without thiamine:carbon source glucose
△ without thiamine:carbon source xylose

Figure 51

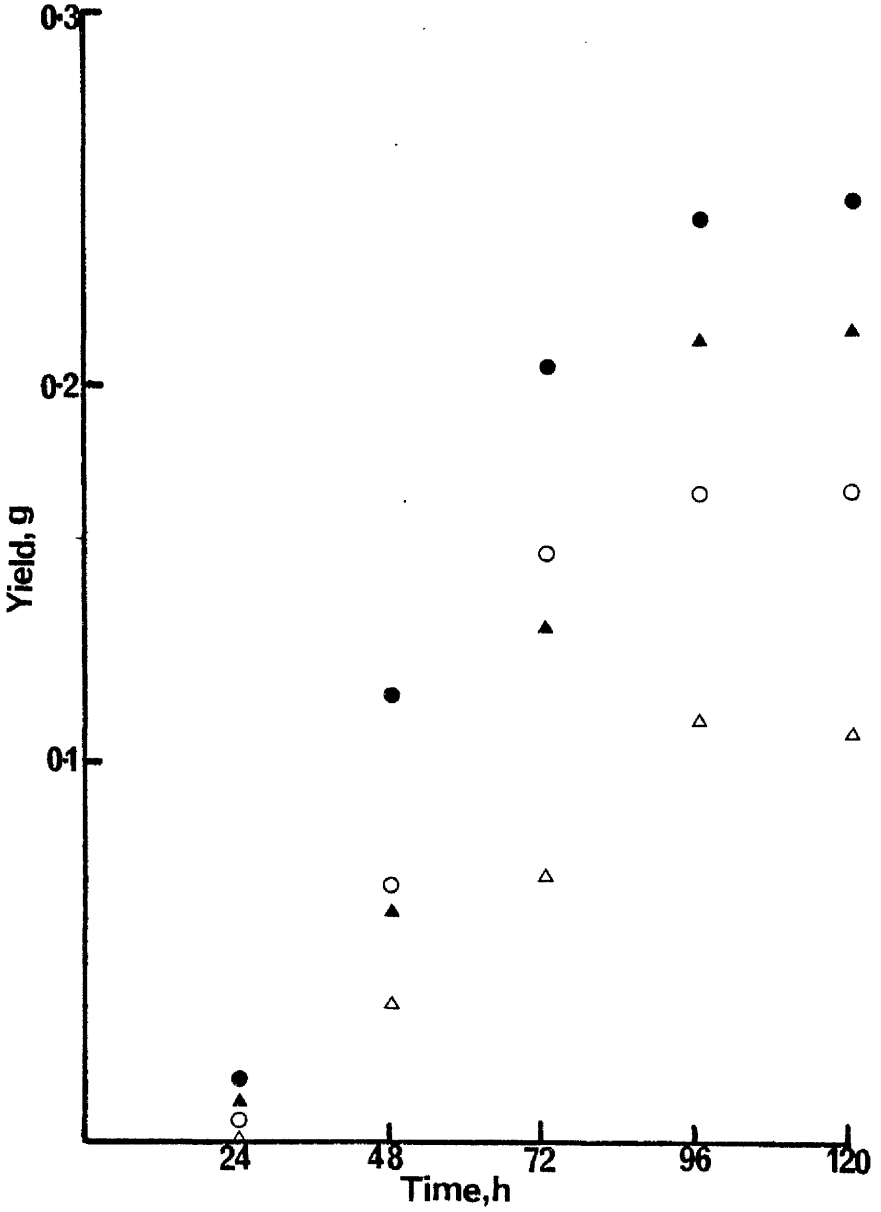


FIGURE 52 The effect of pH on the yield of biomass in $g.g^{-1}$ measured available carbohydrate obtained when *Sporotrichum pulverulentum* is grown in an orbital incubator on cellulose/ mineral salts medium.

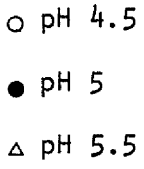
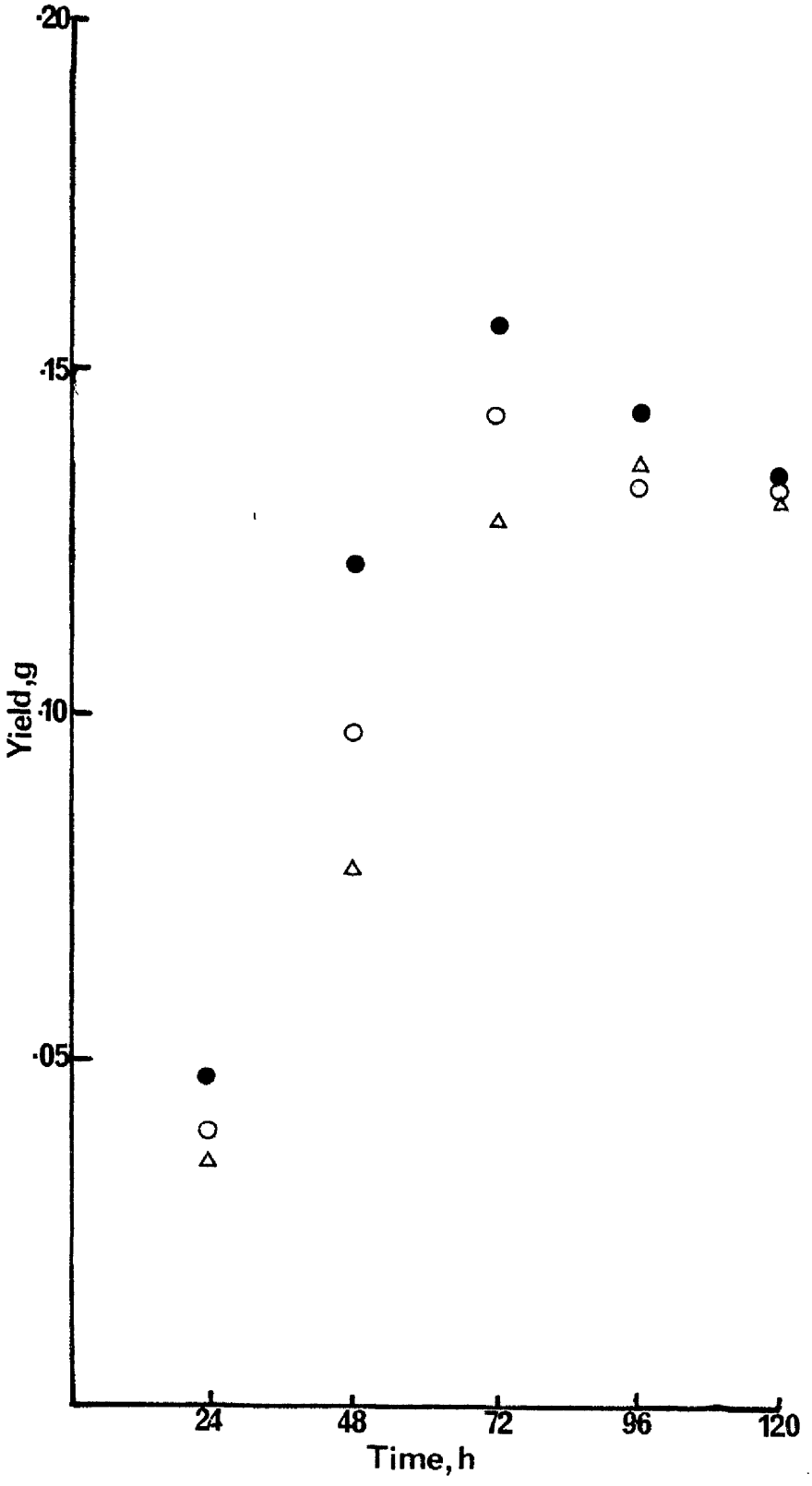


Figure 52



at an initial pH of 5 were investigated. Even after 5 days incubation at 40°C there appears to be no growth of *Sporotrichum pulverulentum* (Figure 53) and the yield of fungal biomass at 35°C is significantly lower than the yields produced at 25°C and 30°C (Figure 53). The maximum yields obtained at 25°C and 30°C are similar, but 30°C has the advantage that a maximum is reached after 72 h, whereas this is achieved after 96 h at 25°C. Thus, an incubation temperature of 30°C and a starting pH of 5 were adopted as standard conditions for all further growth experiments.

Preliminary Growth Trials to Establish the Ability of *Sporotrichum pulverulentum* to Grow on Pig Slurry Hydrolysate

As it was the initial aim of these growth trials to use the liquid hydrolysate recovered after yeast growth as a source of nitrogen and minerals for fungal growth (Figure 49), it was necessary to establish whether *Sporotrichum pulverulentum* was inhibited by the phenol/enolic compound known to be present in the hydrolysate (pages 80-82). Qualitative TLC analysis of the hydrolysate before and after the cultivation of *Rhodosporidium toruloides* (page 82) had indicated that the concentration of the inhibitory compound remained constant throughout the yeast growth period. The effect of the inhibitory compound on the growth of *Sporotrichum pulverulentum* was therefore tested using the hydrolysate before and after yeast growth.

Preliminary trials (pages 96-99) had established that *Sporotrichum pulverulentum* has a requirement for thiamine for optimum growth, and this vitamin was therefore added to all growth media. It was possible, however, that the hydrolysate medium contained thiamine or a precursor of the vitamin, especially after yeast growth. Thus, the need to add thiamine to the hydrolysate after yeast growth was also tested.

MATERIALS AND METHODS

Sporotrichum pulverulentum was grown on the 5[90]3 hydrolysate undiluted and diluted 7:3, 3:2, 1:1, 2:8 and 3:7 v/v with mineral salts solution. *Candida tropicalis* was grown on the 1:1 v/v dilution of the 5[90]3 hydrolysate by the standard method (page 88), and

FIGURE 53 The effect of incubation temperature on the yield of biomass in $g.g^{-1}$ measured available carbohydrate obtained when *Sporotrichum pulverulentum* is grown in an orbital incubator on cellulose/mineral salts medium.

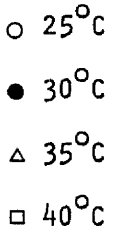
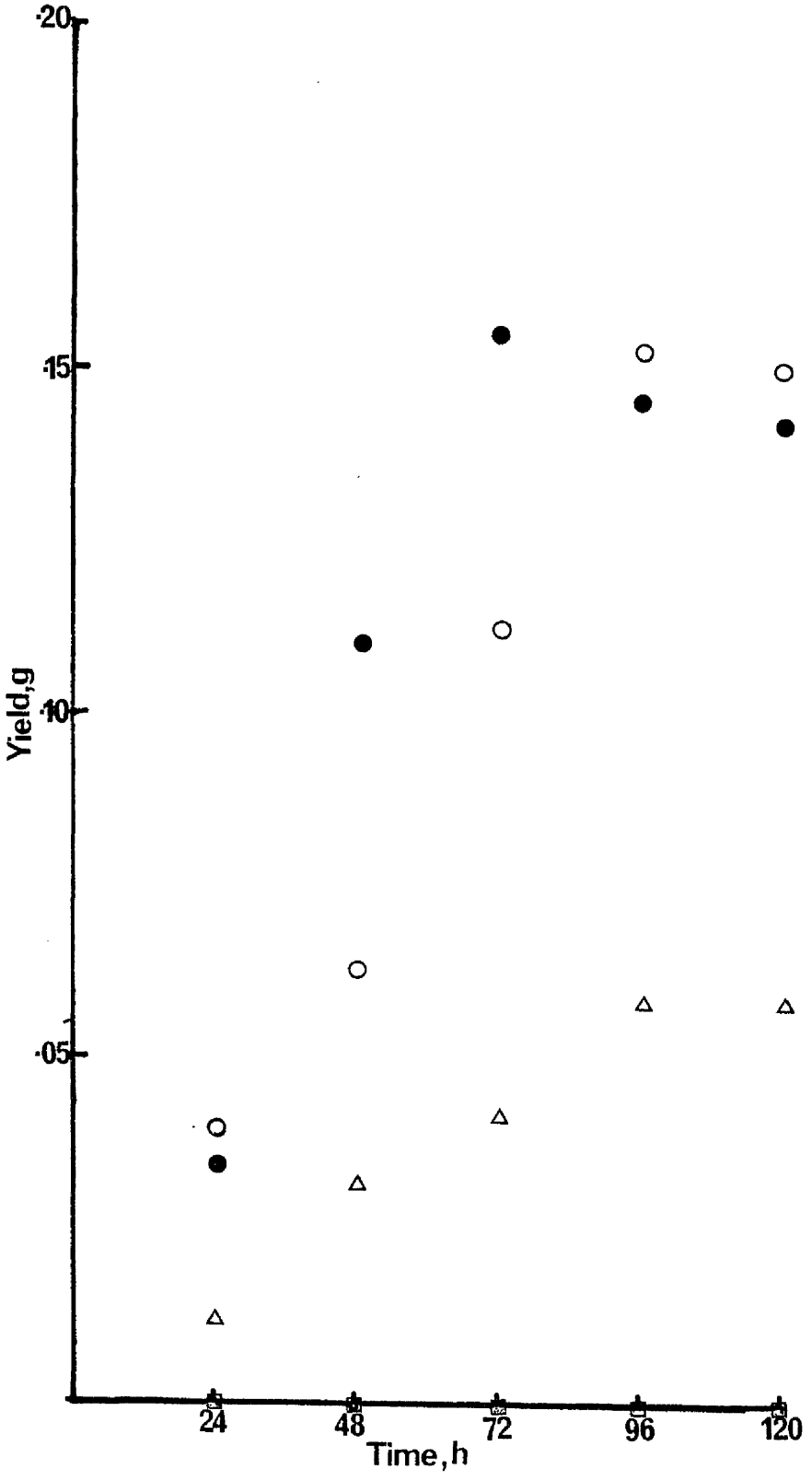


Figure 53



Sporotrichum pulverulentum was then grown on the residual hydrolysate which was undiluted and diluted 4:1 and 3:2 v/v with mineral salts solution. This gave concentrations equivalent to 1:1, 2:3 and 3:7 v/v dilutions of the original hydrolysate. 1% w/v glucose was used as the carbon source in the dilutions of the hydrolysate after yeast growth. Thiamine was normally added to test solutions at the standard rate, except in growth trials in which the need to add thiamine to the hydrolysate recovered after yeast growth was tested.

Flasks containing 20 ml of medium were inoculated with *Sporotrichum pulverulentum* and duplicate flasks of each preparation were analysed at each sampling time.

RESULTS AND DISCUSSION

Figure 54 indicates that the best yield of fungus is obtained when the 5[90]3 hydrolysate is diluted 1:1 v/v. At concentrations of the hydrolysate above 1:1 v/v the yield of fungus progressively decreases. This is probably due to an inhibitory effect similar to that observed during the yeast growth trials (page 89). Dilution of the hydrolysate beyond the 1:1 v/v level results in a drop in biomass yield. The dilution of some essential nutrient is thought to be responsible for this phenomenon. Growth of *Sporotrichum pulverulentum* in the residual hydrolysate, using glucose as a carbon source (Figure 55), confirmed that dilution beyond the 1:1 v/v level results in a lower biomass yield. Thus, it appears that a 1:1 v/v dilution of the residual hydrolysate must be added back to the solid residue in order to promote the maximum possible fungal growth. There are two advantages gained by using this dilution of the hydrolysate. Firstly, the residual hydrolysate after yeast growth can be added directly to the solid residue without further dilution. Secondly, adding this dilution of the residual hydrolysate to the corresponding amount of hydrolysed solids gives a final solids concentration of approximately 1% w/v, which is the normal concentration used for the growth of *Sporotrichum pulverulentum* on fibrous substrates. At higher concentrations the suspensions have been found to be too thick to allow adequate oxygen transfer (Eriksson & Larsson, 1975).

FIGURE 54 The yield of biomass in $g.g^{-1}$ measured available carbohydrate obtained when *Sporotrichum pulverulentum* is grown in an orbital incubator on dilutions of liquid hydrolysate medium 5 [90] 3.

□ 10:0 v/v dilution

○ 7:3 v/v dilution

△ 3:2 v/v dilution

■ 1:1 v/v dilution

● 2:3 v/v dilution

▲ 3:7 v/v dilution

Figure 54

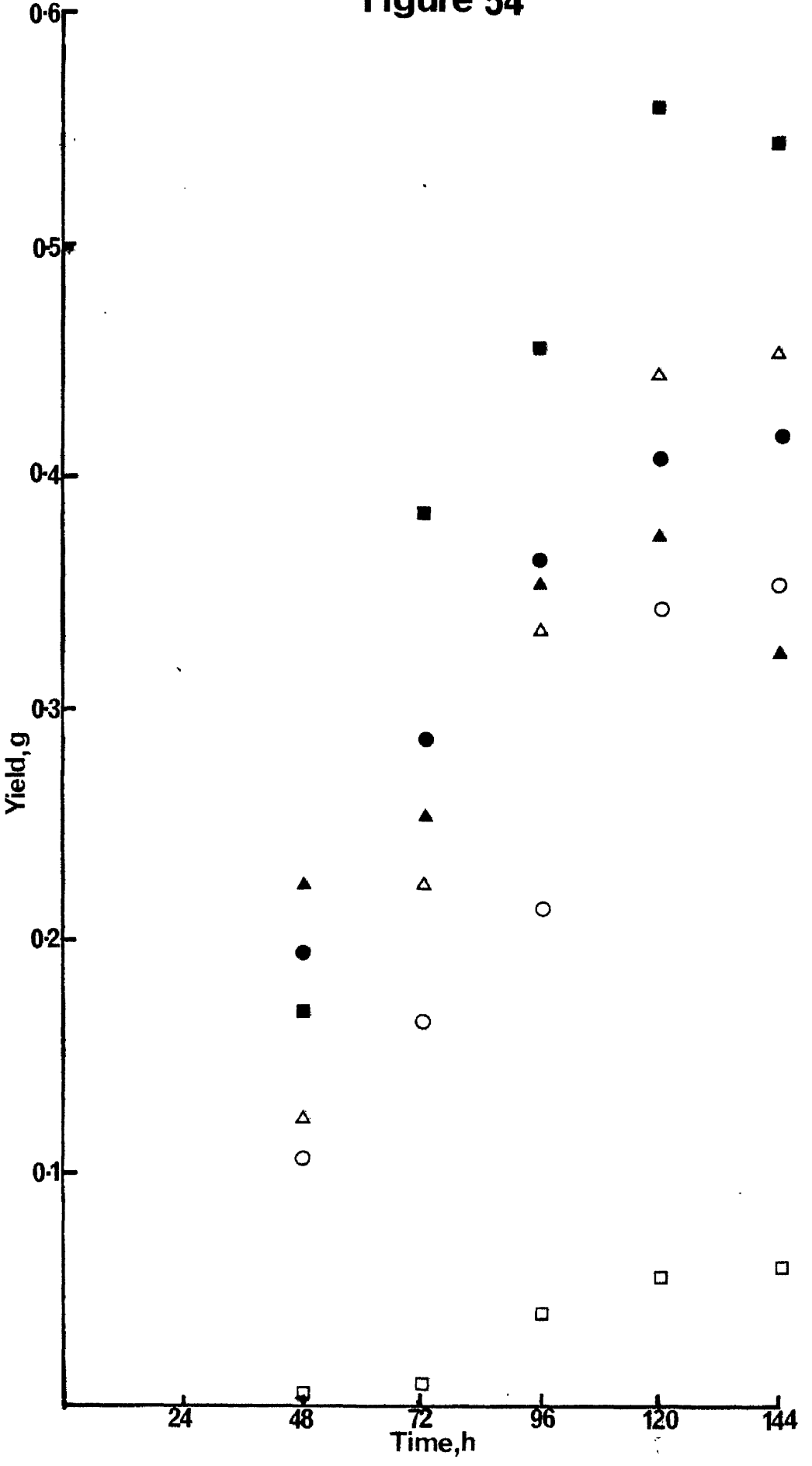


FIGURE 55 The yield of biomass in $g.g^{-1}$ measured available carbohydrate obtained when *Sporotrichum pulverulentum* is grown in an orbital incubator on dilutions of liquid hydrolysate medium 5 [90] 3 recovered after yeast growth and using 1% w/v glucose as a carbon source.

■ 1:1 v/v dilution

● 2:3 v/v dilution

▲ 3:7 v/v dilution

Figure 55

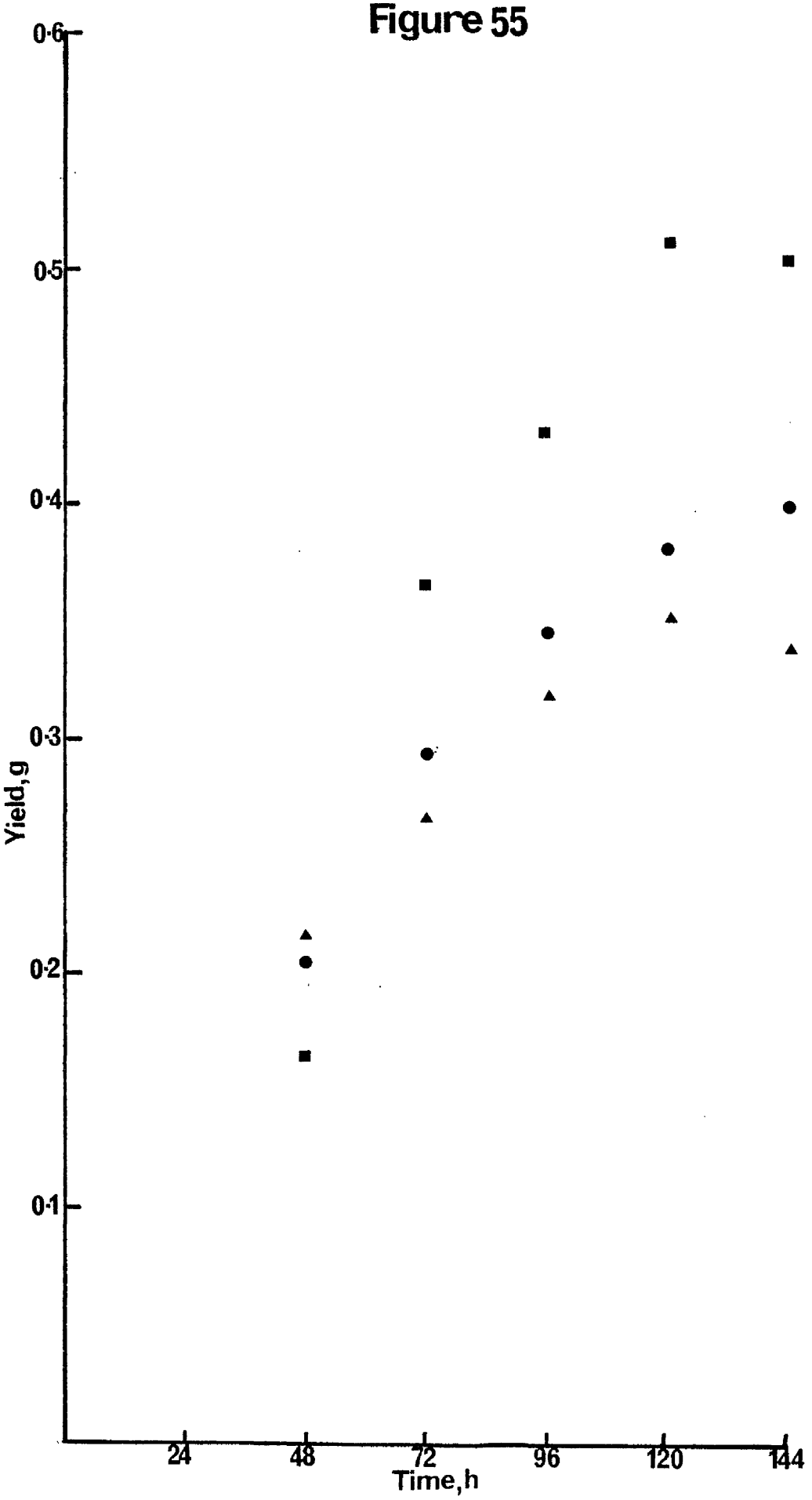
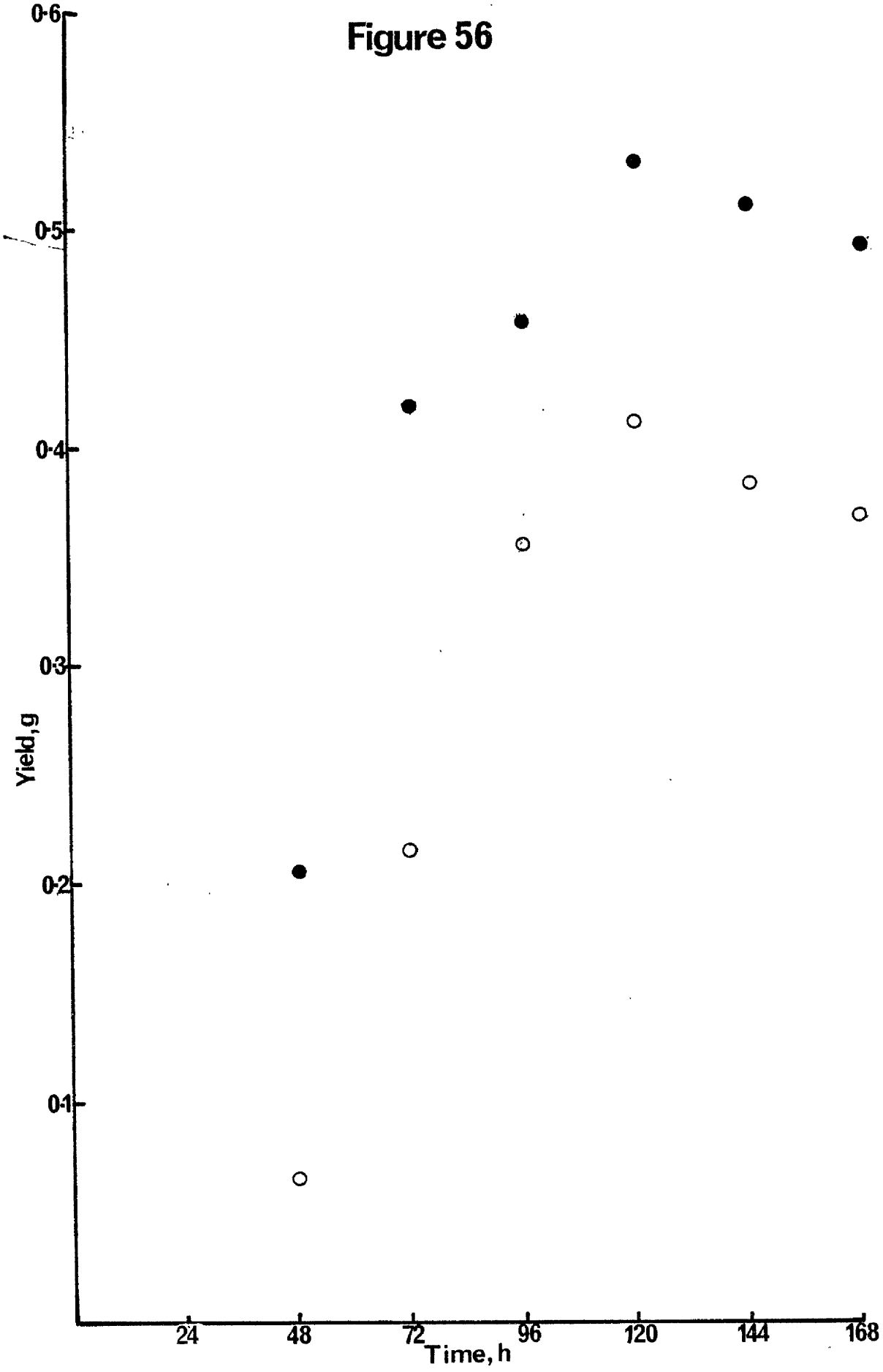


FIGURE 56 The yield of biomass in $g.g^{-1}$ measured available carbohydrate obtained when *Sporotrichum pulverulentum* is grown in an orbital incubator on a 1:1 v/v dilution of liquid hydrolysate medium 5 [90] 3 recovered after yeast growth, using 1% glucose as a carbon source and with and without the addition of thiamine.

● with thiamine

○ without thiamine

Figure 56



Growth of *Sporotrichum pulverulentum* on the 1:1 v/v dilution of the hydrolysate after yeast growth, with and without added thiamine (Figure 56), shows that the addition of the vitamin enhances the biomass yield. Thus, thiamine was added in all further growth trials.

Growth of *Sporotrichum pulverulentum* on Hydrolysed Pig Slurry Solids

MATERIALS AND METHODS

Experiments and Conditions of Cultivation

Prior to carrying out growth trials on hydrolysed slurry solids, the extent to which *Sporotrichum pulverulentum* can attack raw untreated slurry solids was established. For this purpose a sample of raw slurry was diluted to a solids concentration of 1% w/v and the pH was adjusted to 5. This medium was sterilised by steaming for several hours, the requisite amount of thiamine added and the standard procedure for growth trials in the orbital incubator used.

Although the solid residues of hydrolysis have similar cellulosic compositions (pages 56-58), it is possible that the different acid treatment systems, viz 1 [100] 24, 1 [90] 56 and 5 [90] 3 disrupt the structure of the crystalline cellulose to varying degrees, and thus the residues may have different susceptibilities to fungal attack. This was tested by adding these solid residues to the standard mineral salts medium at the rate of 1% w/v.

Further growth trials were then conducted in the orbital incubator in which the most susceptible solid residue was resuspended in the hydrolysate liquid after the yeast has been cultivated and harvested. These trials were then conducted on a larger scale in a 15 l BIOTEC fermenter containing 10 l of growth medium. An aeration rate of 3 $l.min^{-1}$ was shown to maintain a dissolved oxygen level of above 90% saturation throughout the cultivation period. This aeration rate was similar to that used by von Hofsten & Ryden (1975) for the growth of *Sporotrichum pulverulentum* on cereal flours. The temperature in the fermenter was controlled at 30°C, and the pH of the medium was recorded every 24 h when 200 ml samples were taken from the fermenter by the method described on pages 88 and 89.

Determination of Biomass Yield

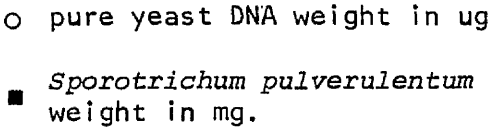
The use of the hydrolysed slurry solids as the cellulosic growth substrate for the fungus introduced a major difficulty in

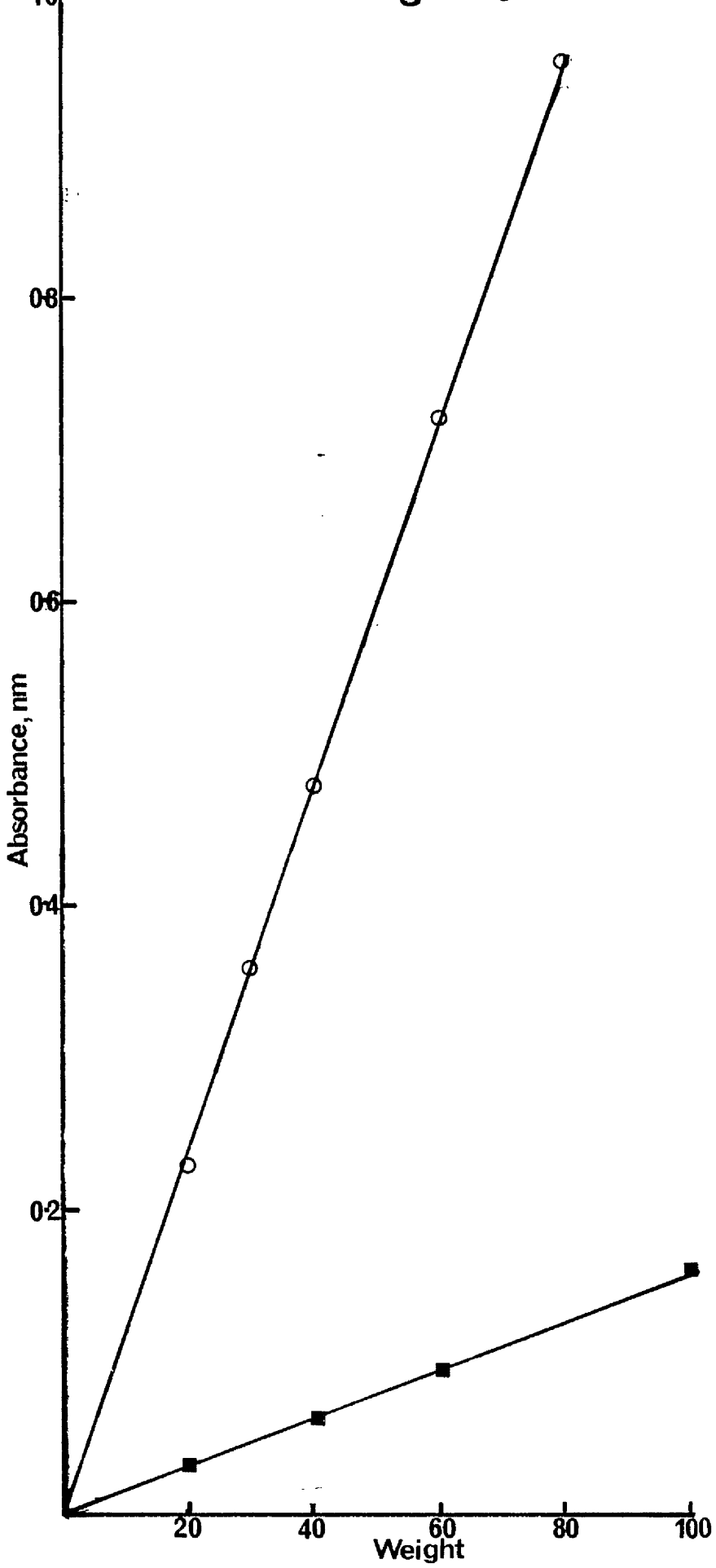
the use of the Biuret reaction for the estimation of yield, as during the protein extraction procedure a brown coloured substance was extracted from the slurry solids. This colour completely masked the blue colour developed during the Biuret reaction. Attempts to remove this colour with agents such as activated charcoal, added at various stages in the Biuret procedure, were shown to remove protein as well as the colour. It was therefore necessary to measure some other cellular component of the fungus which accurately reflected the biomass yield, and was not affected by the colour. For this purpose, analysis of the deoxyribonucleic acid (DNA) content of the solid residue before and after fungal growth was adopted. The method used to determine DNA was basically that described by Herbert *et al*, 1971).

Samples to be analysed for DNA content were separated from the residual liquid portion of the growth substrate by centrifugation. Each sample was then washed twice with an ice cold (0.5°C) 1 millimolar solution of $MgCl_2$. After washing, the samples were freeze-dried and stored over anhydrous $CaCl_2$ prior to analysis. Duplicate 0.2 g samples of the freeze-dried material were weighed directly into 10 ml capacity centrifuge tubes, and 5 ml of ice cold 0.25 N perchloric acid ($HClO_4$) added. This mixture was allowed to stand in an ice bath for 30 min with occasional shaking, and then separated by centrifugation. 4 ml of 0.5 N $HClO_4$ were thoroughly mixed into the solid sample, and it was immersed in a 70°C water bath for 15 min. The solid and liquid portions were separated and retained. A similar extraction was repeated twice using 3 ml quantities of $HClO_4$, and the combined extracts were then made up to 10 ml with 0.5 N $HClO_4$. 2 ml of this extract were mixed with 2 ml of diphenylamine reagent (Herbert, 1971), and incubated at 30°C for 16-20 h. A reagent blank was prepared using 0.5 N $HClO_4$ and when measured against water this blank must have an absorbance of not more than 0.02-0.03. Measured amounts of pure DNA were added to 0.5 N $HClO_4$ and the absorbance of these solutions was measured at 600 nm against the reagent blank to obtain a standard curve (Figure 57).

It was shown that the other components of hydrolysed slurry solids did not interfere with the DNA determination by adding known amounts of pure DNA to the hydrolysed solids, and comparing the increase in absorbance to the normal absorbance expected for the

FIGURE 57 Standard curve relating weight of sample to absorbance in nm obtained in the diphenylanine reaction.




0·6
0·6
Absorbance, nm
0·4
0·2
20
40
60
80
100
Weight

quantity of DNA added. It was also shown that as the quantity of hydrolysed solid analysed increased, the absorbance increased linearly up to an absorbance of 1.0.

A standard curve, relating absorbance in the DNA/diphenylamine reaction to dry weight of *Sporotrichum pulverulentum* was constructed (Figure 57). For this purpose the fungus was cultivated on glucose mineral salts medium. By comparing these values to a similar standard curve prepared with samples of pure DNA (Figure 57), it was calculated that *Sporotrichum pulverulentum* contained 2.45 mg of DNA.g^{-1} of dry fungus.

Initially, it was hoped to use a determination of the decrease in the cellulose content of the hydrolysed slurry solids as a measure of the success of fungal attack. However, chitin is a component of the cell walls of basidiomycetes such as *Sporotrichum pulverulentum* (Bartnicki-Garcia, 1968) and as this β-linked polymer of N-acetylglucosamine is analogous to cellulose (Fieser & Fieser, 1956), it seemed likely that the $NaClO_2$ digestion procedure used to isolate cellulose (pages 51-52) would yield a mixture of chitin and cellulose from a sample containing fungal biomass and cellulosic residue. This hypothesis was confirmed in the laboratory. Methods of extracting pure chitin were also considered and tested (Mahadevan & Tatum, 1975). Again, however, the cellulose and chitin were isolated in the same fraction.

It was possible to use the $NaClO_2$ digestion method (pages 51-52) to determine the chitin content of pure fungal biomass which had been grown on a medium containing a soluble carbon source. In this way the chitin content of pure fungal biomass was estimated as 12.5% w/w of the dry biomass. Thus, by assessing the amount of fungal biomass present in a sample of cellulosic residue a correction for chitin could be applied to the estimated cellulose content. Although the result of this determination may not be entirely accurate, it provides a useful indication of overall efficiency of cellulose utilisation.

As 10 g of material were needed for accurate cellulose determinations by the digestion method, and the solid concentration in fermenter was only 1% w/v, this type of sampling was impracticable throughout the growth period. However, in order to obtain some indication of the overall extent of cellulose breakdown, a determin-

ation of the cellulose content of hydrolysed solids prior to fungal growth was compared to the cellulose content of the solids at the end of the growth period, after a correction for chitin content had been applied.

RESULTS AND DISCUSSION

When raw slurry was inoculated with *Sporotrichum pulverulentum*, growth as evidenced by microscopic examination or by DNA analysis was not detected, even after several days incubation. Thus raw slurry appears to be unable to support fungal growth, without some kind of pre-treatment.

Figure 58 shows the yield of fungal biomass produced when each of the solid residues of hydrolysis, viz 1 [90] 56, 1 [100] 24 and 5 [90] 3 were used as carbon sources in mineral salts medium. These results indicate that the residue of 5 [90] 3 is most susceptible to fungal attack. There is a significantly lower yield of fungal biomass from the residue of 1 [100] 24, and lower still from the residue 1 [90] 56. This indicates that the more severe acid treatment systems break up the structure of the cellulosemore efficiently, and allow greater penetration of fungal hyphae. The residue of the 5 [90] 3 treatment system was therefore selected for further growth trials with the fungus. It was fortunate that this acid treatment produced the solid residue that was most susceptible to fungal attack, as the highest yield of *Candida tropicalis* was obtained from the corresponding liquid residue.

When the solid residue 5 [90] 3 was resuspended in the 1:1 v/v dilution of the corresponding hydrolysate after the yeast had been grown, a maximum yield of 0.218 g of fungal biomass.g^{-1} available cellulosic constituents was recorded after 168 h incubation in the orbital incubator (Figure 59). When this was scaled up in the fermenter (Figure 60), similar yields of 0.211 g and 0.216 $g.g^{-1}$ available cellulosic constituents were achieved after 168 h in two separate trials.

Initially, the solid residue of hydrolysis contained 34% w/w cellulose. In both growth trials in the fermenter 43% w/w of this residual cellulose was degraded by the fungus. It was also calculated that for every litre of pig slurry (dry weight 110 $g.l^{-1}$)

FIGURE 58 The yield of biomass in $g.g^{-1}$ measured available carbohydrate obtained when *Sporotrichum pulverulentum* is grown in an orbital incubator on the solid residues from the H_2SO_4 hydrolysis of pig slurry suspended in a mineral salts solution.

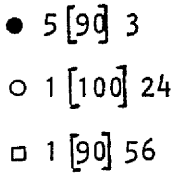


Figure 58

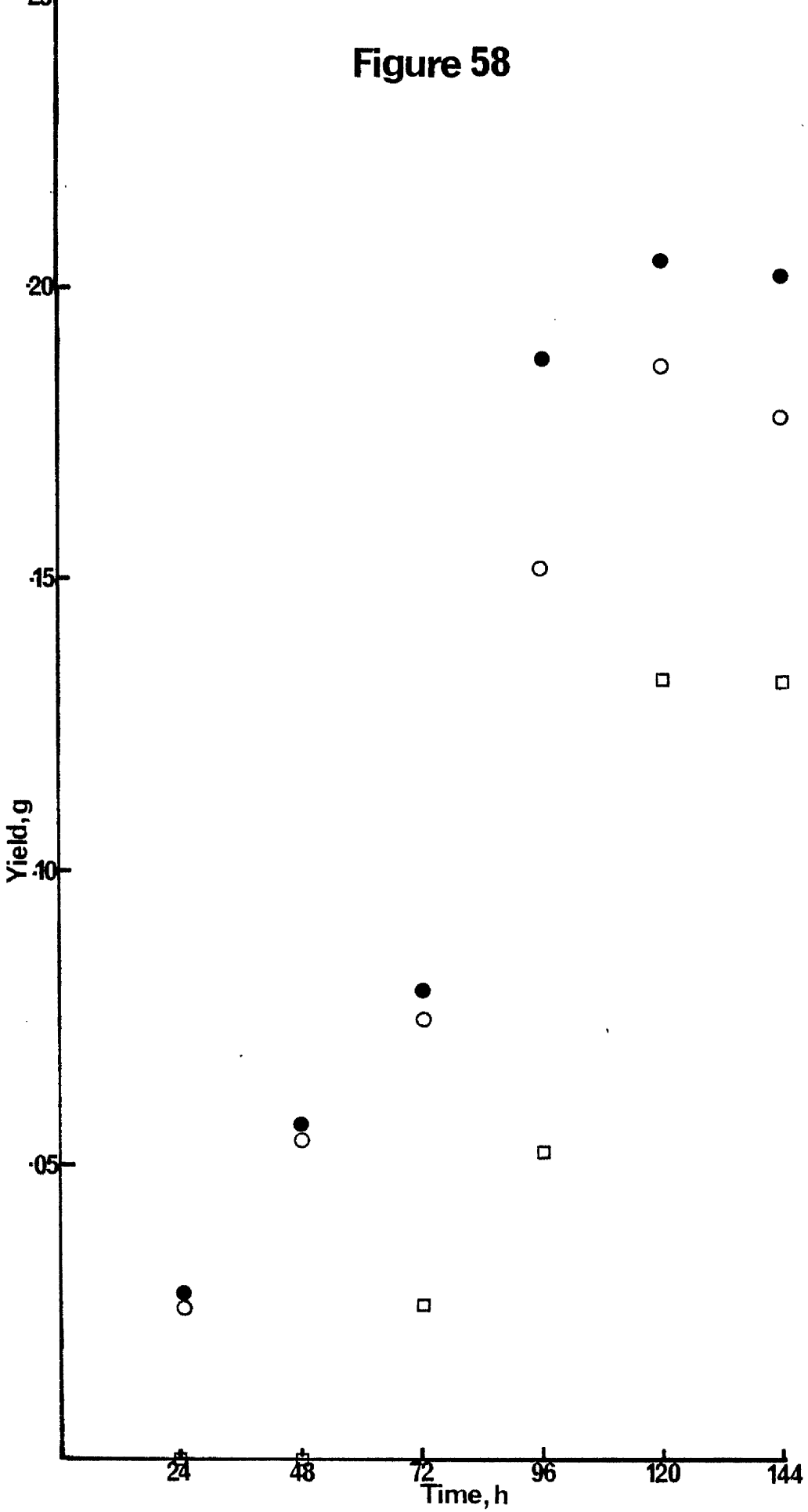


FIGURE 59 The yield of biomass in $g.g^{-1}$ measured available carbohydrate obtained when *Sporotrichum pulverulentum* is grown in an orbital incubator on the solid residue 5[90]3 using the residual liquid hydrolysate medium 5[90]3 recovered after yeast growth as a source of nitrogen and minerals.

Figure 59

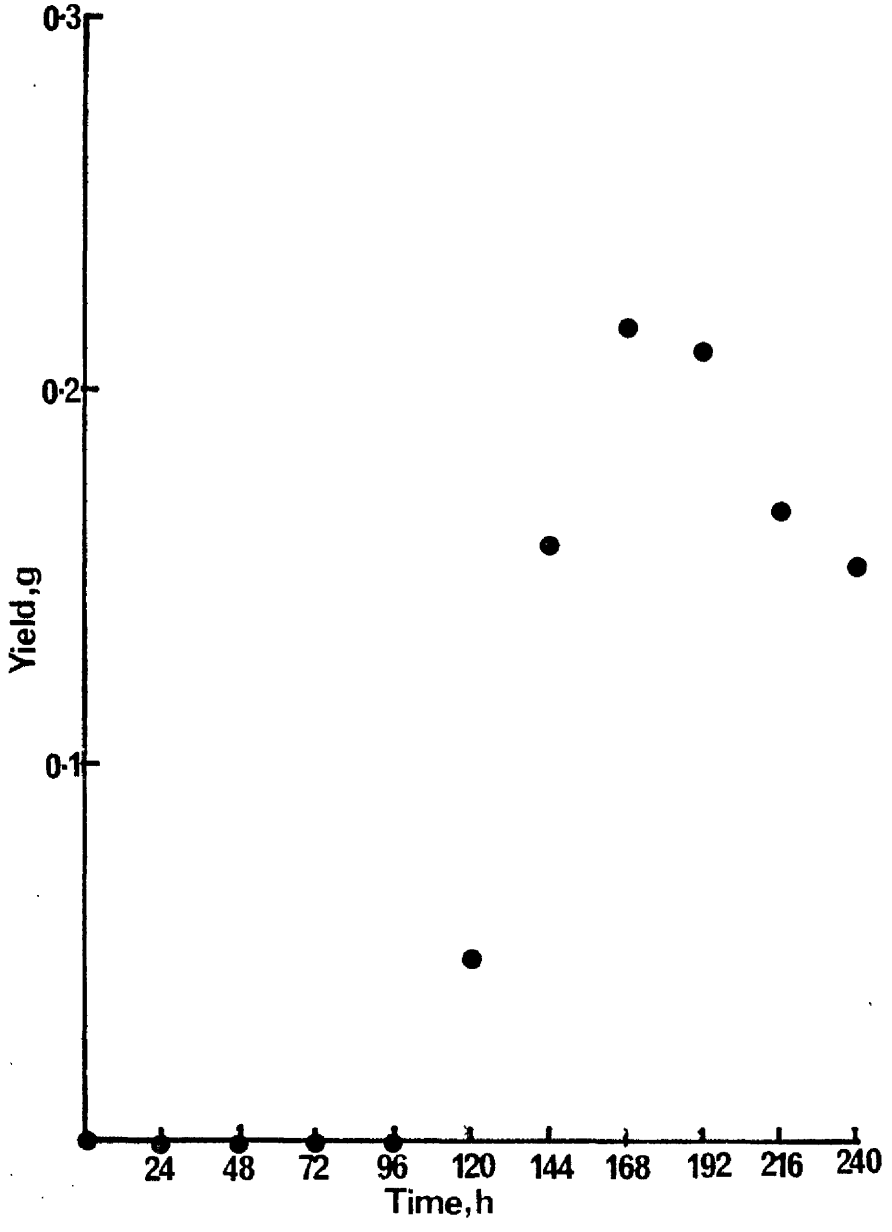
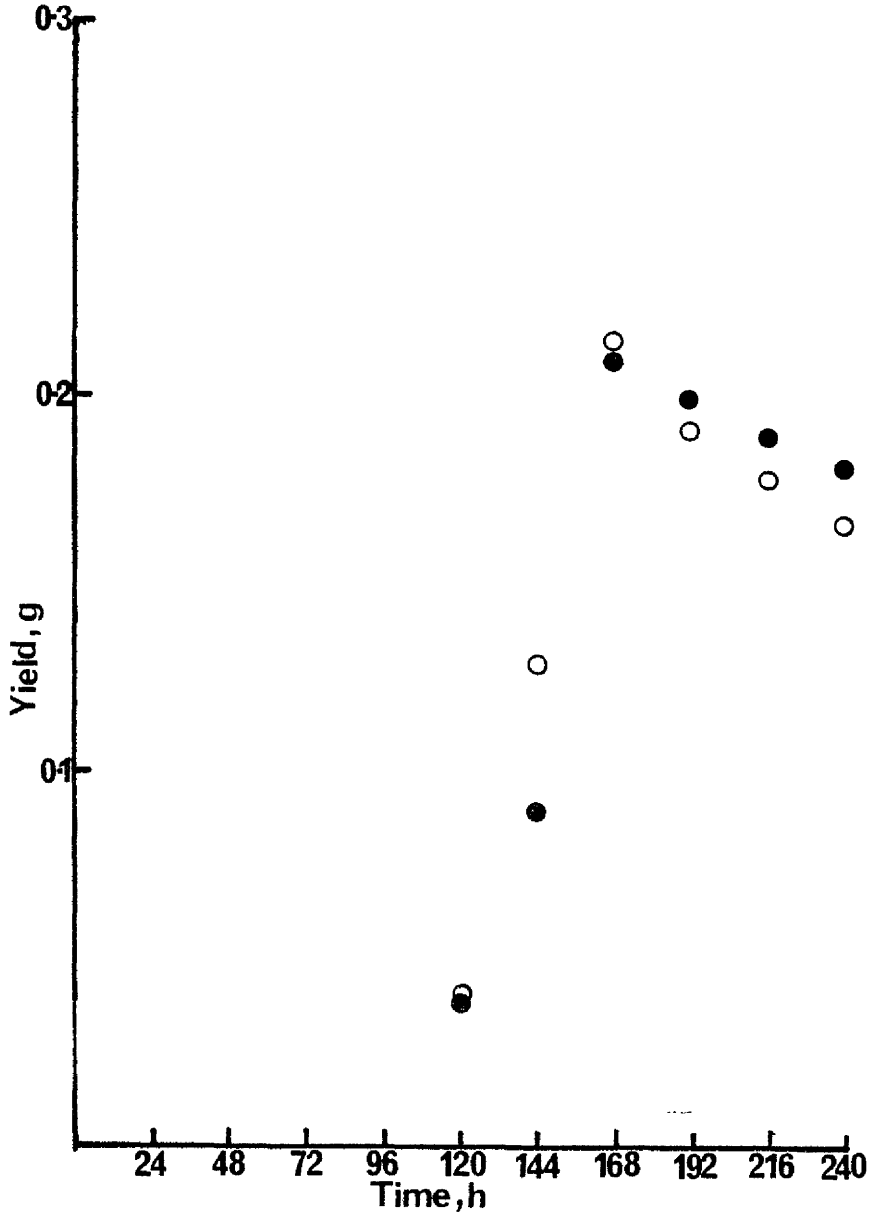


FIGURE 60 The yield of biomass in $g.g^{-1}$ measured available carbohydrate obtained when *Sporotrichum pulverulentum* is grown in a 15 litre fermenter on the solid residue 5 [90] 3 using the residual liquid hydrolysate medium 5 [90] 3 recovered after yeast growth as a source of nitrogen and minerals.

● run 1

○ run 2

Figure 60



hydrolysed, 19 g of cellulosic compounds are left in the solid residue, and of this 8.2 g are utilised to produce 4.6 g of fungal biomass. This does not take into account any other carbohydrates or lignin utilised by the fungus.

The effect of the growth of *Sporotrichum pulverulentum* on the pH biological oxygen demand (BOD), chemical oxygen demand (COD), ammonium and total nitrogen levels of the ultimate liquid residue are presented on pages 116-117.

Growth in the Fermenter of *Sporotrichum pulverulentum* on the Unseparated Liquid and Solid Fractions of Acid Hydrolysed Pig Slurry

The need to separate the liquid and solid fractions of the hydrolysed slurry for the growth procedure outlined in Figure 49 is a major disadvantage. The possibility of omitting the yeast growth stage and growing *Sporotrichum pulverulentum* directly on the unseparated liquid and solid residues of hydrolysis diluted 1:1 v/v with water (Figure 61) was therefore investigated.

MATERIALS AND METHODS

Although preliminary studies (pages 101-102) in the orbital incubator had shown that *Sporotrichum pulverulentum* could grow on the 1:1 v/v dilution of the 5[90]3 hydrolysate, it was considered to be advantageous to establish the contribution that the soluble carbohydrates of the hydrolysate would make to the biomass yield in the 15 l fermenter. Growth of the fungus on charcoal treated hydrolysate was also tested. The biomass yield was estimated by dry weight (page 71) and after fungal growth the residual liquid was tested for the presence of unused monosaccharide by HPLC.

Finally, *Sporotrichum pulverulentum* was grown in the fermenter on the unseparated liquid and solid residues from the hydrolysis process. The growth medium was diluted so that the concentration of solutes in the liquid portion was the same as that in a 1:1 v/v dilution of the separated hydrolysate. Biomass yield was determined by DNA analysis.

RESULTS AND DISCUSSION

The results of growth trials with *Sporotrichum pulverulentum* in the 1:1 v/v dilution of the 5[90]3 hydrolysate are presented in

FIGURE 61 Outline of the process developed for the utilisation of the unseparated liquid and solid residues of acid hydrolysed slurry.

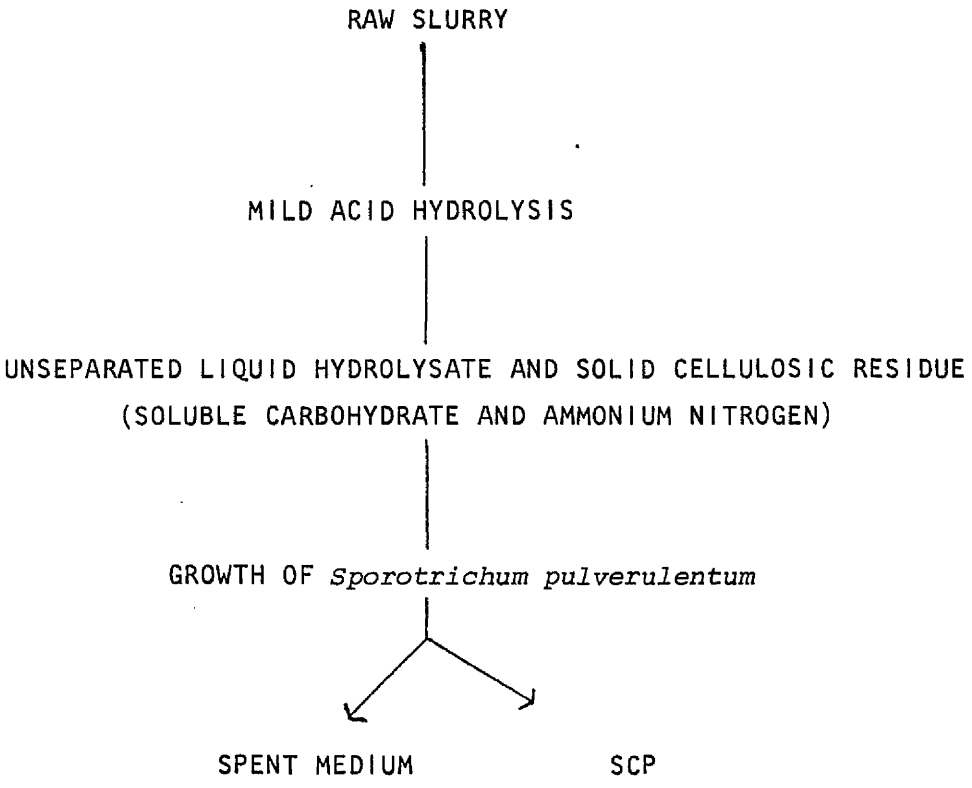


Figure 62. Maximum yields of 0.56 and 0.57 g of fungal biomass.g^{-1} measured available carbohydrate were obtained in consecutive fermenter trials. Thus, for every litre of raw slurry with an initial solids content of 110 g.l^{-1}, approximately 14.4 g of dry fungal biomass can be obtained when the liquid hydrolysate is used as a substrate. This compared favourably with the 9 g of yeast (page 91) produced in a similar trial. The high biomass yield of fungus on the separated liquid hydrolysate made the process (Figure 61), omitting the yeast growth phase, more attractive than consecutive growth of the yeast and fungus (Figure 50).

HPLC analysis indicated that no measurable carbohydrate remained in the hydrolysate after fungal growth. This means that the fungus is able to utilise the xylose, arabinose and traces of glucose initially present in the hydrolysate.

Several attempts to grow the fungus on hydrolysate that had been treated with activated charcoal, to remove the inhibitory compound, were unsuccessful. No fungal growth was observed, even after 11 days incubation, and it is thought that the charcoal treatment adsorbed some substance essential for the growth of *Sporotrichum pulverulentum*.

The yields of fungal biomass achieved when *Sporotrichum pulverulentum* is grown on the unseparated liquid and solid residues of the acid hydrolysis of pig slurry can be seen in Figure 63. In this process the fungus can utilise both the soluble carbohydrate of the liquid fraction and the cellulosic compounds of the solid residue. Yields of 0.407 and 0.412 g of dry fungal biomass.g^{-1} total measured available carbohydrate are obtained after 168 h using this system. Thus, 19.8 g of dry fungus can be produced for every litre of raw slurry hydrolysed. It was estimated that the fungus degraded 56% w/w of the cellulosic constituents of solids remaining after hydrolysis. When *Sporotrichum pulverulentum* was grown on the cellulosic residue resuspended in the liquid recovered from yeast growth, only 43% w/w of the cellulosic constituents were utilised (page 105). There appears to be no obvious reason for the increased degradation of cellulose that occurs when the fungus was grown on unseparated liquid and solid hydrolysis products. It is

FIGURE 62 The yield of biomass in $g.g^{-1}$ measured available carbohydrate obtained when *Sporotrichum pulverulentum* is grown in a 15 litre fermenter on a 1:1 v/v dilution of the liquid hydrolysate medium 5 [90] 3.

○ run 1

□ run 2

Figure 62

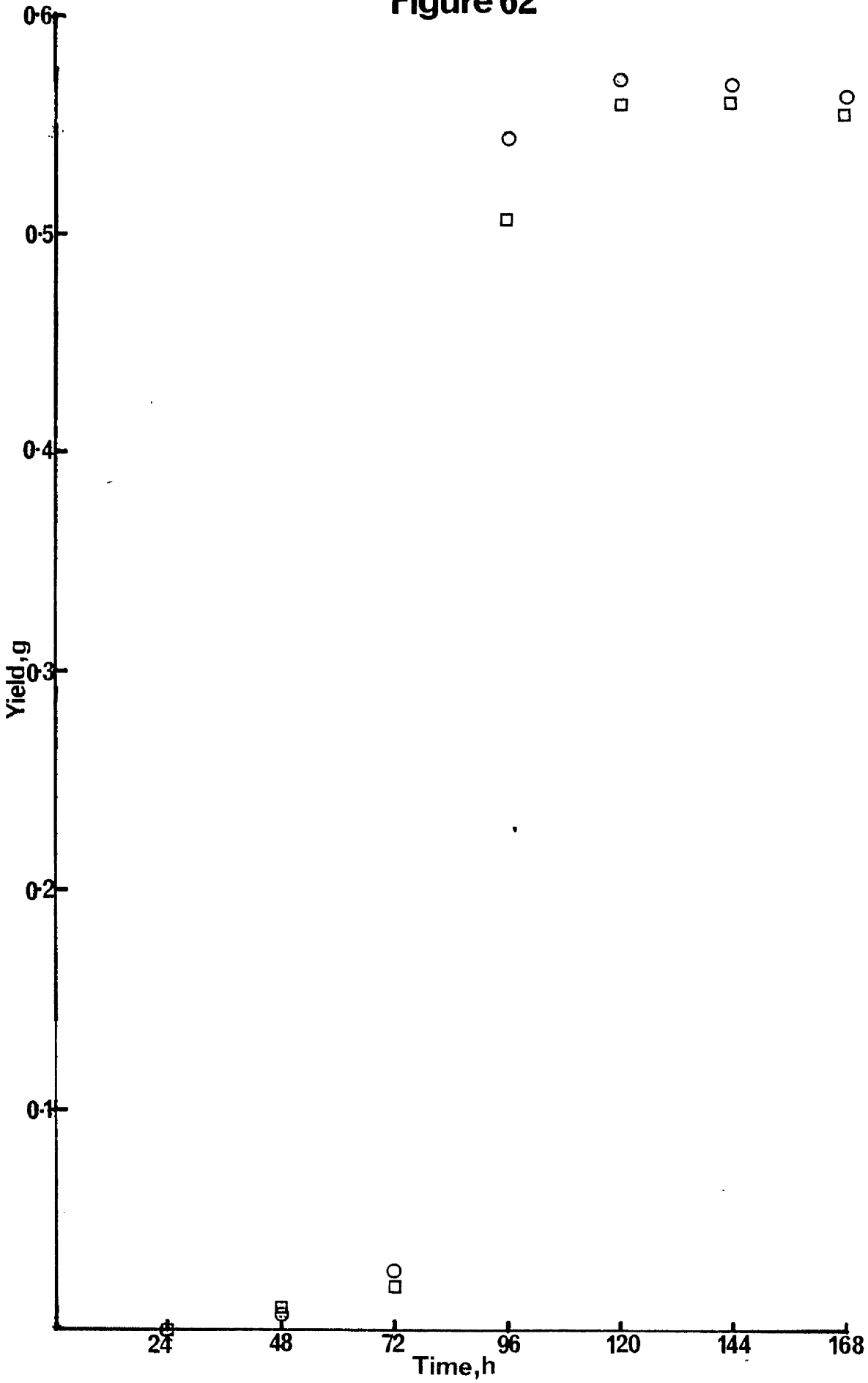
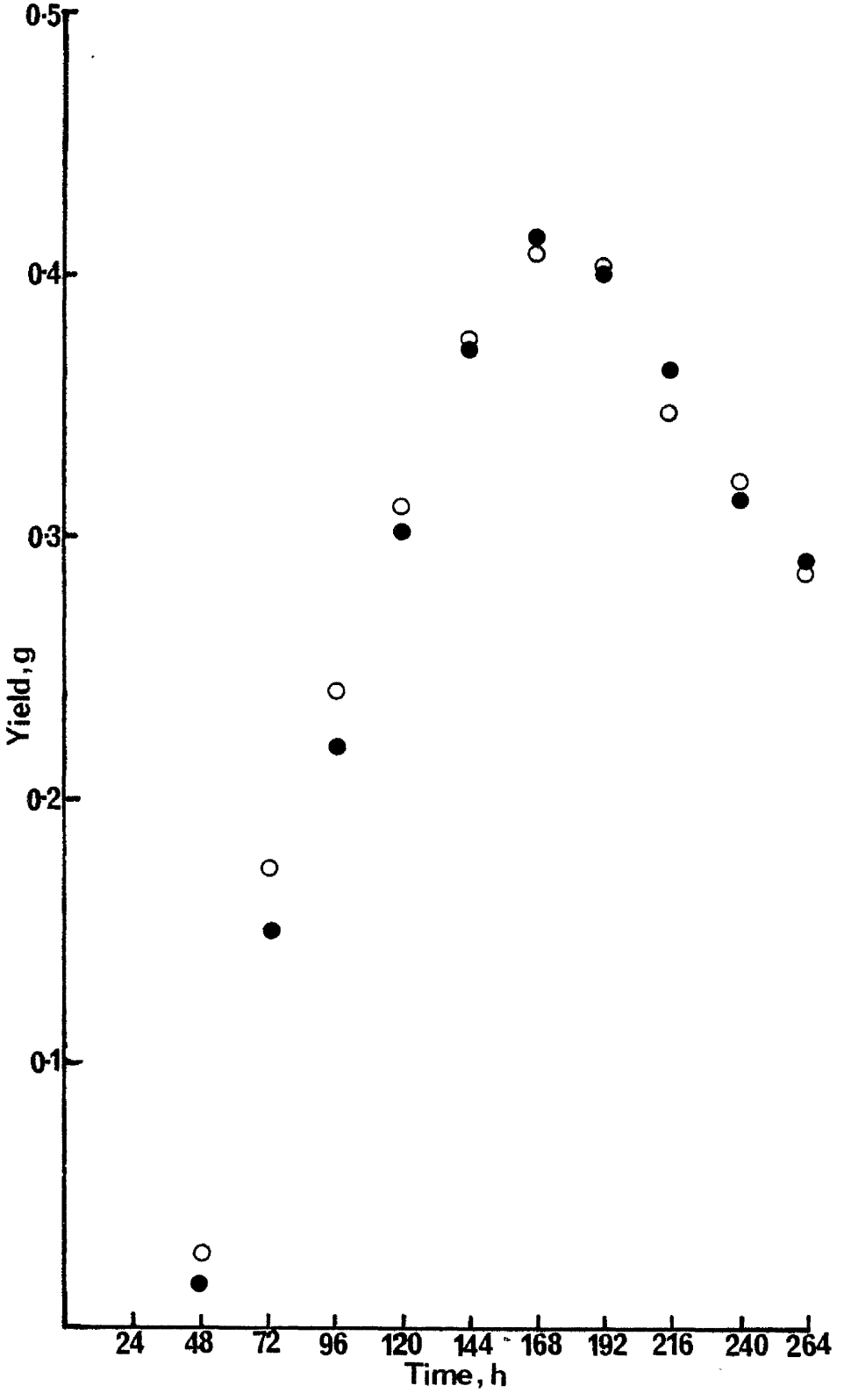


FIGURE 63 The yield of biomass in $g.g^{-1}$ measured available carbohydrate obtained when *Sporotrichum pulverulentum* is grown in a 15 litre fermenter on the unseparated solid residue 5[90]3 and the liquid hydrolysate medium 5[90]3 that has been diluted to the 1:1 v/v level.

● run 1

○ run 2

Figure 63


0·5
0·4
0·3
0·2
0·1
Yield, g
24
48
72
96
120
144
168
192
216
240
264
Time, h

postulated that utilisation of the monosaccharides present in the liquid hydrolysate produces such an actively growing culture of *Sporotrichum pulverulentum* that more cellulose is degraded. It is also possible that production of the lignin or cellulose degrading enzymes is enhanced by some substance normally utilised during the yeast growth stage. Another more likely explanation is that, when the two stage growth procedure is used, the separated solids have to be stored while the yeast is cultivated on the liquid hydrolysate, and that during this time the structure of the cellulose, disrupted by acid treatment, reverts to a more crystalline formation.

The results of analyses for the pH, BOD, COD, ammonium nitrogen, total nitrogen and monosaccharide levels of the ultimate liquid residue produced by the growth of *Sporotrichum pulverulentum* on the unseparated products of acid hydrolysis of pig slurry are presented on pages 116-117.

CONCLUSIONS

1. In a simple mineral salts medium *Sporotrichum pulverulentum* degrades cellulose most efficiently when the starting pH is 5 and the incubation temperature is 30°C.
2. The solid residue of the hydrolysis system 5 [90] 3 is more susceptible to attack by the cellulolytic fungus *Sporotrichum pulverulentum*, than either the residue from the 1 [100] 24 or the residue from the 1 [90] 56 treatment systems.
3. The growth of *Sporotrichum pulverulentum* is inhibited in the hydrolysate 5 [90] 3. This inhibition can be overcome by dilution with water.
4. *Sporotrichum pulverulentum* grows well on the 5 [90] 3 hydrolysate when it is diluted 1:1 v/v with water, and all the component pentose sugars are utilised during fungal growth.
5. A yield of fungal biomass of 14.4 $g.l^{-1}$ of raw slurry can be obtained by growing *Sporotrichum pulverulentum* on the liquid pig slurry hydrolysate 5 [90] 3.
6. When the residual hydrolysate from yeast growth is added to the solid residue of hydrolysis, the cultivation of *Sporotrichum pulverulentum* yields 4.6 g fungal biomass.l^{-1} of raw slurry and 43% w/w of the cellulosic constituents of the solid residue are degraded.

7. Growing *Sporotrichum pulverulentum* on the unseparated hydrolysate and solid residue of hydrolysis produces an overall yield of 19.8 g fungall biomass.l^{-1} of raw slurry. In this process 56% w/w of the cellulosic constituents of the solid residue are degraded.

4. ASSESSMENT OF THE PROTEIN CONTENT, PROTEIN QUALITY AND HEAVY METAL CONTENT OF THE PRODUCTS OF MICROBIAL GROWTH ON ACID HYDROLYSED PIG SLURRY.

Nutritional value and freedom from toxic substances are important criteria in determining the usefulness of an SCP product as food or feed. It is therefore of value to chemically determine the protein content, amino acid patterns and heavy metal content of the products of microbial growth on acid hydrolysed pig slurry.

MATERIALS AND METHODS

Six samples were analysed for amino acid and protein content. These samples were as follows:-

1. Raw slurry solids separated from the liquid by centrifugation.
2. The solid residue recovered after hydrolysis with 5% H_2SO_4 at 90°C for 3 h (pages 54-55).
3. The residual solids from the hydrolysis process 5 [90] 3 on which *Sporotrichum pulverulentum* had been cultivated utilising the cellulosic constituents of the solids as a source of carbon (pages 102-106).
4. The residual solids from the hydrolysis process 5 [90] 3 on which *Sporotrichum pulverulentum* had been cultivated utilising both the cellulosic constituents of the solids and the carbohydrates of liquid hydrolysate as sources of carbon (pages 106-108).
5. *Candida tropicalis* grown on liquid hydrolysate 5 [90] 3 (page 100).
6. *Sporotrichum pulverulentum* grown on liquid hydrolysate 5 [90] 3 (pages 100-102).

All samples were thoroughly washed in a 1 millimolar solution of $MgCl_2$ prior to freeze-drying. Amino acid analysis was carried out by the standard method of Spackman *et al* (1958) using a JEOL model JLC 5-AH Automatic Amino Acid Analyser (JEOL (UK) Ltd., JEOL House, Grave Park, Collindale, London, NW9 0JN). The amino acids tryptophan, cystine and methionine are not detected by the method used. In samples which contain significant quantities of soluble

1. Amino acid analysis was undertaken by Dr W Manson of The Hannah Research Institute, Ayr.

carbohydrates it is virtually impossible to obtain accurate values for sulphur-amino acids. Values for threonine and serine were corrected for hydrolytic losses.

The protein content of the samples was calculated from the amino acid values and is therefore likely to be lower than the true value, due to hydrolytic losses. The standard Biuret method (Herbert *et al*, 1971) was also used to estimate the protein content of samples 5 and 6. Coloured compounds extracted during the Biuret procedure precluded its use for samples 1 to 4. Sample 4 was subjected to analysis for the heavy metals copper (Cu), zinc (Zn), manganese (Mn) and iron (Fe) using standard atomic absorption spectroscopy[2] with an air/acetylene flame.

RESULTS AND DISCUSSION

The values for the amino acid content of microbial protein sources reported in the literature show considerable variation (Birch *et al*, 1976; Davis, 1974; Omstedt *et al*, 1973). These differences can be attributed to analytical errors, strains of organisms, growth media or environmental conditions.

In Table 24 the amino acid composition of *Candida tropicalis* cultivated on pig slurry is compared to the values reported for this organism grown on n-alkanes (Senez, 1972) and straw hydrolysate (Volfova *et al*, 1979). Similarly, the amino acid patterns of *Sporotrichum pulverulentum* grown on pig slurry hydrolysate and barley flour (von Hofsten, 1976) are compared. Normally, the usefulness of a microbial protein is limited by the levels of the sulphur-amino acids, methionine and cystine, present. This deficiency is demonstrated in Table 24, where the levels of sulphur-amino acids in *Candida tropicalis* (Senez, 1972; Volfova *et al*, 1979) are compared to the WHO/FAO (1973) provisional pattern of essential amino acids for humans. *Sporotrichum pulverulentum* (von Hofsten, 1976), however, has been reported to contain high levels of methionine and cystine. Unfortunately, as has already been stated, no values are available for the sulphur-amino acid content of these microorganisms cultivated on pig slurry hydrolysate.

2. *Heavy metal analysis by atomic absorption spectroscopy was performed by Dr J Dixon of the Chemistry Department, The West of Scotland Agricultural College.*

TABLE 24 Amino acid content of *Candida tropicalis* produced by The West of Scotland Agricultural College (W.S.A.C.) compared to that reported by Senez (1972) and Volfova *et al* (1979) and amino acid content of *Sporotrichum pulverulentum* produced by W.S.A.C. compared to that reported by von Hofsten (1976).

Amino acid	g amino acid.100 g^{-1} protein					
		Candida tropicalis	*Candida tropicalis*	*Candida tropicalis*	*Sporotrichum pulverulentum*	*Sporotrichum pulverulentum*
	FAO/WHO 1973	W.S.A.C.	Senez, 1972	Volfova *et al* 1979	W.S.A.C.	von Hofsten, 1976
Isoleucine*	4.0	4.8	5.3	4.7	4.8	3.9
Leucine*	7.0	7.9	7.8	6.8	7.5	6.9
Lysine*	5.5	10.0	7.8	6.6	6.8	6.2
Phenylalanine*	[6.0	4.3	4.8	4.2	4.4	[9.6
Tyrosine*		2.3	4.0	3.8	2.8	
Valine*	5.0	6.6	5.8	4.8	7.3	5.0
Threonine*	4.0	5.5	5.4	4.8	6.1	4.9
Tryptophan*	1.0	N/A	1.3	1.1	N/A	1.5
Methionine*	[3.5	N/A	[2.5	[2.0	N/A	[4.4
Cystine*						
Histidine		TRACE	-	2.0	TRACE	-
Arginine		5.6	-	-	4.6	-
Aspartic acid		8.7	-	-	9.7	-
Serine		5.5	-	-	5.9	-
Glutamic acid		13.6	-	-	13.8	-
Proline		3.8	-	-	4.0	-
Glycine		5.4	-	-	5.7	-
Alanine		7.1	-	-	7.5	-

* FAO/WHO essential amino acids

Homb (1976) has reviewed the data for the essential amino acid requirements for growing finishing pigs and this information is summarised in Table 25. Insufficient information is as yet available to enable the requirements of pigs for arginine, histidine, phenylalanine, leucine and valine to be estimated. However, it is unlikely that under practical conditions these five essential amino acids are limiting (Cuthbertson & Baskett, 1969; Homb, 1976). For most practical purposes, lysine and the sulphur amino acids are likely to be limiting in a pig's diet and it can be seen from Table 25 that pigs have an even greater requirement for the sulphur-amino acids than humans. Thus, the sulphur-amino acid content of micro-organisms is even more important when the microbial protein is to be used in pig nutrition.

The main value of microbial protein in the diet of animals is the high lysine level. Cereals, which normally provide most of the protein in animal diets, are adequate in sulphur-amino acids, but extremely deficient in lysine. The lysine content of wheat is reported to be as low as 2.6 g.100 g^{-1} protein (Kent, 1970). The growth of *Candida tropicalis* on xylose rather than glucose has been reported to almost double the lysine content of the yeast protein (Worgan, 1974), and this may explain the high levels of lysine that can be seen in the *Candida tropicalis* and *Sporotrichum pulverulentum* grown on pig slurry hydrolysate (Table 24) which has a high xylose content. The other significant difference in amino acid pattern demonstrated by both the yeast and fungus grown on the hydrolysate medium compared to these organisms grown on other media, is a decrease in the phenylalanine/tyrosine content (Table 24). These amino acids are normally considered together, as tyrosine can be converted to phenylalanine in the body. The lower values for the phenylalanine/tyrosine content of both organisms may be attributable to differences in the growth medium.

Apart from protein quality, the crude protein content of the product is also an important feature. The Biuret reaction gives a protein content of 43% w/w dry matter for *Candida tropicalis*. When the total amino acid content is used as the basis for protein determination, a value of 40% w/w is obtained. This difference

TABLE 25 Estimates of the essential amino acid requirements of pigs between 20 kg and 90 kg liveweight compared to the FAO/WHO pattern for humans.

Amino acid	g amino acid.100 g^{-1} protein		
	Amino acid requirements of pigs[a]		FAO/WHO pattern for humans[b]
	20-50 mg	50-90 mg	
Isoleucine	3.8-3.6	2.7	4.0
Leucine			7.0
Phenylalanine + Tyrosine			6.0
Threonine	3.5-3.2		4.0
Tryptophan	0.93-0.9	0.9-0.8	1.0
Valine			5.0
Lysine	5.5-5.0	5.0-4.6	5.5
Cystine + Methionine	4.0-3.9	3.9-3.6	3.5

a. Calculated from Homb (1976)

b. Taken from FAO/WHO (1973)

can be attributed to hydrolytic losses, during the amino acid determination, especially of sulphur-amino acids and tryptophan. With *Sporotrichum pulverulentum*, the discrepancy between the Biuret value for protein content of 35% w/w dry matter and the protein content calculated from the amino acid analysis of 25% w/w is less easily accounted for. However, it is known that the presence of compounds such as free carbohydrates can cause significant hydrolytic losses in the amino acid determination.

The protein content and protein quality of the raw slurry solids, hydrolysed slurry solids and hydrolysed solids on which the fungus had been cultivated were also analysed. The results for this analysis are given in Tables 26 and 27 respectively. The protein content of raw slurry is 15.5 g.100 g^{-1} dry weight (Table 26) and is likely to be a mixture of residual plant protein and microbial protein.

During acid hydrolysis, almost 50% of the dry weight of raw slurry solids is solubilised, and the resulting solids have a protein content of 17.5 g.100 g^{-1} dry weight (Table 26). Growth of *Sporotrichum pulverulentum* on the residual hydrolysed solids increases the protein content to 21.5 g.100 g^{-1} (Table 26). In this case the nitrogen source is supplied by the residual hydrolysate after yeast growth. When the yeast growth stage is omitted, and the fungus is grown on a mixture of the hydrolysate and hydrolysed solids, the protein content of the product is 22.7 $g.g^{-1}$ of the dry matter (Table 26). The minimum crude protein requirements for pigs have been reported by Cuthbertson & Baskett (1969) and are given in Table 28. It can be seen from this Table that the minimum requirements for fattening pigs of 50-90 kg weight is 15.5 g protein.100 g^{-1} dry matter, and that smaller pigs require higher protein contents. Thus, the diets of fattening pigs normally contain 17% crude protein (O'Callighan *et al*, 1971).

The growth of fungus on the hydrolysed slurry solids produces materials that have a more than adequate protein content for pig feeding. Furthermore, this protein is likely to be more available to the pig than the plant and microbial protein of raw slurry. The importance of protein quality has already been discussed, and it can be seen in Table 27 that the products of fungal growth are well above the requirements of fattening pigs given in Table 25.

TABLE 26 The overall protein content of samples of raw and treated pig slurry solids calculated from the amino acid content.

	Protein content g.100 g^{-1} dry weight
Raw slurry solids	15.5
Hydrolysed slurry solids	17.5
Hydrolysed slurry solids + *Sporotrichum pulverulentum*[1]	21.5
Hydrolysed slurry solids + *Sporotrichum pulverulentum*[2]	22.7

1. Carbon source cellulosic constituents of hydrolysed solids.

2. Carbon source cellulosic constituents of hydrolysed solids + monosaccharides of liquid hydrolysate.

TABLE 27 The amino acid content of raw slurry solids, hydrolysed slurry solids and hydrolysed slurry solids on which fungus has been cultivated.

Amino acid	Raw slurry solids	Hydrolysed slurry solids	Hydrolysed slurry solids[1]+ *Sporotrichum pulverulentum*	Hydrolysed slurry solids[2]+ *Sporotrichum pulverulentum*
	g amino acid.100 g^{-1} protein			
Isoleucine	5.7	5.46	5.47	5.99
Leucine	7.04	7.89	9.10	8.90
Lysine	7.56	6.59	5.80	5.73
Phenylalanine	5.36	4.73	4.81	5.02
Tyrosine	3.36	3.27	3.35	3.44
Valine	5.81	5.92	6.89	6.78
Threonine	5.88	5.13	5.09	5.90
Tryptophan	N/A	N/A	N/A	N/A
Methionine	N/A	N/A	N/A	N/A
Cystine	N/A	N/A	N/A	N/A
Histidine	TRACE	2.25	2.12	2.11
Arginine	6.52	5.69	4.72	4.23
Aspartic acid	9.04	8.34	8.77	10.09
Serine	5.17	5.75	4.48	5.24
Glutamic acid	12.67	14.14	12.83	11.98
Proline	4.52	4.17	5.47	3.44
Glycine	6.20	5.58	5.33	4.93
Alanine	6.52	6.03	5.90	7.27

1. Carbon source cellulosic constituents of hydrolysed solids

2. Carbon source cellulosic constituents of hydrolysed solids + monosaccharides in liquid hydrolysate.

TABLE 28 Estimates of the crude protein requirements of pigs (Cuthbertson & Baskett, 1969).

Weight of pig (Kg)	Requirements as a percentage of the dry matter of the diet
1.4-4.5	32
4.5-9.0	26
9.0-20.0	20
20.0-50.0	18.5-20
50.0-90.0	15.5-16.5

Crude protein content and protein quality indicate that the products of fungal growth on hydrolysed slurry solids are potentially good sources of nutrition for fattening pigs.

Finally, the product of fungal growth on the combined hydrolysate and hydrolysed solids was analysed for heavy metals. The results are given in Table 29. The levels of Cu and Zn are approximately twice those reported by Evans *et al* (1978) for raw slurry solids. This is accounted for by the fact that almost half the slurry solids are solubilised during hydrolysis, and it indicates that Cu and Zn are not solubilised to any great extent during the hydrolysis procedure. This confirms the results reported earlier (page 74), where analysis of the liquid hydrolysate shows very low levels of heavy metals. These levels of heavy metals should not present a problem when the products are used as feed supplements for fattening pigs, which are normally fed large amounts of Cu and Zn salts in their diets (Table 3).

Overall, these chemical analyses indicate that the products of microbial growth on the liquid and solid residues of acid hydrolysis of pig slurry are potentially useful as animal feed supplements. This can only be confirmed, however, by intensive nutritional and toxicological feeding trials, which are outwith the scope of the present study.

TABLE 29 The heavy metal content of hydrolysed pig slurry solids on which *Sporotrichum pulverulentum* has been cultivated.

Heavy metal	Concentration ($mg.l^{-1}$)
Fe	833
Cu	922
Zn	1830
Mn	125

5. CHEMICAL ANALYSIS OF THE RESIDUAL LIQUID FRACTIONS PRODUCED WHEN MICROORGANISMS ARE GROWN ON ACID HYDROLYSED PIG SLURRY.

The products of microbial growth on the liquid and solid residues from the hydrolysis of pig slurry with 5% H_2SO_4 at 90°C for 3 h are yeast and/or a fungal biomass that is inextricably mixed with residual solids. The ultimate disposable residue of microbial cultivation is therefore a liquid fraction. Thus, it is considered necessary to establish the quality of this liquid, and for this purpose the changes that occur in BOD_5, COD, total-N, ammonium-N, pH and soluble carbohydrate were measured. Unfortunately, due to pressure of work in our analytical laboratories, the results reported for BOD_5, COD, total-N and ammonium-N were based on one set of determinations only. It is considered, however, that these results are sufficient to give a general indication of the effect of microbial growth on these chemical parameters.

MATERIALS AND METHODS

Chemical parameters of the liquids recovered during the fermenter growth trials of *Candida tropicalis* (page 89) *Sporotrichum pulverulentum* (pages 106 & 108) were measured. These were the levels of residual monosaccharides, Kjeldahl nitrogen (total-N)[1], ammonium nitrogen (ammonium-N)[1], chemical oxygen demand (COD)[1], 5 day biological oxygen demand (BOD_5)[1] and pH.

Residual monosaccharides were quantified by the standard HPLC technique (pages 39-47).

BOD_5 and COD were determined by standard methods (American Public Health Association, 1971).

Ammonium-N was extracted by steam distillation and measured by back titration of the absorbing acid (American Public Health Association, 1971).

Total-N was determined by the standard Kjeldahl method (American Public Health Association, 1971) using the zirconium dioxide catalyst of Glowa (1974).

The pH was measured on a Pye pH meter model 11085 (W.G. Pye & Co. Ltd., Cambridge).

1. *Chemical analysis was performed in the Analytical Chemistry Section of the Microbiology Department, The West of Scotland Agricultural College.*

RESULTS AND DISCUSSION

It has to be appreciated that the BOD_5, COD, total-N and ammonium-N levels of raw slurry vary significantly from batch to batch (Evans *et al*, 1978). This is especially true of the ammonium-N level which is influenced by the urine content of the slurry. Another difficulty in comparing raw and hydrolysed slurry is the fact that acid treatment causes changes in the chemical parameters. Probably the most obvious example of this phenomenon is the increase in BOD_5 caused by the hydrolysis of the cellulosic fractions, as they do not normally contribute to the BOD_5 of raw slurry, but the monosaccharides produced by acid treatment form part of the soluble BOD_5 of the resulting liquid hydrolysate.

In Figures 64 and 65, the utilisation of monosaccharides and soluble nitrogen compounds is depicted during the growth of *Candida tropicalis* on charcoal treated 5[90]3 hydrolysate and a 1:1 v/v dilution of 5[90]3 pig slurry hydrolysate respectively. These results indicate that the available glucose disappears almost immediately and that the xylose and arabinose levels progressively decrease throughout the growth period. Arabinose seems to be the most slowly utilised monosaccharide, and even after 72 h incubation a proportion of this carbohydrate is still detected in the residual liquid. After 96 h, however, all the arabinose is utilised by the yeast. In both cases the ammonium-N level gradually falls throughout the growth period. At the point where the maximum yield of yeast biomass is attained, namely after 30 h incubation in the charcoal treated hydrolysate and 42 h in the 1:1 v/v dilution (Figure 45), the ammonium-N level has decreased by 180 $mg.l^{-1}$ and 70 $mg.l^{-1}$ respectively. Similarly, decreases of 370 $mg.l^{-1}$ and 150 $mg.l^{-1}$ respectively have been recorded for total-N content. The level of total-N is seen to increase again when the yeast cultures are incubated beyond the point of maximum yield. This indicates that a certain amount of autolysis of yeast cells is occurring. During yeast growth the BOD_5 has been observed to fall from 7100 $mg.l^{-1}$ to 5615 $mg.l^{-1}$ after 42 h in the 1:1 v/v diluted hydrolysate and 14160 $mg.l^{-1}$ to 10920 $mg.l^{-1}$ after 30 h in the charcoal treated

FIGURE 64 The effect of the growth of *Candida tropicalis* on the chemical parameters of pig slurry hydrolysate 5 [90] 3 pre-treated with activated charcoal.

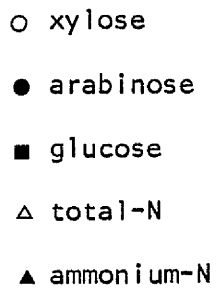


Figure 64

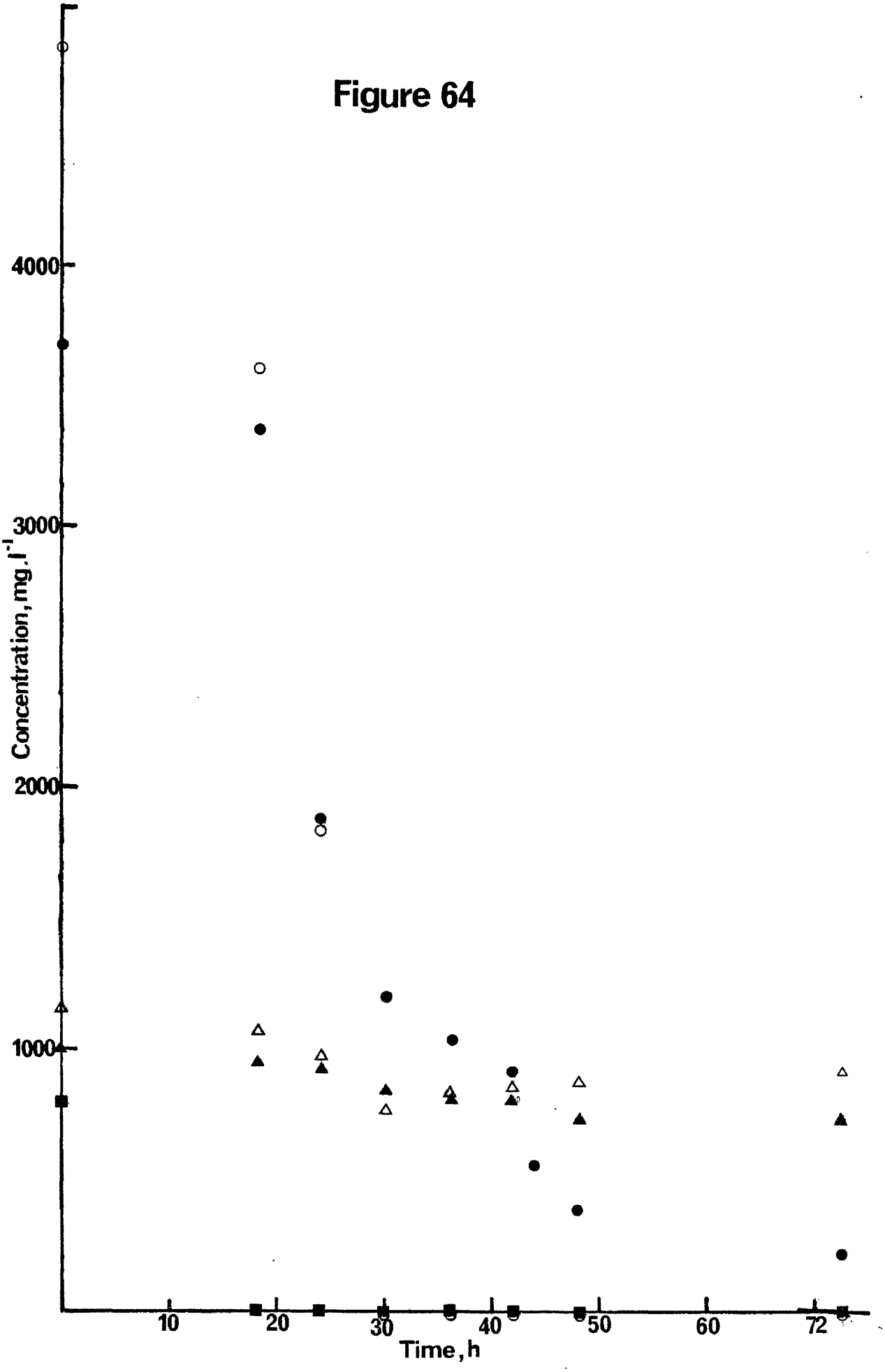

4000
3000
2000
1000
Concentration, mg.l-1
10
20
30
40
50
60
72
Time, h

FIGURE 65 The effect of the growth of *Candida tropicalis* on the chemical parameters of pig slurry hydrolysate 5[90]3 diluted 1:1 v/v.

○ xylose

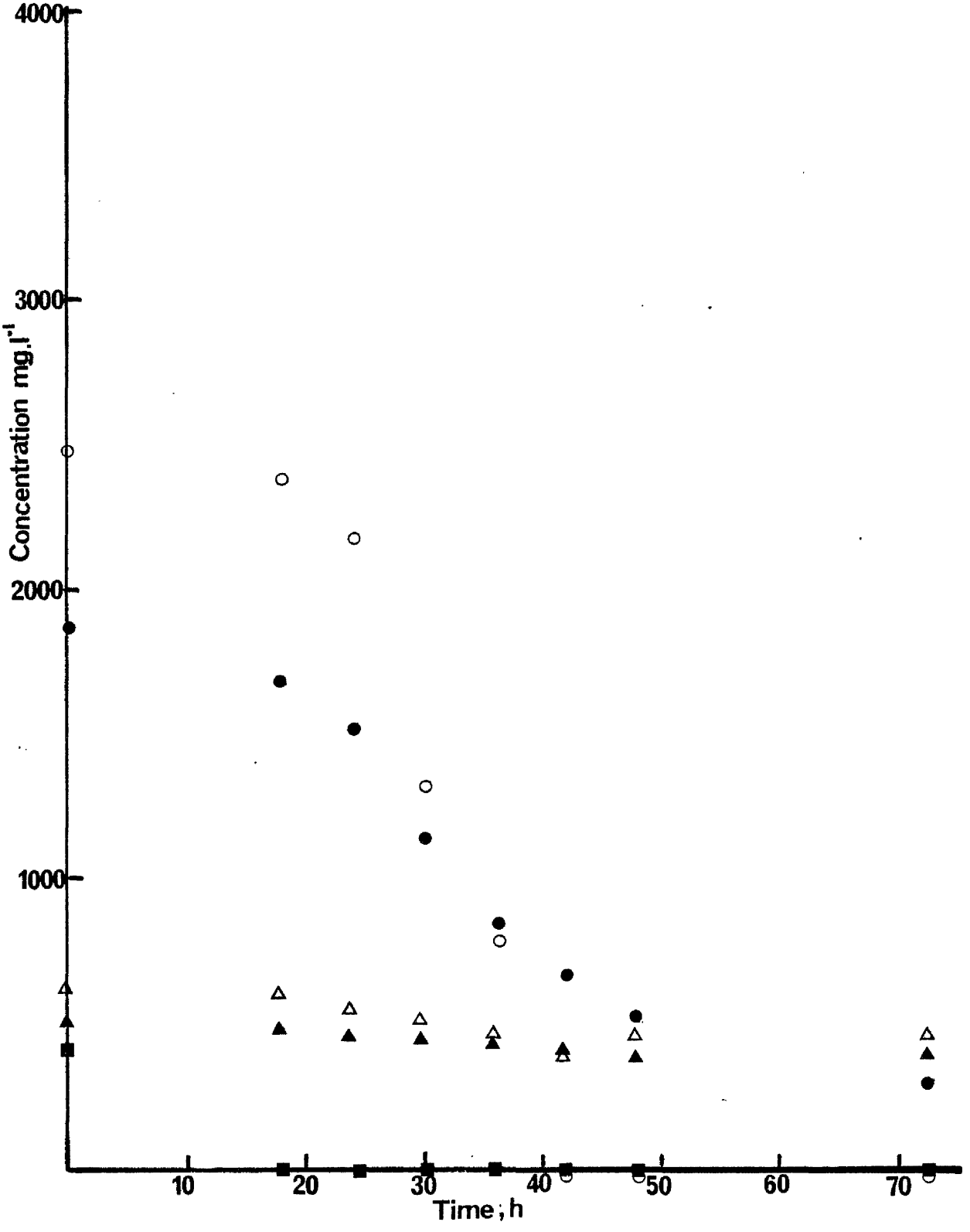
● arabinose

■ glucose

△ total-N

▲ ammonium-N

Figure 65


4000
3000
2000
1000
Concentration mg.l-1
10
20
30
40
50
60
70
Time; h

hydrolysate. The pH rises from 4.5 to 5 in both cases.

When the fungus, *Sporotrichum pulverulentum* is grown on a 1:1 v/v dilution of the hydrolysate, a maximum yield is attained after 120 h incubation (Figure 62) analysis of the residual liquid shows no trace of soluble carbohydrates, and the ammonium-N and total-N levels fall by 250 $mg.l^{-1}$ and 480 $mg.l^{-1}$ respectively during fungal growth. The BOD_5 falls from 9694 $mg.l^{-1}$ to 6312 $mg.l^{-1}$ and the COD from 11200 $mg.l^{-1}$ to 6900 $mg.l^{-1}$ (Figure 66).

The BOD_5, COD, total-N and ammonium-N levels of the residual liquid have also been measured when *Sporotrichum pulverulentum* is grown on the cellulosic constituents of hydrolysed pig slurry. The residual hydrolysate from yeast growth is used to provide the nitrogen source. After 168 h incubation, a maximum fungal yield can be harvested (Figure 60). The results of chemical analysis of the residual liquid are presented in Figure 67. In this case, the BOD_5 decreases by 2500 $mg.l^{-1}$, the COD by 3300 $mg.l^{-1}$, the total-N by 200 $mg.l^{-1}$ and the ammonium-N by 150 $mg.l^{-1}$ during fungal growth.

When the yeast growth stage is omitted, and the fungus is grown on the monosaccharides of the hydrolysate and the cellulosic constituents of the solid fraction simultaneously, a maximum yield of fungal biomass is again detected after a 168 h incubation period (Figure 63). This cultivation produces decreases of 480 $mg.l^{-1}$ in total-N, 250 $mg.l^{-1}$ in ammonium-N 5175 $mg.l^{-1}$ in BOD_5 and 7200 $mg.l^{-1}$ in the COD level of the hydrolysate (Figure 68). In all growth trials using *Sporotrichum pulverulentum* the pH has been observed to remain constant at 5 throughout the incubation period.

It is of interest to compare the chemical parameters of the ultimate residue to the average values obtained from raw slurry. These values are given in Table 30. It can be seen from these results that acid hydrolysis of pig slurry, followed by microbial cultivation on the products, decreases the measured chemical parameters of the waste by factors of between 2 and 5.5. Thus, although the residual liquids produced by these procedures still have polluting potential, they contain significant amounts of ammonium nitrogen, and it is possible that this could be usefully sprayed onto agricultural land.

FIGURE 66 The effect of the growth of *Sporotrichum pulverulentum* on the chemical parameters of pig slurry hydrolysate 5 [90] 3 diluted 1:1 v/v when the fungus utilises the soluble monosaccharides of the hydrolysate as a carbon source.

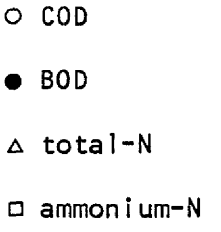


Figure 66

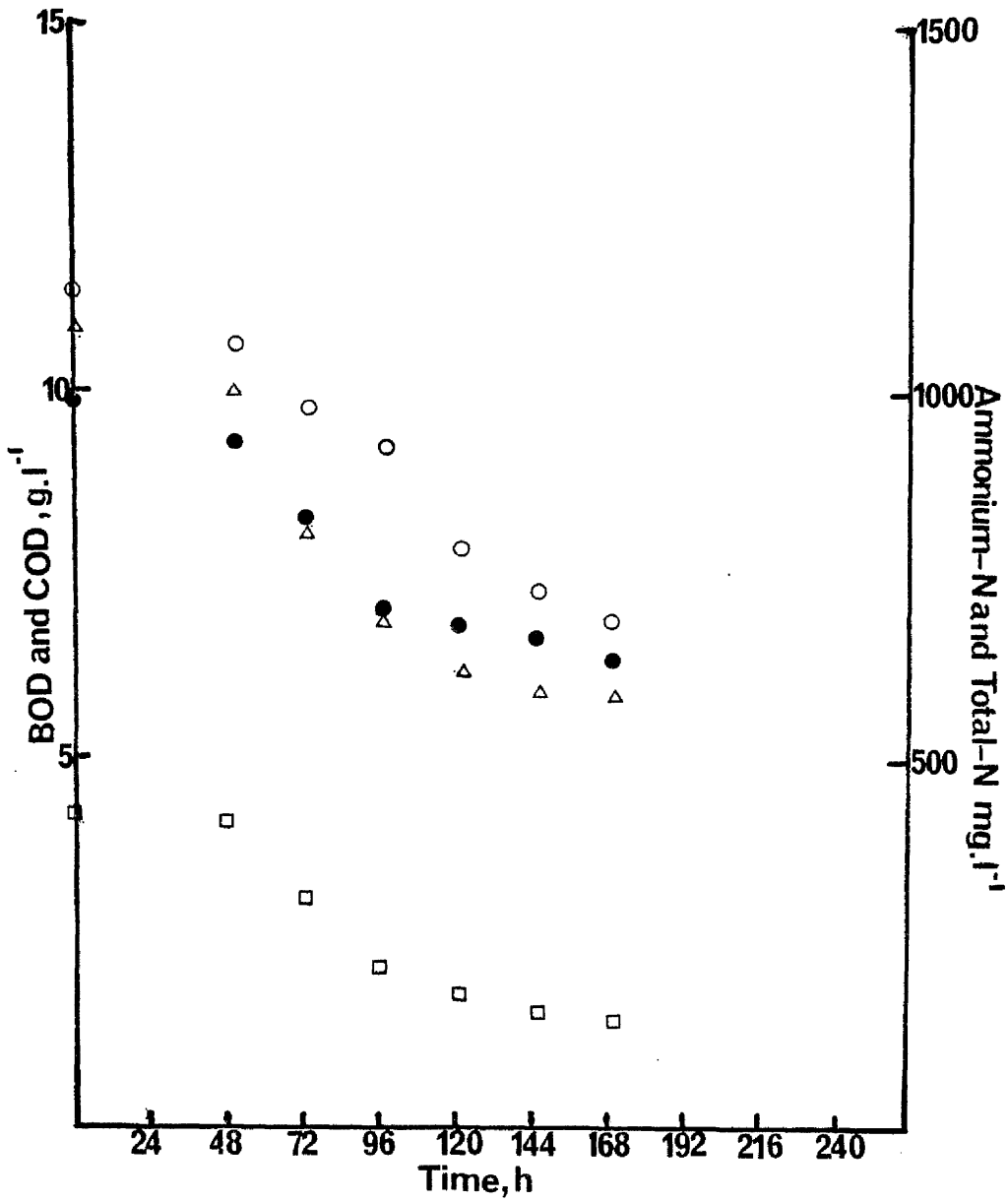

15
10
5
1500
1000
500
BOD and COD, g.l⁻¹
Ammonium-N and Total-N mg.l⁻¹
24
48
72
96
120
144
168
192
216
240
Time, h

FIGURE 67 The effect of the growth of *Sporotrichum pulverulentum* on the chemical parameters of the residual liquid 5[90]3 from the growth of *Candida tropicalis* when it is added back as a source of nitrogen for fungal cultivation in the cellulosic constituents of hydrolysed slurry.

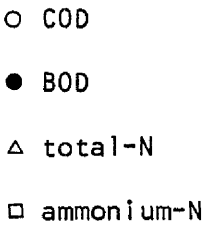


Figure 67

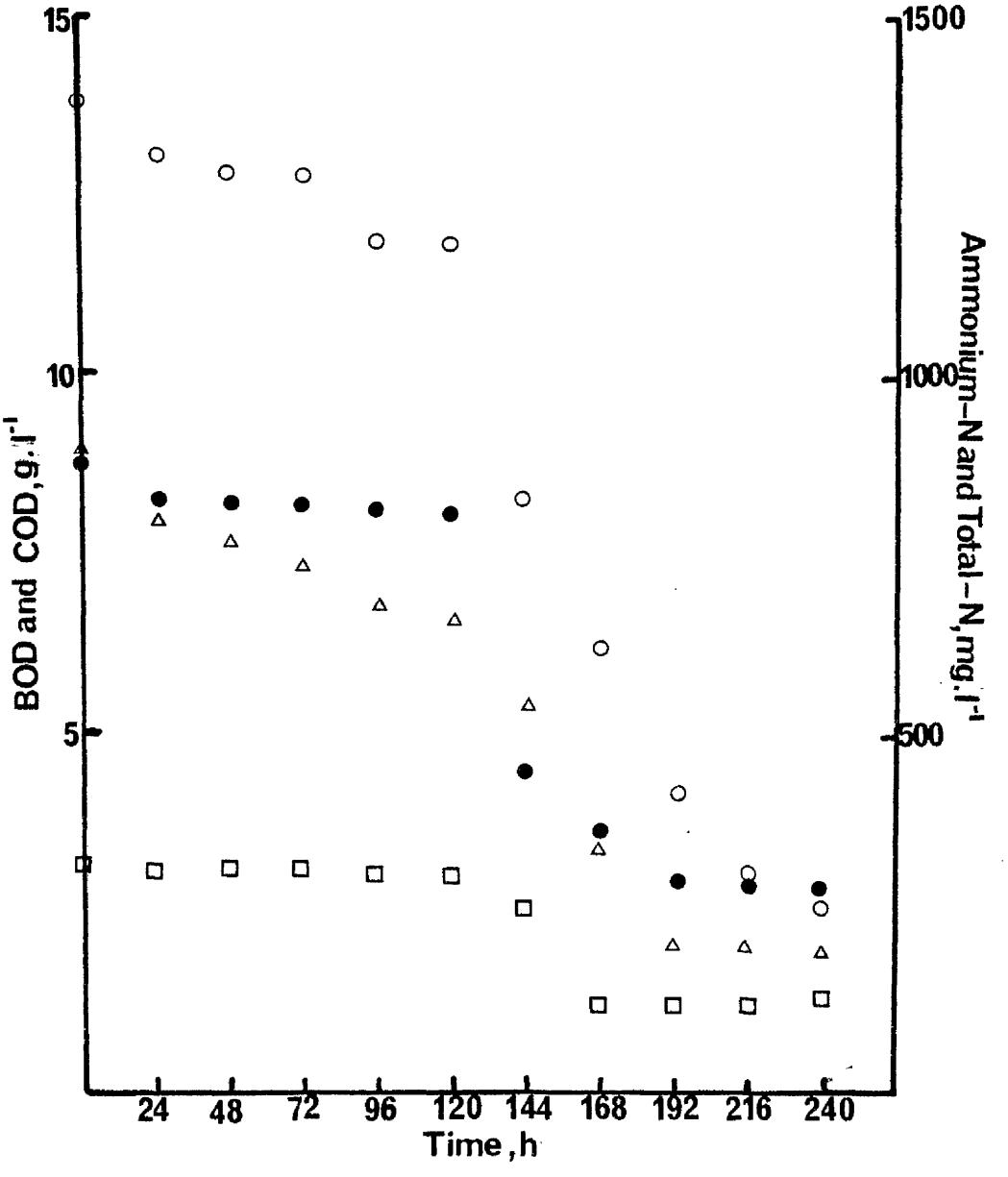

15
10
5
1500
1000
500
BOD and COD, g.l-1
Ammonium-N and Total-N, mg.l-1
24
48
72
96
120
144
168
192
216
240
Time, h

FIGURE 68 The effect of the growth of *Sporotrichum pulverulentum* on the chemical parameters of pig slurry hydrolysate 5[90]3 diluted 1:1 v/v when the fungus utilises both the cellulosic constituents of the solid residue and the monosaccharides of the hydrolysate as carbon sources.

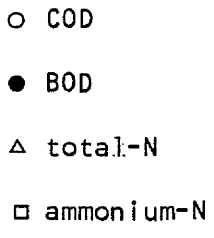


Figure 68

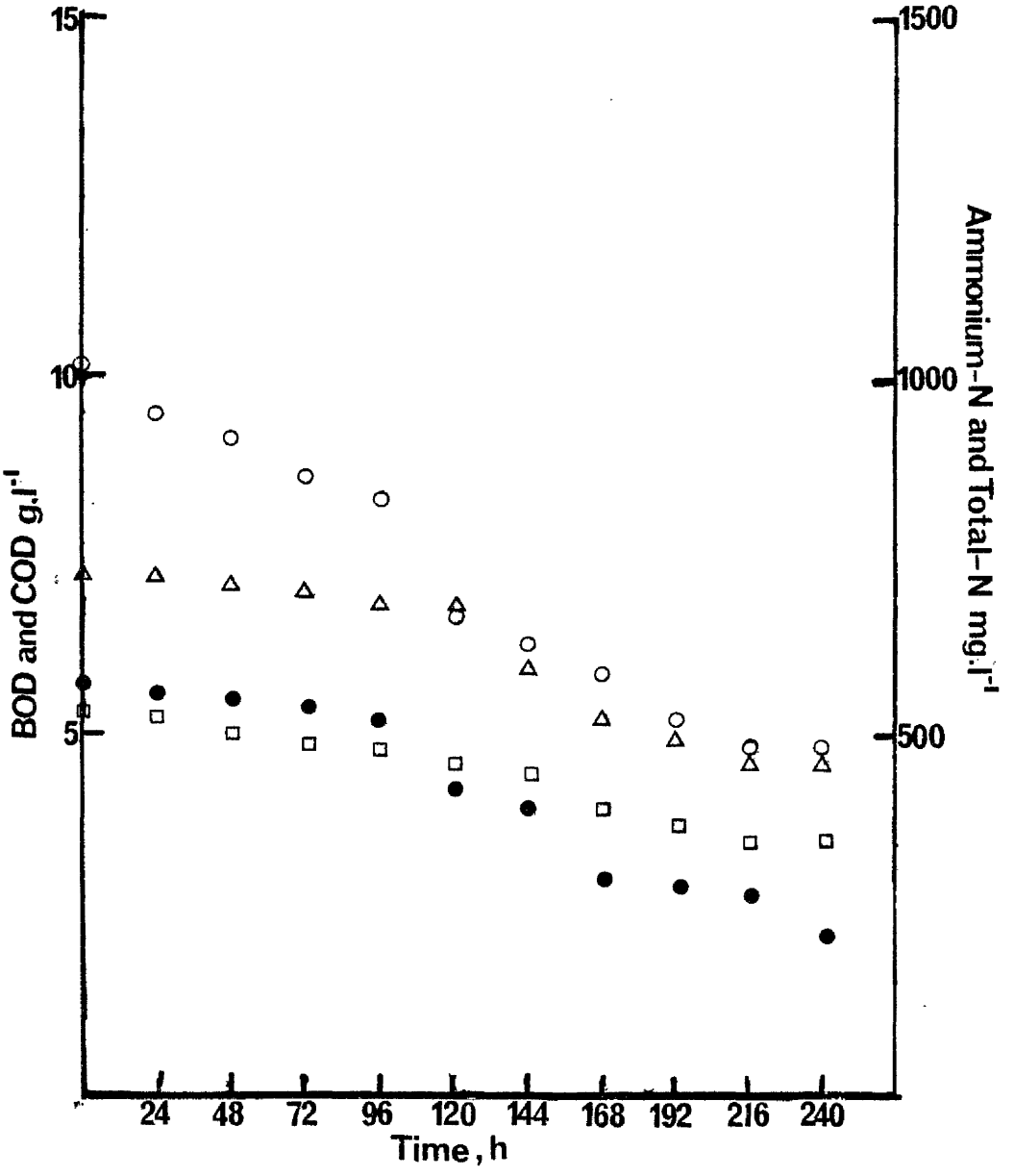

15
10
5
1500
1000
500
BOD and COD g.l⁻¹
Ammonium-N and Total-N mg.l⁻¹
24
48
72
96
120
144
168
192
216
240
Time, h

TABLE 30 Characteristics of excreta (faeces and urine) from fattening pigs compared to the liquid residues produced when microorganisms are grown on the liquid and solid fractions of acid hydrolysed pig slurry.

Physical and chemical parameters	Raw slurry[a]	Liquid residue[b] after the growth of *Candida tropicalis* and *Sporotrichum pulverulentum*	Liquid residue[c] after the growth of *Sporotrichum pulverulentum*
Volume (l)	1	5	5
Conc. ($g.l^{-1}$)	110 $g.l^{-1}$	-	-
BOD_5 ($mg.l^{-1}$)	146,000	5,900	6,400
COD ($mg.l^{-1}$)	38,000	3,026	3,510
Total-N ($mg.l^{-1}$)	7,190	514	300
Ammonium-N ($mg.l^{-1}$)	3,650	398	129

a. Overall values for raw slurry taken from Evans *et al* (1978)

b. *Sporotrichum pulverulentum* grown on the solid residue of hydrolysis and the residual liquid after the cultivation of *Candida tropicalis*.

c. *Sporotrichum pulverulentum* grown on the combined liquid and solid residues of acid hydrolysis.

CONCLUDING DISCUSSION

This research project has shown that the carbohydrate of pig slurry is present as α-cellulose and hemicellulose, which constitute 17.5% w/w and 22.5% w/w of the dry weight respectively. In this form it is not readily available to microorganisms. Mild H_2SO_4 treatment resulted in hydrolysis of the hemicellulose fraction, while the α-cellulose remained almost intact in the solid residue. Although it was not degraded, the acid treatment systems employed are likely to have rendered the α-cellulose more amenable to attack by cellulolytic microorganisms. The resulting liquid hydrolysate contained xylose, arabinose, trace amounts of glucose and a considerable amount of ammonium nitrogen. Several acid treatment systems were tested, of which a process employing 5% H_2SO_4 at 90°C for 3 h produced the highest yield of monosaccharides. In order to facilitate the growth of microorganisms on the products of hydrolysis, the pH was adjusted to 4.5 with NaOH, and the solid and liquid fractions separated by centrifugation.

Initial attempts to grow yeast species on the liquid fraction were unsuccessful, due to the presence of an inhibitory compound. It was established that this inhibitory agent was polyphenolic in nature, and was produced by the breakdown of pentoses in the acid conditions prevailing during the hydrolysis process. This inhibitory effect was, however, overcome by diluting the liquid hydrolysate with water, or by absorbing out the compound with activated charcoal.

By growing a yeast species later identified as *Candida tropicalis* on the diluted hydrolysate, a maximum biomass yield of 9 g was achieved for every litre of raw slurry hydrolysed (solids content 110 $g.l^{-1}$). This biomass had a protein content of 43% w/w with a favourable amino acid pattern and was especially rich in lysine, which is the limiting amino acid of cereals. It has to be accepted, however, that the potential pathogenicity of *Candida tropicalis* may limit its usefulness as a source of SCP. Nonetheless, the cultivation of this yeast species has established the principle of biomass production from pig slurry hydrolysate, and it is possible that another species, such as the non-pathogenic *Candida maltosa*, could be directly substituted into the process.

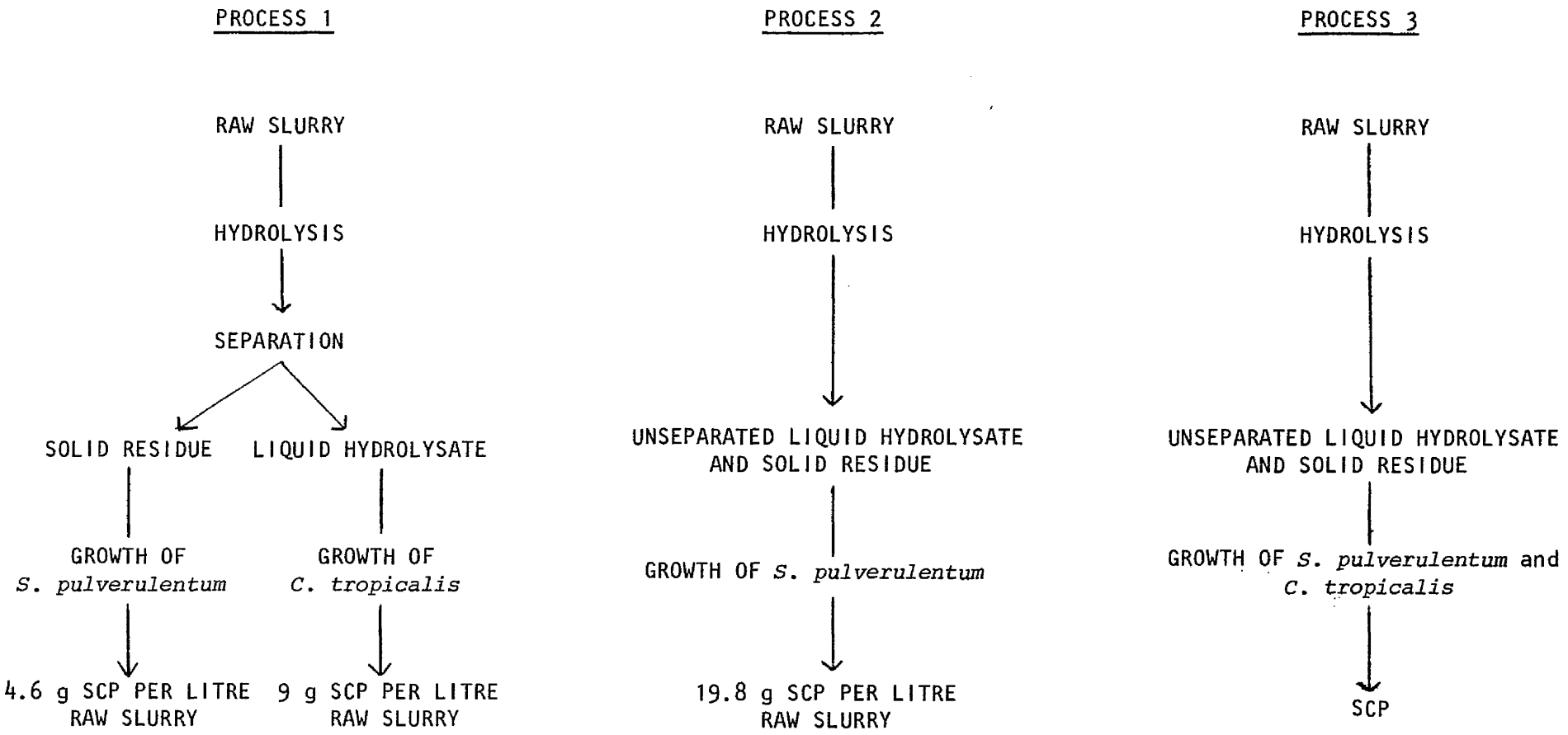
After the yeast biomass was harvested, the residual liquid still contained significant amounts of soluble nitrogen. Resuspension of

the cellulosic residue from hydrolysis in this liquid produced a medium suitable for the growth of the cellulolytic fungus *Sporotrichum pulverulentum*. The successful cultivation of this organism resulted in the breakdown of 43% w/w of the residual α-cellulose and the production of 4.6 g of fungal biomass for every litre or raw slurry hydrolysed. As the fungal biomass is inextricably mixed with the residual solids, it is envisaged that the mixed product would be used as an animal feed, which provides both protein and roughage. The protein content of the pure fungal biomass was shown to be between 25 and 35% w/w, and the amino acid pattern was adequate, although the lysine content was lower than that of *Candida tropicalis*. The growth of *Sporotrichum pulverulentum* on the hydrolysed solids increased the overall protein content, and appeared to produce a product which could be usefully fed back to fattening pigs. This process is summarised in Process 1, Figure 69.

One of the major disadvantages associated with Process 1 was the need for centrifugal separation of the liquid and solid residues of hydrolysis. Thus, Process 2 (Figure 69) was developed, in which *Sporotrichum pulverulentum* was grown on the unseparated liquid and solid residues from the hydrolysis process. This process had the added advantage of omitting the yeast growth and harvesting stages. Process 2 produced 19.8 g of fungal biomass.l^{-1} of raw slurry hydrolysed, which is 6.2 g more than the combined yeast and fungal biomass yields in Process 1. However, the yield of yeast biomass was estimated by dry weight, whereas that of the fungus was estimated by DNA analysis, and therefore the values may not be directly comparable. Process 2 utilised 13% w/w more of the cellulose of the solid residue than the first process. The protein content of the product was more than adequate for pig feeding, and the amino acid pattern was again favourable. Estimation of the nutritional quality of the products of microbial growth on hydrolysed pig slurry was based purely on chemical analysis, which only gives an indication of their feed potential. Only more extensive biological testing of their nutritional and toxicological characteristics would establish the nutritional value and safety of these products as animal feeds. This type of feeding trial was, however, outwith the scope of this work.

The ultimate residue of both processes was a liquid which had

FIGURE 69 Outline of the processes for the production of SCP from acid hydrolysed pig slurry



considerably less polluting potential than raw slurry, although it still contained significant amounts of soluble nitrogen. It is therefore thought that it could be usefully sprayed on agricultural land.

Although not investigated, a third process (Figure 69) is worthy of consideration. In this process it is proposed that the yeast and cellulolytic fungus are simultaneously cultivated on the unseparated solid and liquid residues of hydrolysis. This concept of combined growth has been reported to increase cellulose utilisation (Callihan & Dunlap, 1969; Dunlap, 1975; Peiterson 1975a and 1975b) and also to increase the breakdown of starch in the "Symba" process (Skogman, 1976). It is postulated that actively growing cultures of both organisms would be rapidly established in the hydrolysate media, and that when the *Sporotrichum pulverulentum* degraded the α-cellulose of the solid residue the glucose would be quickly utilised by *Candida tropicalis* thus preventing any product inhibition of the cellulase enzyme system of the fungus. In order to monitor yields in such a system, a more sophisticated method of independently determining yeast and fungal biomass would have to be developed. This might involve the estimation of some cellular component peculiar to each microorganism.

Several other methods have been developed to produce SCP from piggery waste (Henry & Thomson, 1979; McGill, 1973; Seal & Eggins, 1976). Although the approach to the problem is completely different in each case, there appears to be a serious disadvantage common to all the processes, in that the initial growth medium for the protein-producing microorganism is not sterile. The control of temperature and/or pH is used to control the growth of the mixed indigenous flora and establish the dominance of the desirable organism. Thus, unlike the processes described in Figure 69 , the composition, nutritional quality and pathogenicity of the final product will inevitably vary from batch to batch. It is considered completely unacceptable to produce SCP for human food or animal feed in this way.

Recently, Brown *et al* (1980) have described a procedure in which the soluble nitrogen components of pig slurry are extracted by washing the solids with water, and the resulting liquid sterilised prior to inoculation with a pure culture of *Aspergillus niger*. This process

requires the addition of a source of carbon, such as waste sugar beet, maize or potatoes. Since the waste carbon and the pig slurry are unlikely to be produced on the same site, the problem of transporting at least one of these wastes is inherent in the process.

Finally, although the successful cultivation of *Sporotrichum pulverulentum* on the acid treated α-cellulose of the solid residue was relatively successful, it is possible that other forms of pre-treatment could further enhance the efficiency of cellulose utilisation. The swelling of the crystalline cellulose matrix in solutions of dilute alkali at elevated temperatures (Han & Callihan, 1974) is probably the most practicable of those described in the literature. Alkali treatment of the α-cellulose in the solid residue from acid hydrolysis could increase its susceptibility to degradation by *Sporotrichum pulverulentum*. Alternatively, a new process omitting the acid hydrolysis stage altogether could be developed in which raw slurry is initially treated with alkali. It is postulated that this treatment would solubilise the hemicellulose fraction, swell the α-cellulose and sterilise the medium for the growth of a pure culture of *Sporotrichum pulverulentum*. This alkali pre-treatment would appear to have advantages and disadvantages when compared to the system employing mild H_2SO_4. It is similar to Process 2 (Figure 69) in that it avoids the need for separation of the liquid and solid residues. Furthermore, the use of alkali is likely to overcome the problem of the production of the polyphenolic inhibitory compound. However, it is apparent that only a cellulolytic fungal species could be grown on the solubilised hemicellulose and the swollen α-cellulose produced by alkali treatment. Depending on overall biomass yields, and on the relative nutritional quality and availability of the protein of yeast species and fungal species in animal feeding trials, the inability of a system based purely on alkali pre-treatment to produce yeast biomass may ultimately prove to be disadvantageous. However, it is considered that systems incorporating alkali pre-treatment are worthy of further investigation.

Overall, the results of this research project indicate that pig slurry, which poses a formidable disposal problem, contains significant amounts of carbon and nitrogen that can be successfully utilised to produce SCP for the purpose of animal feeding.

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